Study association of bPRL (Bovine Prolactin) gene and milk production trait in Indonesian Holstein dairy cattle

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Abstract. This study was aimed to analyze the genetic variation of bPRL (Bovine Prolactin) gene and its association to milk production in Indonesian Friesian Holstein (IFH) dairy cattle. In total 49 DNA from whole blood were extracted and followed with PCR-RFLP to distinguish the genotype difference between individual samples. The fragment of bPRL gene was amplified using pair of primer forward 5’-GAGTCCTTATGAGCTTGATTCTT-3’ and reverse 5’-GCCTTCCAGAAGTCGTTTGTTTTC-3’, resulting in 156 bp PCR product size. DNA digestion then performed using RsaI restriction enzyme that recognizes 5’-GT↓AC-3’ sites with blunt-end termini. According to the study, two genotypes were discovered that are AA and AB, with respective frequencies of 0.84 and 0.16. Based on the genotype grouping of milk production data, AB genotype yielded 4016.63±1344 kg while AA genotype yielded 3314.32±1153 Kg of milk per lactation. However, there is no statistical (P>0.05) difference between both genotypes on the amount of milk yielded. It is concluded that bPRL gene was not associated with milk production traits in the studied Indonesian Dairy Cattle population.

1. Introduction
Friesian Holstein cattle are dairy cows that are widely bred in Indonesia and currently known as Indonesian Friesian Holstein (IFH). Their milk production capacity can reach to 17.7 kg per day [1]. However due to some factors its productivity need to be improved to account the national milk demand which still struggle to fill the gap between production and consumption [2,3].

One of the methods for increasing dairy cattle milk production is using genetic selection based on DNA markers also known as Marker-Assisted Selection (MAS). Bovine Prolactin gene or bPRL is one of genes that responsible for milk production and could be the candidate for genetic marker of milk production [4]. According to NCBI database, bPRL gene is located in Bos taurus autosome (BTA) 23 in NC_037350.1 (35332693-35341308, complement) with gene ID 280901. This gene consists of 5 exons and encodes 229 amino acids [5]. bPRL gene reported to regulates milk production by regulating the development of the mammary glands (mammogenesis), initiation of milk secretion (lactogenesis), and maintaining the stability of milk secretion (galactopoiesis) [6,7]. Earlier study shows that the
variation of bPRL|RsaI locus in exon 3 was found to be significantly associated with the amount of milk production and milk fat [8–10]. Variations in the genome could affect the gene function and change the gene products resulting in phenotypic variations [11]. For that, study aimed to determine the variation of bPRL gene and its association to milk production in IFH dairy cattle.

2. Materials and methods

2.1. Blood sample collection
Forty-nine samples of IFH lactated cow were collected from one of dairy cow breeding stations at Central Java. Blood was obtained from coccygeal vein by venipuncture using 21GA×1” BD Vacutainer® Flashback Blood Collection Needle (Becton, Dickinson USA) attached to vacuum tube containing EDTA K3 (Onemed). The blood sample was gently shaken to homogenize and immediately stored at 4°C temperatures before being transported to the laboratory for DNA Extraction.

2.2. DNA extraction
DNA Extraction was carried out using Wizard® Genomic DNA Purification Kit (Promega, USA) based on default protocol provided by the company. The DNA quality then tested in electrophoresis using agarose 1.5% stained with ethidium bromide and visualized by UV-transilluminator.

2.3. bPRL gene amplification and genotyping
The bPRL gene was amplified and genotyped using the Polymerase Chain Reaction-Restriction Restriction Fragment Length Polymorphism (PCR-RFLP) method. The experiment was carried out at the Laboratory of Animal Production, Animal Science Department, Faculty of Agriculture, Universitas Sebelas Maret, using pair of primer and enzyme as shown in Table 1.

| Primers (5’-3’) | Tm (℃) | Product Size (bp) | Restriction Enzyme | Reference |
|----------------|--------|------------------|-------------------|-----------|
| F GAGTCCTTATGAGCTTGATTCTT | 55     | 156              | RsaI              | [12]      |
| R GCCTTCCAGAAGTCGTGTTTC |        |                  |                   |           |

The amplification of bPRL gene fragment of target is in exon 3 and contained SNP namely rs211032652 which then referred as bPRL|RsaI (Figure 1). Each PCR reaction was consisted of 10 µl Promega Green PCR Master Mix (Promega, USA), 7 µl nuclease-free water (Promega, USA), 1 µl primer (Integrated DNA Technologies, Singapore) in each forward and reverse, and 1 µl DNA template. The PCR was performed in SelectCycler™ II Thermal Cycler (Select Bioproduct, Taiwan) and reaction is set at initial denaturation step on 95℃ for 5 minutes followed by 35 cycles of denaturation step on 95℃ for 30 seconds, annealing step on 55℃ for 30 seconds, 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 RsaI restriction enzyme (recognition site 5’-GT|AC-3’). In each reaction, it was contained 5 µl of PCR product, 3 units RsaI (Promega, USA), 1.3 µl nuclease-free water (Promega, USA), and 0.5 µl buffer C 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). To confirm the PCR product is the intended target, DNA sequencing was performed by Sanger Sequencing Method at PT Genetika Science, Jakarta.
Figure 1. Target Amplification and Digestion. (A) bPRL Gene map at exon 3-4 and amplification site for primers (A1); (B) Detail region for amplification bPRL Gene and SNP rs211032652 for genotyping, region for amplification target (B1), location of SNP rs211032652 and RsaI restriction Site sequence (B2).

