Indigenous goat breeds might be utilized as the fundamental elements required in a breeding program. The goat’s productivity could be increased through molecular breeding. Bone morphogenetic protein 4 gene plays a vital role in cumulus expansion, ovulation, follicular growth, and differentiation, which can operate directly on the granulosa and result in a significant alteration in the follicle-stimulating hormone activity. The study was carried out to investigate the polymorphism in the bone morphogenetic protein 4 gene and to analyze the factors that correlate with follicle-stimulating hormone levels in Indonesian native goat breeds. To our knowledge, no studies have examined the bone morphogenetic protein 4 gene in Indonesian indigenous goat breeds. This study was conducted in three regencies that represented different goat breeds. The polymerase chain reaction fragments were digested using PvuII restriction enzymes, and deoxyribonucleic acid sequencing was performed for further analysis. The restriction site showed a similar pattern, indicating merely one allele (G/GG genotype existed. Further, nucleotide sequence alignment analysis of the intron 2 bone morphogenetic protein 4 gene revealed that single-nucleotide polymorphisms were not detected. In other words, the bone morphogenetic protein 4 gene was monomorphic in the screened goat breeds. The frequency of genotype GG and allele G was reported to be 1.0, respectively. The locations and permanent teeth classification showed a significant distinction among categories, whereas the oestrus cycle was not associated significantly. Therefore, the present research could not reveal the association of reproductive traits with the polymorphisms of the bone morphogenetic protein 4 gene in Indonesian native goat breeds.

**Keywords:** BMP4 gene, FSH level, indigenous breeds, monomorphic, reproductive trait

**Introduction**

There has been a huge imbalance between the supply and demand of livestock products in recent years. The discrepancy between demand and output, as well as a program to improve the biological and economic efficiency in small ruminant production, can be addressed by genetic improvement of reproductive traits (Ahlawat et al., 2015; Mohammadabad, 2016; Tao, 2011). Increasing reproductive traits, particularly a rise in litter size, may be a good strategy to enhance the population of goats, thus resulting in greater profit (Febriana et al., 2021; Islam et al., 2020). Yet the fundamental data on goat reproduction and production as the basis for increasing productivity are mostly undocumented in many countries, including Indonesia (Adhianto et al., 2019). Small ruminants, particularly indigenous goat breeds, contribute a significant part to the livelihoods of society in the tropical region (Jafari Ahmadabadi et al., 2023). Indigenous goat breeds in Indonesia have strong genetic structure (Vahidi et al., 2014) and high genetic diversity (Periasamy et al., 2016), which might be utilized as one of the fundamental elements required in a breeding program for the advancement of livestock production in Indonesia (Astuti et al., 2007).

Utilization of breed resources, selection within breeds, and use of major genes are three methods for genetically increasing litter size in small ruminants (Land & Robinson, 1985). There are many local goat breeds in Indonesia as the national genetic resources (Kurnianto et al., 2013); unfortunately, many goat breeds have not yet been genetically characterized (Barker et al., 2001; Batubara et al., 2011). Three well-known indigenous goat breeds in Indonesia, namely, Kacang goat (KAC), Senduro goat (SEN), and Kejebong goat (KEJ), have a high prolificacy trait (Ministry of Agriculture, 2012, 2014, 2017). In addition, to generate excellent livestock with better production ability and well adapted to the environment, breeding selection within indigenous breeds is required (Subasinghe, 2016).

