Allele and genotype variation of follicle stimulating hormone receptor gene of Indonesian Friesian Holstein in Boyolali District, Central Java

G Pambuko1, R Vanessa2, Purwadi3, N Widyas1, and S Prastowo1,*

1Animal Science Department, Agriculture Faculty, Sebelas Maret University, 57126, Indonesia  
2Graduate School of Bioscience, Faculty of Mathematics and Natural Sciences, Sebelas Maret University, 57126, Indonesia  
3Animal Husbandry Faculty, Boyolali University, 57314, Indonesia  
Corresponding author e-mail: prastowo@staff.uns.ac.id

Abstract. Follicle Stimulating Hormone Receptor (FSHR) is a gene that has function to regulate reproductive performance by controlling oogenesis in females and also spermatogenesis in male. Since dairy cattle operation lies on the reproductive trait efficiency, therefore it’s became a logical reason to use FSHR genes as part of selection criteria. This study aimed to determine allele and genotype variation of FSHR gene in local Indonesian Friesian Holstein (IFH) dairy cattle in Boyolali District, Central Java. In total 20 IFH cattle we sampled for DNA source. Allele and genotype variation was determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) using AluI restriction enzyme. The methods started with DNA extraction, PCR, and DNA digestion, followed by allele and genotype frequencies calculation. Two alleles were observed in this study, C and G with respective frequencies 0.75 and 0.25. According to that, we found three genotype combinations that are CC, CG, and GG, the frequencies were 0.56, 0.38, and 0.06, respectively. To use that result as a part of selection criteria in dairy cattle, especially in females, further association study with reproductive trait needs to be done.  
Keywords: allele and genotype variation, FSHR gene, Indonesia Friesian Holstein, PCR-RFLP

1. Introduction
Dairy milk consumption in Indonesia is increasing over time. The national demand for milk in 2019 was reached 4.3 million tons, while the domestic fresh milk production was only able to meet 22% of national needs. Such amount of milk production was produced from 561,061 head of dairy cow, with total milk production as amount of 996,442 tons [1]. This demand gap can be solved by increasing population and improve the productivity of dairy cattle [2]. One of area which has large population of dairy cattle in Indonesia is located in Boyolali District. This district represents the potential of dairy cattle farms business that have been practised since 1990 [3], and was dominated by smallholder farmer as the most characteristic of Indonesia animal production system.

Follicle-stimulating hormone (FSH) is a reproductive hormone which stimulate the growth and maturity of de Graaf follicle in ovarium and spermatogenesis in testis [4]. In females, granulose cells are responsible for FSH secretion. FSH is the glycoprotein hormone that needs receptor to affect the target cells [5] namely Follicle Stimulating Hormone Receptor (FSHR). It’s a G-Protein Coupled
Receptor (GPCR) that receive, regulate and transfer FSH to target cells and generate respond of the target cells [6]. Moreover, this receptor is coded by FSHR Gene. FSHR gene is a negative-strand (complement) gene located in Bos taurus Autosome (BTA) 11 and consists of 10 exons and 9 introns [7]. The bovine FSHR has gene ID 281172 and Ensembl version ENSBTAG00000032424.4 located at NC_037338.1 (3125549..31450537) in complement strand [8,9]. FSHR gene is reported to have association with reproductive traits [10,11]. Previous report shows the association of FSHR gene with superovulation response in Friesian Holstein, total ova, estrogen level, embryo viability, and the number of fertilized oocyte [12–15]. All these reports bring a background to use FSHR gene as molecular marker to improve reproductive efficiency trait in dairy cattle. To know its allele and genotype variation in the distinct dairy cattle population would be a potential effort for further selection and/or breeding programs.

For that this research aims to investigate the allele and genotype and in FSHR gene in IFH at Boyolali District, Central Java as local dairy industry.

