Polymorphism of follicle stimulating hormone beta sub-unit (FSH-β) gene as a molecular marker for reproductive status in Peranakan Ongole x Bali crossbred (POBA) cattle

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Abstract. POBA cattle is a crossbred cattle from Peranakan Ongole (PO) and Bali cattle, which is developed by the Beef Cattle Research Institute (BCRI), Ministry of Agriculture of the Republic of Indonesia. This study aimed to identify the genetic and phenotypic characteristics of POBA cattle raised in the BCRI. Genetic diversity of FSH-β gene was also determined in order to identify a possible marker for reproductive status in POBA cattle. Rambon cattle collected from Banyuwangi regency were used as comparison. A 313 bp fragment of the FSH-β gene was amplified using the polymerase chain reaction (PCR). The PCR products were sequenced and aligned to detect polymorphism. As results, a polymorphism (SNP g.2583C/T) in the FSH-β gene, which produced three genotypes (TT, CC, and CT) was detected. The frequency of each allele was 0.13 (allele C) and 0.87 (allele T). However, the FSH-β gene polymorphism did not significantly affect sperm quality, body weight, body size, and cervical condition of POBA cattle.

1. Introduction
Indonesian native and local cattle are known to have a superior adaptive capability to tropical conditions and a good reproductive capability even under poor-quality feed conditions. Indonesia has several local cattle breeds, which are valuable genetic resources that are needed to be conserved. Each breed may have unique genetic materials, which are valuable for designing crossbreeding to increase the productivity and population of local cattle in the country. Indonesian local beef cattle include Bali, Peranakan Ongole (PO), Madura, Aceh, and other local breeds of cattle [1]. PO x Bali (POBA) crossbred cattle have been firstly developed in Banyuwangi regency, but it is known as Rambon cattle by local livestock farmers. To date, there are two types of Rambon cattle, namely Rambon Rivet and Rambon Tes cattle. Both types of cattle have good productive and reproductive performance [2].

POBA is a crossbred cattle derived from PO (male) and Bali (female) cattle, which was developed by the Beef Cattle Research Institute (BCRI) in 2015. In 2018, POBA cattle (F1; 2 bulls and 10 females) were mated to produce offspring (F2). Natural mating was applied with a ratio of 1 : 5. The population of POBA cattle at the BCRI in 2019 was 24 heads. There was no pregnant cow based on a pregnancy test in the fourth month after being collected in the mating cage. Therefore, it is important to conduct a study in order to determine the reproductive performance of POBA cattle, both males and females.

It is known that most reproductive traits have low heritability. Genetically improving reproductive traits like twin birth through traditional selection is small due to long-term improvement and interval
generation [3]. Likewise, the reproductive performance of bulls can be evaluated using a progeny test, of which the parents of progeny with higher performance for desirable traits are selected for future breeding. However, this method only provides phenotypic information, such as fertility traits, which requires high costs and takes a long time for sperm analysis until it can be used as a selection tool. Furthermore, the phenotypic performance of superior cattle does not guarantee their reproductive superiority and thus, the use of molecular markers as a selection tool may be useful to increase the accuracy and efficiency of traditional selection, especially for reproductive traits like sperm fertility. Therefore, it is imperative to conduct research exploring genes controlling reproductive traits in POBA cattle, such as follicle stimulating hormone subunit beta (FSH-β) gene.

The FSH gene is located on chromosome 15 and consists of two heterodimers, namely alpha (FSH-α) and beta (FSH-β). FSH is a glycoprotein hormone secreted by the pituitary gland and has a function to control reproductive activity in mammals [4]. FSH can stimulate the growth of ovarian follicles, as well as initiates and improves the activity of Sertoli cells in the process of spermatogenesis [5].

Studies have been carried out to identify candidate genes for sperm quality traits. A polymorphism in exon 3 of the FSH-β gene resulting in three alleles (A, B, and C) significantly affects the volume, quality, and motility of both liquid and frozen semen in Limousin, Hereford, and Friesian Holstein cattle. A polymorphism is also detected in intron 2 and exon 3 of the FSH-β gene. This mutation significantly affects fertility and semen quality in Charolais, Simmental, and Limousin cattle [6]. Furthermore, a significant association between FSH gene polymorphism and superovulation has been identified in Chinese cattle [7]. A polymorphism in the FSH-β gene (two alleles detected; A and B alleles) is detected in Brahman, Friesian Holstein, Simmental, and Limousin cattle. However, the gene is found to be monomorphic in Bali cattle (A allele is absent) [8].