Several candidate genes for prolificacy have been reported (GH, GnRHR, AA-NAT, GDF9, BMPR1B, BMP15, POU1F1, PRLR, KISS-1, GPR54, INH, CART, GnrH, LH, BMP4, KiT1G, FSH, FshR, MT2, and CYP21), which may contribute through molecular breeding to increased productivity in the goats (Ahlawat et al., 2015). The transforming growth factor beta (TGF-β) superfamily contains 30 members known as BMP members, with the bone morphogenetic protein 4 (BMP4) gene being the most crucial of these (Sarma et al., 2019). It has been demonstrated that the BMP4 gene plays a vital role in cumulus expansion, ovulation, follicular growth, and differentiation. Bone morphogenetic protein 4 expression is extremely high in healthy follicles, which can operate directly on the granulosa and result in a significant alteration in the follicle-stimulating hormone (FSH) activity (Shimasaki et al., 1999). This may prevent progesterone production and secretion and thereby eliminate FSH-stimulating action either in cattle (Fatehi et al., 2005; Knight & Glister, 2003) or sheep (Juengel et al., 2006; Pierre et al., 2004).
Due to the BMP4 gene’s major role, further research of different BMP4 gene intron 2 regions A for potential effects on reproduction is required. To our knowledge, no studies have examined the BMP4 gene in Indonesian indigenous goat breeds. Hence, this research was carried out to explore the polymorphism in BMP4 genes intron 2 and to examine the factors associated with FSH levels in Indonesian native goat breeds.

**Materials and Methods**

**Ethics Committee Approval**

The Animal Ethics Committee of the Faculty of Animal and Agricultural Sciences Universitas Diponegoro gave its approval to all of the methods used on the experimental animals in the present study (number: 57-06/A-4/KEP-FPP).

**Animals and Sample Collections**

The studied animals were reared by the farmer’s group in three different regions under relatively homogenous conditions on Java Island in Indonesia. In this study, 90 whole blood samples from adult female goats of three distinct breeds (KC, SEN, and KEJ goats) were used, 30 samples from each breed. Blood samples (3 mL) were collected from their jugular veins and stored in a sterile ethylamine (2–5), age (18–60 months), and unrelatedness with other animals. According to Millard (2002), the age of the goats was determined using the dentition method. When a goat has four permanent teeth, it is 2 years old. Six and eight permanent teeth appear between the ages of 3 and 4 years. The farmers involved in the research were informed and allowed permission for the utilization of animals in the current investigation.

**Estrus Synchronization Treatment**

To induce oestrus in goats, sponges impregnated with 0.30 mg medroxyprogesterone acetate (aka MAP) were inserted intravaginally (considered day 1). The sponges were withdrawn on day 14. Subsequently, the goats were maintained in a stall, and the blood samples were collected from their jugular veins at 0, 3, 6, 9, and 12 hours after the sponge removal. Hour 0 was considered to be the moment of sponge withdrawal. A total of 3 mL of blood samples per animal was obtained, and they were centrifuged at 3000 rpm for 5 min. The plasma was maintained at a cool temperature and stored at −20°C in Eppendorf tubes until FSH profile analysis. The goat follicle-stimulating Hormone Kit (Bioassay Technology Laboratory Cat. No. E0006Go Shanghai, China) and a microplate reader (ZENIX-320, USA) were utilized to appraise and calculate the FSH hormone levels, respectively. The range of the standard curve was from 0.05 mIU/mL to 15 mIU/mL.

**Deoxyribonucleic Acid Extraction and Polymerase Chain Reaction**

Genomic deoxyribonucleic acid (DNA) was extracted utilizing a GeneJET Kit (Thermo Scientific, USA) from whole blood samples under the manufacturer’s instructions. A 50 µL total reaction volume was used for the polymerase chain reaction (PCR) amplification reaction, which contained 20–30 ng/µL DNA extraction (4 µL), 10 pmol/µL of each primer (1 µL), ddH2O (19 µL), and MyTaq Red Mix Bioline (25 µL). The extracted DNA was checked for quality and integrity by electrophoresis using 1% agarose.

Primers were designed for amplification of intron 2 of the BMP4 gene (1063 bp) based on the BMP4 gene of Capra hircus (GenBank Accession Number EU104684.1) using Primer3Plus software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primers designed for this region were: forward (5’-GTGAGGAGCTTCCACCGG-3’) and reverse (5’-CTCAGCAAGATCCGAGGAGAAA-3’) were specifically synthesized by first BASE. The PCR reaction program applied was first denaturation at 95°C for 5 minutes, followed by DNA amplification for 35 cycles with 30 seconds of denaturation at 94°C, 30 seconds of annealing at 61°C, 30 seconds of extension at 72°C, and 9 minutes of final extension at 72°C. Amplified products were detected by electrophoresis in 2% agarose gel (Invitrogen, Life Technologies Co., CA, USA) for 30 minutes at 100 volts, aside 100 bp DNA marker (Geneaid, 1st BASE) recorded by digital gel imaging equipment (UV Transilluminator, Uvitec Cambridge).