2. Methods

2.1. Blood sample collection
Twenty blood sample of IFH cow were obtained from Boyolali District, Central Java by random selection. Blood was collected by venipuncture from coccygeal vein using 21GA×1” BD Vacutainer® Flashback Blood Collection Needle (Becton, Dickinson USA) attached to vacuum tube containing EDTA K3 (Onemed). Blood sample then homogenized and stored immediately at 4℃ temperatures, then transported to the laboratory for DNA Extraction.

2.2. DNA extraction
DNA Extraction was performed in Biomedical Laboratory, Medical Faculty, Universitas Sebelas Maret. The extraction was performed using Wizard® Genomic DNA Purification Kit (Promega, USA), and carried out using protocol supplied by the company.

2.3. FSHR gene fragment amplification and genotyping
Amplifications of FSHR gene fragment was done using PCR method and were performed in Animal Science Department, Faculty of Agriculture, Universitas Sebelas Maret, using pair of primers as listed in Table 1.

| Table 1. Primer and enzyme for PCR-RFLP in FSHR gene. |
|--------------------------------------------------------|
| Primers | Tm (℃) | Product Size | Restriction Enzyme | Reference |
| F  5’-CTGCCTCCCTCAAGGTGCCCTC-3’ | 65℃ | 306 bp | AluI | [7] |
| R  5’-AGTTCTTGGCTAAATGTCCTTGGGGG-3’ | | | |

Each PCR reaction was consisted of 10 µl Promega Green PCR Master Mix (Promega, USA), 7 µl nuclease-free water (Promega, USA), primer (Integrated DNA Technologies, Singapore) 1 µl in each forward and reverse, and 1 µl DNA template in Axygen® 0.2 mL thin-wall 8 strips (Corning, USA). The PCR was performed in SelectCycler™ II Thermal Cycler (SelectBioproduct, Taiwan) and set with initial denaturation step on 95℃ for 5 minutes followed by 33 cycles of denaturation step on 95℃ for 30 seconds, annealing step on 65℃ for 30 seconds, 72℃ extension steps on 72℃ for 30 seconds, and final extension on 72℃ for 10 minutes. A negative control in every performed PCR reaction was always set by replacing the DNA template with 1 µl nuclease-free water. Following amplification, PCR product then digested using Atrhobacter luteus I (AluI) restriction enzyme (recognition site 5’-AG(CT-3’)). In every digestion reaction, it was contained 5 µl of PCR product, 3 units AluI (Promega, USA), 1.3 µl nuclease-free water (Promega, USA), and 0.5 µl buffer B 10X (Promega, USA). The mixtures were then incubated in 37℃ for 120 minutes.
All the PCR and resulted digestion product were visualized using electrophoresis reaction using Mupid-ExU (Mupid, Japan) on agarose 2% stained with ethidium bromide at 100 V for 30 minutes. The DNA bands were visualized under UV-transilluminator (Avegene, Taiwan). DNA sequencing was also performed to confirm the PCR product sequence, by the Sanger Sequencing Method. In total 40 µl of PCR product for each sample was sequence. The sequence was done in PT. Genetika Science Jakarta, Indonesia.

2.4. Data analysis
Allele and genotype and frequency were analyzed according to previous study [16]. The formula of genotype and allele was described in formulas (1) and (2).

\[ x_i = \frac{2n_{ii} + \sum n_{ij}}{2N} \]  
(1)

Where: \( x_i \) is allele frequency i, \( n_{ii} \) is the sum of genotype ii individual, \( n_{ij} \) is the sum of heterozygote genotype ij, \( N \) is total individual.

\[ x_{ii} = \frac{n_{ii}}{N} \]  
(2)

Where: \( x_{ii} \) is genotype frequency ii, \( n_{ii} \) is the sum of homozygote genotype ii, \( N \) is total individual.

The distribution of genotype in population to determine Hardy-Weinberg Equilibrium (HWE) was analysed by chi-square test (\( \chi^2 \)) [16].

Following PCR product sequencing, the DNA sequencing was interpreted using Unipro Ugene v. 39 software [17]. Subsequently, the result was confirmed by BLASTN on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [18], followed by the SNP confirmation that was performed by using Variant Effect Predictor (VEP) in the Ensembl database website (https://www.ensembl.org/Tools/VEP) [8,19].