It is known that identification of candidate genes associated with economic traits is useful in designing breeding programs. Therefore, this study aimed to identify the polymorphism of FSH-β gene and its association with reproductive traits in POBA cattle.

2. Materials and methods
In total, 11 Rambon cattle (collected from Banyuwangi regency) and 24 POBA cattle (collected from BCRI) were used for blood sample collection. DNA analysis was carried out at the Laboratory of Molecular Genetics, BCRI and the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada.

2.1. Sample collection and DNA extraction
Blood samples (3 ml each) from 11 Rambon cattle and 24 POBA cattle were taken from the jugular vein using a vacutainer tube containing K3EDTA. The samples were transferred to the laboratory using an ice box and stored at 4°C before being analyzed. Genomic DNA was extracted from the blood samples using a gSYNCTM DNA extraction kit (Geneaid, New Taipei City, Taiwan) and stored at −20°C before analysis. The extracted DNA was run on 1.5% agarose gel electrophoresis and visualized under ultraviolet (UV) light.

2.2. Polymerase chain reaction (PCR) amplification
A specific DNA fragments of the FSH-β gene was amplified using primer forward: 5'-AGTATAAAAGATTTACCTGCTGTCGCT-3' [6,8] and primer reverse: 5'-TGTATTATTCTTATATTGCTTCTACTA-3'. The PCR reaction was performed using SensoQuest (Germany) and consisted of 2 µL of template DNA, 0.5 µL of each primer, 12.5 µL PCR kit diluent (2x My Taq HS Red Mix gSYNCTMPCR Kit-Bioline-London), and 9.5 µL ddH2O for a total volume of 25 µL. The thermal cycling included an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension step at 72°C for 10 min. The PCR products were checked on 1.5% agarose gel electrophoresis and visualized under ultraviolet (UV) light.
2.3. Sequencing analysis
Nucleotide sequences of the FSH-β gene were determined using the Sanger method. Sequencing analysis was carried out to determine point mutations in the targeted sequences of the FSH-β gene. All PCR product samples were sequenced. Each PCR product sample was used as a template for the sequencing reactions [9]. The sequencing results were analyzed to determine polymorphism of the FSH-β gene using Bioedit software.

2.4. Calculation of allele and genotype frequency
Allele frequency (Xi) is the relative frequency of an allele at a particular locus in a population. Allele frequency was calculated using the following formula based on Nei and Kumar [10]:

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

Genotype frequency is the number of individuals with a given genotype divided by the total number of individuals in the population [11]:

\[ X_{ii} = \frac{n_{ii}}{N} \]

Note:
- \( X_{ii} \) = the frequency of the ii genotype
- \( X_i \) = the frequency of the ith allele
- \( n_{ii} \) = the number of individuals with genotype ii
- \( n_{ij} \) = the number of individuals with genotype ij

2.5. Hardy-Weinberg equilibrium
Hardy-Weinberg equilibrium was analyzed using the \( x^2 \) (Chi-square) test based on Noor [12]:

\[ x^2 = \frac{\sum (O - E)^2}{E} \]

\( x^2 \) = Chi-square value
O = observed frequency
E = expected frequency

3. Result and discussion
3.1. FSH-β gene amplification
The 313 bp fragment of the FSH-β gene sequences were successfully amplified for all the samples from Rambon and POBA cattle (Figure 1). The results of the PCR amplification were in accordance with previous reports [6,8].

![Figure 1. Visualization of the amplified FSH-β gene (313 bp) on a 1.5% agarose electrophoresis](image-url)
The FSH-β gene in pigs, cattle, and humans consists of three exons [13]. FSH-β gene consists of a non-coding (untranslated) and two protein-coding (translated) exons and two introns. The original sequence of the gene is around 1547 bp and its complete sequence in Bos taurus is 6601 bp (GenBank Acc. No. M83753) [14].

3.2. FSH-β gene polymorphism

The polymorphism of the FSH-β gene in Rambon and POBA cattle was analyzed using the sequencing method. The sequencing results from all the samples were aligned with a reference sequence from GenBank Acc. No. NC_037342.1. The results showed polymorphism in the FSH-β gene (SNP g.2583C/T). The chromatogram of the FSH-β gene is shown in Figure 2.

![Chromatogram of the FSH-β gene (SNP g.2583C/T)](image)

Figure 2. Chromatogram of the FSH-β gene (SNP g.2583C/T)

The results of this study showed that the FSH-β gene in Rambon and POBA cattle was polymorphic. Three genotypes, namely TT, CC, and CT were observed. The genotype of each animal was determined and used to calculate allele and genotype frequency (Table 1).