**Genotyping**

The amplicons were digested using PvuII restriction enzymes in the thermal cycling process to identify the genetic variant of the BMP4 gene in all animals. The digestion procedure, as a result of the optimization protocol, was carried out in a 15 µL volume with 5 µL of the amplified DNA, 0.5 µL of PvuII restriction enzyme, 8 µL of ddH2O, and 1.5 µL of buffer and incubated at 37°C for 4 hours as per protocol. Restriction fragments were resolved by electrophoresis on a 3% agarose gel stained with Flurosafe DNA stain (1st BASE).

**Sequence Analysis**

Polymerase chain reaction product is sent to 1st BASE for sequencing. The obtained data were aligned with the reference sequence of the BMP4 gene of C. hircus (GenBank Accession Number EU104684.1) and analyzed by MEGA-X software (Kumar et al., 2018).

**Follicle-Stimulating Hormone Analysis**

The enzyme-linked immunosorbent assay (ELISA) analysis was applied to assess the FSH level using a goat FSH kit (Bioassay Technology Lab., China). The ELISA was carried out according to the kit’s instructions.

A previous study on Jining Grey goats found a correlation between prolificacy and genetic variations in BMP4 (Chu et al., 2011). In contrast, numerous studies failed to identify a variation in the BMP4 gene and its association with reproductive traits (Sarma et al., 2019; Sharma et al., 2013). Improving the reproductive efficiency of various goat breeds may be obtained from the identification of several genetic polymorphisms (single-nucleotide polymorphisms (SNPs)) associated with prolificacy (Mishra et al., 2017). The BMP4 gene has a length of 4.30 kb. The transcript of the BMP4 gene is 2284 bp long and encodes for 409 amino acids. This gene was found on chromosome 10 in goats, which has three exons and two introns (ENSCIT0000031538.1). The vast majority of the genes are composed of introns. Although SNPs in the noncoding area do not directly affect proteins, they have an advantageous effect on gene expression through regulating stability and transcription rate (Cui et al., 2018; Jing et al., 2019; Shaul, 2017). Furthermore, the noncoding region can alter the binding sites of regulatory gene expression elements or the splicing of this gene (Bai et al., 2021). Moreover, some intronic variations could be in perfect LD with known trait-associated mutations (Nakaoka et al., 2016), i.e., linkage among rs648407208, novel g.2436A>G, and rs655206996 in intron 1 of KISS1 gene (Febriana et al., 2022).

According to Millard (2002), the age of the goats was determined by the Animal Ethics Committee of the Faculty of Animal and Agricultural Sciences Universitas Diponegoro gave its approval to the utilization of animals in the present study (number: 57-06/A-4/KEP-FPP).
Statistical Analysis

Observed heterozygosity (Ho), expected heterozygosity (He), and Hardy–Weinberg equilibrium (HWE) were estimated by Arlequin Software Package version 3.5 (Excoffier & Lischer, 2010). The Basic Local Alignment Search Tool (BLAST) tool from The National Center for Biotechnology Information (NCBI) (http://https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find the level of similarity among selected sequences (Pearson, 2013).

Since the present research did not find any polymorphisms, the association between genetic variants of the BMP4 gene and reproductive traits was not evaluated. The correlation between location, permanent teeth classification and estrus cycle on FSH levels was counted using a fixed general linear model of SAS software (SAS University Edition, 2018). Multiple comparisons of the means were analyzed using Tukey’s post hoc.