3. Result and discussion
The FSHR gene fragment was successfully amplified yielding 306 bp of product size (Error! Reference source not found.), and there was no amplified product in the negative control. The DNA sequence confirmed the PCR product was belongs to exon 10 Bos taurus FSHR gene as shown in Table 2. This result was in agreement with the previous studies [15,20–23]. The absence of PCR product in negative control reaction, shows no contaminations were present in the PCR mixture [24,25], therefore it can reduce the false positive as well as could be used to validate the success of the PCR reaction [26].

![Figure 1. FSHR gene PCR Result. Note: (L) Ladder 100bp, (1-5) PCR Product, (NC) Negative Control.](image-url)
Table 2. The similarity of FSHR sequence against reference sequence in NCBI database.

| Sample | Similarity | Accession Number | Gene ID |
|--------|------------|------------------|---------|
| #1     | 99.00%     | EU148061.1       | Bos Taurus Follicle Stimulating Hormone Receptor (FSHR) Gene, Exon 10 and Partial cds |
| #2     | 99.00%     | EU148061.1       | Bos Taurus Follicle Stimulating Hormone Receptor (FSHR) Gene, Exon 10 and Partial cds |
| #3     | 100.00%    | EU148061.1       | Bos Taurus Follicle Stimulating Hormone Receptor (FSHR) Gene, Exon 10 and Partial cds |

The digestion of the FSHR PCR product by \textit{AluI} obtaining 2 alleles (C, G) and 3 genotypes (CC, CG, GG) as shown in (Error! Reference source not found.). The PCR product had two \textit{AluI} recognition sites at base numbers 192–195 (A site) and base numbers 242–245 (B site). The A site had polymorphism C\textless{}G at base number 193 thus the \textit{AluI} was recognized partially on the site. Allele G had both of the sites otherwise, Allele C just had A site. CC genotype had two fragments (243 bp and 63 bp), GG genotype had three fragments (193 bp, 63 bp and 50 bp) and CG genotype had four fragments (243 bp, 193 bp, 63 bp and 50 bp). The 63 bp and 50 bp fragments were ignored since the 243 bp and 193 bp were clearly detected and could distinguish the genotypes.

![Figure 2](image_url)

The chromatogram map and alignment with the reference sequence (NM_174061) revealed the presence of polymorphism (Error! Reference source not found.). The polymorphism is located at BTA 11 nucleotide number 31255991 in the genomic sequence and c.1973C\textless{}G in coding sequence because of the substitution of C to G base. Both, allele C and G were found in this study. The CC genotype sequence had one peak of C nucleotide in the base call and the absence of \textit{AluI} recognition site in the sequence, otherwise the GG sequence had one peak of G nucleotide in the base call and \textit{AluI} recognition site was present. The CG genotype had double peak C and G in the base call because there were two different allele combinations in the sequence and \textit{AluI} recognized the sequence partially. The FSHR polymorphism has SNP ID rs209882669 and affected the FSHR protein at ENSBTAP00000048971.2:p.Thr658Ser by missense mutation by change the amino acid of threonine to serine. A similar result was reported in previous studies [14,15].
Figure 3. Map Chromatogram FSHR Gene with Reference and Consensus Sequence. Note: (S) C>G Nucleotide Substitution Site, (*) AluI Restriction Site.

Genotype and allele frequency in IFH cattle in this study were shown in Table 3. The highest genotype and allele frequency were found at CC genotype (0.56) and C allele (0.75), meanwhile GG genotype (0.06) and G allele (0.25) was the lowest genotype and allele frequency. The HWE test was not significant ($\chi^2=0.0044$ and P-Value 0.99), then it can be said that the IFH population in this study is in equilibrium.

| Cattle | n  | CC  | CG  | GG  | C   | G   | $\chi^2$ | P-Value |
|--------|----|-----|-----|-----|-----|-----|----------|---------|
| IFH    | 20 | 0.56| 0.38| 0.06| 0.75| 0.25| 0.0044   | 0.99    |

Note: HWE in nonequilibrium significant at P<0.05.