There were two alleles (C and T) in Rambon and POBA cattle, which indicated an absence of selection in the both cattle populations. The Chi-square test for both cattle populations was calculated (Table 2). Only Rambon cattle were in Hardy-Weinberg equilibrium, while POBA cattle showed a deviation from HWE. This deviation may indicate a negative selection that can lead to inbreeding in the population. Therefore, new bulls are needed to be introduced and mated with productive females to avoid the adverse impact of inbreeding in POBA cattle. The heterozygosity value was also estimated, which indicated the level of crossbreeding in the population.

**Table 1.** Allele and genotype frequency for SNP g.2583C/T of the FSH-β gene in Rambon and POBA cattle

| Population | Genotype | Genotype frequency | Allele frequency |
|------------|----------|--------------------|------------------|
|            | CT       | TT                 | CC               |
| Rambon     | 4        | 6                  | 1                | 0.42 | 0.52 | 0.06 | 0.27 | 0.73 |
| POBA       | 2        | 20                 | 2                | 0.76 | 0.22 | 0.02 | 0.13 | 0.87 |
Table 2. Hardy-Weinberg equilibrium for SNP g.2583C/T of the FSH-β gene in Rambon and POBA cattle

| Population | Genotype | Allele frequency | X² value | HWE |
|------------|----------|------------------|----------|-----|
|            | CC       | TT               | CT       |     |
| Rambon     | Observed | 1                | 6        | 4   | 0.27 | 0.73 | 0.20 | 0.19 |
|            | Expected | 0.71             | 5.71     | 4.57|     |      |      |      |
| POBA       | Observed | 2                | 20       | 2   | 0.13 | 0.87 | 11.11| 6.90 |
|            | Expected | 0.32             | 5.36     | 18.32|     |      |      |      |

Note: X² < 0.05:2 = 5.99
If the X² value was less than X² table (not significant), it indicates that a population is in accordance with the HWE. In POBA cattle, the X² value > X² table indicates a deviation from the HWE.

Table 3. Observed (Ho) and expected (He) heterozygosity for SNP g.2583C/T of the FSH-β gene in Rambon and POBA cattle

| Population | N  | Ho  | He  |
|------------|----|-----|-----|
| Rambon     | 11 | 0.36| 0.4 |
| POBA       | 24 | 0.27| 0.23|

The genetic diversity of a population can be measured based on heterozygosity value. In this study, observed (Ho) and expected heterozygosity (He) were calculated based on the formula of Nei and Kumar [10]. Generally the heterozygosity value, especially He is a good indicator for analyzing the genetic diversity of a livestock population. The He is also an indicator regarding the level of crossbreeding in the research site [15].

3.3. Association between genotype and phenotype

The association between genotype and the quality of spermatozoa in POBA cattle is shown in Table 4.

Table 4. Association between genotype and sperm quality in POBA cattle

| Bull ID | Genotype | Sperm volume | Sperm pH | Motility | Mass movement | Concentration |
|---------|----------|--------------|----------|----------|---------------|---------------|
| PB16/14 | TT       | 5.00         | 6.90     | 0        | 0             | 0             |
| PB16/16 | TT       | 3.67         | 6.93     | 0        | 0             | 0             |
| PB16/20 | CC       | 4.67         | 7.00     | 0        | 0             | 0             |
| PB16/21 | TT       | 6.50         | 7.00     | 0        | 0             | 0             |
| PB16/32 | TT       | 4.75         | 6.90     | 0        | 0             | 0             |

Based on the genotyping results, there was a polymorphism in the FSH-β gene of POBA male cattle (Table 6). Two alleles (T and C) and two homozygous genotypes (TT and CC) were detected. The percentage of TT genotype (80%) was higher than that of CC genotype (20%). The Chi-square test for POBA cattle population showed a deviation from HWE. The genotyping results were associated with sperm quality of POBA cattle. As shown in Table 6, POBA male cattle had poor sperm quality, because the sperm was not found during the storage process. Therefore, there was no value for motility, mass movement, and concentration. The other sperm quality traits, including volume (4.92 ml) and pH (6.95) were observed. The poor sperm quality of POBA male cattle may be due to the low variation in the F1 offspring of POBA male cattle, which can decrease the level of follicle stimulating hormone (FSH) production, which further inhibits the process of spermatogenesis by testis. In addition, the sperm motility is also affected by the treatment after the sperm is ejaculated. A poor sperm treatment after the first ejaculation can reduce the percentage of sperm motility [16]. Furthermore, Dai et al. [6] reported that FSH-β gene polymorphisms also affect semen quality and fertility.
Although the genotyping results of the FSH-β gene in POBA male cattle was polymorphic, the diversity of the gene in this population was very low, as indicated by the absence of CT genotype. This indicated poor semen quality and fertility of POBA bulls. The poor sperm quality is also clarified from the results of the HWE calculation (6.90, as shown in Table 2), which indicated a deviation from HWE in POBA cattle population. This deviation may cause unbalanced selection, because the FSH-β gene has functions to balance selection. Kim et al. [17] reported that the FSH-β gene in mammals has structures and functions to balance selection. The association of FSH-β genotype with body weight and body size of POBA cattle is presented in Table 5.