The fixed model used to analyze FSH level is:

\[ y_i = \mu + F_i + e_i \]

where \( y_i \) is the FSH level of the observed animal; \( \mu \) is the overall mean of FSH level; \( F_i \) is the fixed effect of \( i \)th factor (\( i = 1, 2, 3 \)); and \( e_i \) is the random residual for \( y_i \). The results were significant at \( p \leq 0.05 \).

Results

DNA Extraction and Polymerase Chain Reaction (PCR)

The 1063 bp fragment of the BMP4 gene of KAC, SEN, and KEJ goats was identified and amplified by a specified primer at 1063 bp (Figure 1). The results revealed that amplification fragment sizes conformed to the target region, and clear band patterns showed excellent specificity. Further, the samples could be directly evaluated using restriction fragment length polymorphism (RFLP) or sequencing analysis.

Genotyping

The 1063 bp amplified region of the BMP4 gene was digested using the restriction enzyme PvuII, which recognizes the polymorphism at the recognition site CAGCTG. The RFLP analysis produced two fragments, each 335 bp, and 728 bp for the GG genotype (wild homozygotes), as illustrated in Figure 2. The restriction site showed a similar
pattern, indicating merely one allele (G)/GG genotype existed across all samples.

**Sequences Analysis**
The nucleotide sequences of the \( \text{BMP4} \) gene among the samples and the sequence from GenBank (EU104684.1) were aligned using MEGA X software. Regrettably, nucleotide sequence alignment analysis of 1063 bp of the intron 2 \( \text{BMP4} \) gene revealed that SNPs were not detected (Figure 3). The amplified area of the \( \text{BMP4} \) gene in all of the samples showed 100% sequence similarity.

BLAST analysis was conducted in the Indonesian goat \( \text{BMP4} \) gene’s intron 2 region to compare and determine the degree of nucleotide homology with other species. Huanghuai goats from China (EU104684.1), Bos taurus (AC149774.4), Sus scrofa (EU549864.1), and Homo sapiens (EU518936.1) had the highest levels of similarity, with 100%, 98.18%, 89.18%, and 85.97%, respectively. The sequences from different species and breeds were acquired from the NCBI GenBank database.

**Follicle Stimulating Hormone (FSH) Analysis**
The current study showed the data on the FSH level in three indigenous goat breeds in Indonesia (Table 1). The various regencies represented different goat breeds, i.e., Grobogan for KAC goat, Lumajang for SEN goat, and Purbalingga for KEJ goat.

**Discussion**
The present research used three different indigenous goat breeds in Indonesia. The utilization of various goat breeds to identify the genetic variation could escalate the SNP yield; therefore, a mixed goat breed is an excellent approach to discovering SNPs (Sharma et al., 2013). Unfortunately, the digested amplicons using \( \text{PvuII} \) restriction enzymes show a similar patterns. In other words, the \( \text{BMP4} \) gene was monomorphic in screened indigenous goat breeds from Indonesia, which demonstrates significant genome organization conservation among higher vertebrates (Ansari et al., 2022). DNA sequencing was performed as further analysis to discover the polymorphism in the \( \text{BMP4} \) gene at different sites along the fragment.

Further, these findings justified the conclusion that the nucleotide sequence acquired from this investigation was exactly the predicted intron 2 of goat \( \text{BMP4} \). According to our findings, previous research by Sharma et al. (2013) did not discover polymorphism in the intron 2 \( \text{BMP4} \) gene of nine Indian goat breeds, either in the exon 3 or 3’ untranslated region (UTR) regions. Furthermore, another research by Sarma et al. (2019) using the Haelll restriction enzyme is in agreement with the present study. A previous study revealed the monomorphic pattern in exon 2 in four Chinese goat breeds (Chu et al., 2011). These findings are in agreement with the fact that the \( \text{BMP4} \) gene is one of the most evolutionary conserved genes in various species (Winnier et al., 1995; Zhang et al., 2014). The current condition

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**Table 1.**
The Average of Follicle-Stimulating Hormone Level in Indonesian Native Goat Breeds (Mean ± SE)