Previous research on genotype and allele frequencies in FSHR|AluI in several cattle breeds has been conducted (Table 4. Genotype and Allele FSHR|AluI variation in some cattle breedsTable 4). Genotype CC and allele C had the highest frequency observed in the AR, TG, AB, ZAV, TFH and BT populations that originated Bos taurus species. The highest frequency of the CG genotype was observed in the ZXC, ZXB population, which is the population of a crossbreed between Bos taurus and Bos indicus. In the MDR and BAL populations, only the GG genotype was observed, giving it the highest frequency. FSHR|AluI derived from the Bos taurus species, on the other hand, has the highest CC genotype and C alleles. Crosses between Bos taurus and Bos indicus had the highest CG genotype due to the combination of different alleles from their parents, this will affect the quantitative traits and it is the one of fundamentals for the selection program. The GG genotype and the G allele have the highest frequency in the MDR and BAL (Bos javanicus) populations because the nucleotide sequences in the FSHR gene in Bos javanicus are different from Bos taurus and Bos indicus, so there is no variation in FSHR|AluI, this makes the determination feature between Bos javanicus with other cattle breeds. The genotype and allele frequencies in the population used in this study compared with the population in previous studies (Table 4. Genotype and Allele FSHR|AluI variation in some cattle breedsTable 4) indicated that the genotype and allele frequencies were the same as the genotype and allele frequencies in Bos taurus, which had the highest CC genotype and C allele frequencies.
Table 4. Genotype and Allele FSHR|AluI variation in some cattle breeds.

| Cattle Breed                      | Allele | Genotype | Reference |
|-----------------------------------|--------|----------|-----------|
| East Anatolian Red Cattle (AR)    | 0.68   | 0.32     | 0.51 0.35 0.14 | [23] |
| Turkish Grey Cattle (TG)          | 0.67   | 0.33     | 0.53 0.30 0.18 |
| Anatolian Black Cattle (AB)       | 0.65   | 0.35     | 0.53 0.23 0.23 |
| Zavot Cattle (ZAV)                | 0.72   | 0.28     | 0.55 0.35 0.11 |
| Turkey Friesian Holstein (TFH)    | 0.77   | 0.23     | 0.61 0.31 0.08 |
| 50% Zebu x 50% Continental (ZXC)  | -      | -        | 0.19 0.67 0.14 |
| 50% Zebu x 50% British (ZXB)      | -      | -        | 0.13 0.79 0.08 |
| Madura Cattle (MDR)               | 0.00   | 1.00     | 0.00 0.00 1.00 |
| Bali Cattle (BAL)                 | 0.00   | 1.00     | 0.00 0.00 1.00 |
| Bos taurus*(BT)                   | 0.69   | 0.31     | 0.50 0.38 0.13 |

Note:
a Bos taurus sample from Ensembl Database
b the shown data is inverted data from the source, due to FSHR is negative-strand gene

The FSHR|AluI (c.1973C>G) belongs to candidate of SNP of genetic test analyses and associate to reproductive traits. It could be a candidate for the paternity tests and segregation analyses because of the high heterozygosity [21]. Previous study shows that GG genotype is associated with low yield of embryo and higher unfertilized oocyte [14]. Meanwhile CC genotype gives better ovulation rate than CG and GG genotypes [15]. These result shows the possibility to use FSHR|AluI as molecular marker in the aim to select animals which have better reproductive performance. The more efficient of reproductive performance, is the key of improving population as well as the productivity in dairy cattle industry.

In conclusion, the FSHR gene variation in this study shows 2 alleles and 3 genotypes. C allele and CC genotypes were the highest frequency observed. Due to the variation of FSHR allele and gene have association to reproductive traits, according to previous reports, therefore further association study using local IFH phenotype data population need to be done. In the future specific FSHR allele or genotype could be selected as the aim to improve the reproductive efficiency and milk production in dairy cattle.

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