**Table 5.** The results of genotype and phenotype (body weight, body size) of POBA cattle

| Cattle ID | Body weight (kg) | Body length (cm) | Height at withers (cm) | Back height (cm) | Chest girth (cm) | Cervix | Genotype |
|-----------|------------------|------------------|------------------------|------------------|------------------|-------|----------|
| PB16/01   | 416              | 148              | 132                    | 129              | 191              | 1     | TT       |
| PB16/07   | 428              | 149              | 135                    | 136.5            | 187              | 1     | TT       |
| PB16/08   | 404              | 140              | 134                    | 135              | 184              | 1     | TT       |
| PB16/10   | 290              | 131              | 117                    | 122              | 165              | 1     | TT       |
| PB16/23   | 398              | 129              | 132                    | 132              | 193              | 2     | TT       |
| PB16/29   | 360              | 129              | 128                    | 126              | 172              | 1     | TT       |
| PB16/35   | 356              | 128              | 129.5                  | 129              | 186              | 1     | TT       |
| PB16/02   | 504              | 157              | 139                    | 140              | 196              | 1     | CT       |
| PB16/04   | 538              | 150              | 137                    | 140.5            | 205              | 1     | TT       |
| PB16/05   | 374              | 145              | 131                    | 132.5            | 177              | 1     | TT       |
| PB16/06   | 406              | 141              | 130                    | 132              | 182              | 2     | CT       |
| PB16/12   | 384              | 137              | 128                    | 128              | 178              | 2     | TT       |
| PB16/17   | 296              | 118              | 127                    | 128              | 168              | 1     | TT       |
| PB16/30   | 372              | 122              | 133.5                  | 132.5            | 174              | 1     | TT       |
| PB16/36   | 262              | 137              | 126                    | 126              | 167              | 1     | CC       |

Note: Normal = 1; Abnormal = 2

The genotyping results of POBA female cattle (Table 5) showed that the FSH-β gene was polymorphic, as indicated by the presence of three genotypes (TT, CC, and CT). Furthermore, the association of the FSH-β genotype with body weight and body size in POBA female cattle is summarized in Table 6.

**Table 6.** Association of the FSH-β genotype with body weight and body size in POBA cattle

| Trait               | TT          | CT          | CC          | P value |
|---------------------|-------------|-------------|-------------|---------|
| Body weight         | 384.67±64.28| 455±69.30   | 262         | 0.089   |
| Body length         | 135.50±10.92| 149±11.31   | 137         | 0.307   |
| Weight at withers   | 130.33±5.19 | 134.5±6.36  | 126         | 0.414   |
| Back height         | 131±4.83    | 136±5.66    | 126         | 0.233   |
| Chest girth         | 181.67±11.54| 189±9.90    | 167         | 0.324   |

As shown in Table 6, there was no significant association between FSH-β genotype and body weight, body size, and cervical condition. However, POBA female cattle with CT genotype had relatively higher body weight and body size than those with TT and CC genotype. The cervical condition of POBA female cattle with CT genotype was normal (50%) and thus, cattle with CT genotype are more suitable to be selected than those with CC and TT genotypes when body weight and body size are used as selection criteria. However, a more comprehensive study is needed in the future, because the number of samples investigated in this study was quite small. The number of individuals with TT genotype was higher than
individuals with CT and CC genotypes. We suggest increasing the number of individuals with CC and CT genotypes in the future study to obtain more accurate results.

4. Conclusion
There was a polymorphism in the FSH-β gene in POBA cattle, as indicated by the presence of three genotypes (TT, CC, and CT). The frequency of each allele was 0.13 (allele C) and 0.87 (allele T). The FSH-β genotypes had no significant association with sperm quality, body weight, body size, and cervical condition of POBA cattle. The results of this study do not show accurate data, because the number of the investigated animals is quite small. The number of animals with TT genotype is higher than those with CT and CC genotypes. Therefore, we suggest increasing the number of individuals with CC and CT genotypes in the future study.

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