<table>
<thead>
<tr>
<th>Classification</th>
<th>Category</th>
<th>Mean ± SE (mIU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Grobogan</td>
<td>3.88 ± 0.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(( p &lt; .002 ))</td>
<td>Lumajang</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Purbalingga</td>
</tr>
<tr>
<td>Permanent teeth</td>
<td>4</td>
<td>3.86 ± 0.73&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(( p &lt; .01 ))</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Estrus cycle</td>
<td>Follicular phase</td>
<td>3.21 ± 0.60</td>
</tr>
<tr>
<td></td>
<td>(( p &lt; .50 ))</td>
<td>Luteal phase</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values with different superscripts in the same column differ significantly at \( p < .05 \).
could be due to strong selective pressure resulting in a lower frequency of nucleotide polymorphism (Sharma et al., 2013), breed conservatism (Gipson, 1993), and fixation of the gene (Pandya et al., 2020). This could be associated with BMP4’s critical role in a variety of biological processes (Coucouvanis & Martin, 1999; Lawson et al., 1999; Winnier et al., 1995). However, it is possible that the BMP4 gene is not completely free from selection (Bai et al., 2016).

Earlier investigation identified a polymorphism in the BMP4 gene’s intron 2 at g.1986A>G and g.2203G>A in Xuhuai White goats, Boer goats, and Haimen goats (Fang et al., 2009). Furthermore, Chu et al. (2011) ensured a polymorphism at g.2203G>A in low-fecundity goat breeds (Angora and Inner Mongolia Cashmere goats) either high-fecundity goat breeds (Jining Grey goats) and a monomorphic AA genotype in Boer goats. Interestingly, Indonesian goat breeds represent a GG monomorphic genotype. The variations in genotypic expression could be led by differences in the environments between countries (Ismail et al., 2020), goat breeds, which are subjected to different evolutionary forces, and breed sampling under research (Mahrous et al., 2018). However, the current study was unable to discover SNPs in the BMP4 gene’s intron 2 regions, such as rs64679739, rs647724258, rs668938251, rs643536682, and rs659409637 (https://asia.ensembl.org/).

The frequency of genotype GG and frequency of allele G was reported to be 1.0, respectively, in all Indonesian indigenous goat breeds affected by the monomorphic sequences of the BMP4 gene, while the He and Ho are 0 and 1. The HWE value could not be determined because the gene was monomorphic. Therefore, a meaningful connection between the obtained BMP4 genotypes and reproductive traits could not be determined.

Additionally, the current study showed the data on the FSH level in three indigenous goat breeds in Indonesia (Table 1). The various regencies represented different goat breeds, i.e., Grobogan for KAC goat, Lumajang for SEN goat, and Purbalingga for KEJ goat.

The BLAST analysis represents closely related sequences that are identical at the nucleotide level and might be interpreted as regulatory elements or protein products of gene expression with the same function and structure (Mahdavi & Dashab, 2017). The Huanghui goat BMP4 gene was the closest to the BMP4 gene in KAC, SEN, and KEJ goats and relatively close to B. taurus, S. scrofa, and H. sapiens at the molecular level. Considering that its structures and, frequently, its roles are similar, this gene might be classified as a homologous sequence (Pearson, 2013). The DNA sequences of the samples utilized in the current study did not reveal any similarities to sheep, which are categorized as small ruminants. This condition was driven by the reproductive isolation among species during evolution (Zhang et al., 2014). On the other hand, according to Zheng et al. (2018), goat breeds have a significant degree of similarity with Ovis aries, even though the KiSS1 gene is highly conserved in species.

The Payoya goats in the previous study were given intravaginal sponges containing progestogens for 11–12 days (Arrebola et al., 2022), whereas the goats in the current study were given the treatment for 14 days. This dissimilarity is due to variations in standard durations for treatment periods across several goat breeds or other species’ breeds in terms of productive characteristics or area. Table 1 shows that Lumajang, which represents the SEN goat, has a lower FSH level compared to two other breeds, even though all three goat breeds are classified as highly prolific breeds (Ministry of Agriculture, 2012, 2014, 2017). On the contrary, earlier research by Cui et al. (2009) has shown that FSH levels in prolific breeds (Boer goats), are higher than in nonprolific breeds (Yunling black goats) indicating that FSH levels are closely related to litter size. Many factors influence goat reproduction, including associations between reproduction, genetics, feed, goat breed, and environmental factors, which are required to be addressed clearly (Fatet et al., 2011). Hence, while the BMP4 gene could not have influenced to FSH level in Indonesian indigenous goats significantly, its prominent role should be investigated further.

The permanent teeth classification showed that the distinction among categories (4, 6, and 8 permanent teeth) is significant (Table 1). Many species use the time of eruption of the permanent teeth as an indicator of age (Anwar & Ahmad, 1999; Colomer-Rocher et al., 1987). In the present research, four and eight permanenttooth goats revealed higher FSH levels than six permanent-tooth goats. On the other hand, FSH levels did not significantly vary among the different ages in goats (Mandour et al., 2022). Further Malhi et al. (2005) stated that there were no distinctions in FSH levels between young and old cows in the preovulatory FSH peak concentration or time of occurrence in cattle. The pattern of hormonal events during estrus in goats is similar to that reported in sheep and cows (Chemineau et al., 1982). The current condition could be caused by the difference in goat breed, litter size (Febriana et al., 2021), different goat types used, lactating/nonlactating female goats, the length of postpartum days, and the geographical distribution of animals used.

The FSH level in the recent study did not diverge significantly in either the follicular phase or the luteal phase. The present condition might lead to a short observation period. Febriana et al. (2021) reported that the late luteal phase and early follicular phase demonstrated a low increase in FSH levels. Slower follicle growth prevents equine chorionic gonadotrophin from stimulating ovulation in early-stage follicles and lowering fertility. The FSH peak was detected at 33.91 ± 0.29 h after sponge withdrawal (Arrebola et al., 2022).

Reproductive physiological differences can be associated with the breed; therefore, desirable to know the specific hormone release pattern for each breed (Arrebola et al., 2022). Future research should take into consideration the different categories of goat breeds used, lactating/nonlactating female goats, the length of postpartum days, and the geographical distribution of each goat breed. Hereinafter, the current research could not declare the correlation of FSH level with the polymorphisms of the BMP4 gene in Indonesian native goat breeds because of its monomorphic pattern.

**Conclusion and Recommendations**

In this study, the BMP4 gene showed a monomorphic banding pattern when digested with PvuII restriction enzyme among the studied goat breeds (KAC, SEN, and KEJ goats). The DNA sequencing result also confirms the PCR-RFLP analysis. The FSH level observed in Indonesian indigenous goat breeds was significantly different based on permanent teeth. On the contrary, the FSH levels were equal both in the follicular and luteal phases. Considering the limited quantity of samples tested in this research, there is a need for further investigation of a significantly larger population and various regions of the BMP4 gene to provide new findings. As we know, this is the first
study to investigate the effect of BMP4 gene polymorphisms on reproductive traits in Indonesian indigenous goats.

Ethics Committee Approval: Ethical committee approval was received from the Ethics Committee of Diponegoro University Faculty of Animal and Agricultural Sciences (Approval number: 57-06/A-4/KEP-FPP, Date: June 3, 2021).

Informed Consent: Verbal informed consent was obtained from the farmers who agreed to take part in the study.

Peer-review: Externally peer-reviewed.


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Declaration of Interests: The authors have no conflict of interest to declare.

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References


Ahlawat, S., Sharma, R., Maitra, A., & Tanthing, M. S. (2015). Current status of molecular genetics research of goat fecundity. Small Ruminant Research, 125, 34–42. [CrossRef]


Sharma, R., Ahiwatt, S., Maitra, A., Roy, M., Mandakmale, S., & Tantia, M. S. (2013). Polymorphism of BMP4 gene in Indian goat breeds differing in prolificacy. Gene, 532(1), 140–145. [CrossRef]