2.4. Data analysis
The analytical data used in this study including milk production data, Sanger sequencing data analysis, genotype and allele frequency analysis, and Hardy-Weinberg Equilibrium analysis.

2.4.1. Data collection and milk production analysis. Milk production data was collected from individual milk production records and standardized to 305 2X Mature Equivalent (ME) at the first lactation recorded in kg/lactation.

2.4.2. Hardy-Weinberg equilibrium, allele and genotype frequency analysis. Allele and genotype frequency were analyzed based on previous study from Nei and Kumar [13]. The formula of genotype and allele were described in formulas (1) and (2).

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

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.

The distribution of genotype in population to determine Hardy-Weinberg Equilibrium (HWE) was analyzed by chi-square test (\( \chi^2 \)) [13].

2.4.3. Association of bPRL|RsaI with milk production analysis. The association of milk production and genotype variation was done by comparing between genotype groups with t-test using R-Studio software at \( \alpha = 0.05 \) [14].

2.4.4. Similarity and sequencing analysis. DNA sequencing was interpreted using Unipro Ugene v. 39 software [15]. Subsequently, the result was confirmed by BLASTN on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [16].
3. Result and Discussion

The bPRL gene fragment was successfully amplified, yielding 156 bp of product size (Figure 2), with no bands in the negative control. This result was similar with the previous studies [12,17]. The absence of bands in the negative control reaction indicates that no contaminations were present in the PCR mixture [18], therefore it can diminish the false positive as well as could be used to validate the success of the PCR reaction [19].

![Figure 2](image_url)

**Figure 2.** bPRL Gene Amplification Product. (L) Ladder 100bp; (1-10) Amplicon of gene with 156 bp; (NC) Negative control

Two DNA sample was sequenced to confirm the PCR product amplify the target sequence interest of bPRL|RsaI. The BLAST Analysis shown the sequence of the samples had strong similarity (Table 2) with Bos taurus PRL gene with GenBank accession number AH013356.2 *Bos taurus* chromosome 23 prolactin (prolactin) gene, partial cds. The result was similar with previous study that bPRL gene is located in BTA 23 chromosome [20].

**Table 2.** Similarity Against Reference Sequence in NCBI Database

| Sample | Similarity | Accession Number | Description |
|--------|------------|------------------|-------------|
| 1      | 98.00%     | AH013356.2       | *Bos taurus* chromosome 23 prolactin (prolactin) gene, partial cds |
| 2      | 97.00%     | AH013356.2       | *Bos taurus* chromosome 23 prolactin (prolactin) gene, partial cds |

The digestion bPRL|RsaI found 2 genotype, namely AA and AB genotype (Figure 3). The AA genotype had one band with band size 156 bp. It was similar with PCR product because there was no RsaI site in the sequence thus the sequence did not digest by RsaI. The AB genotype had 156 bp, 74 bp and 86 bp band size, because the RsaI recognized partially at allele B only. The 74 bp and 86 bp bands could not separate clearly in AB genotype because the 2% agarose unable to resolve band size under 100 bp.

![Figure 3](image_url)

**Figure 3.** bPRL|RsaI Digestion Product. (L) Ladder 100 bp; (AA) AA Genotype with single band at 156 bp; (BB) AB Genotype with double band at 156 bp and 74/82 bp

The chromatogram of AA and AB genotype sample revealed the presence of polymorphism (Figure 4). The AA genotype sequence had one-peak on highlight G base call and there was no RsaI recognition
site at this genotype. Otherwise at AB genotype found the double-peak G and A on highlight base call and Rsal could recognize the restriction site partially on B allele only. The polymorphism located at BTA 23 at nucleotide 35333764 in the genomic sequence and c.396G>A in coding sequence because of the substitution G to A base. The bPRL/Rsal polymorphism had variant ID rs211032652. That variant did not affect the translation product because the variant had same amino acid, it was synonymous variant on valine at amino acid number 132 (ENSBTAP00000020313.3:p.Val132=) [21].

![Figure 4. Chromatogram AA and AB Genotype. (AA) the AA Genotype with single-peak at G base in highlight and Rsal restriction site was absence; (AB) the AB genotype with double-peak at G base in highlight, the double peak of G and A base were present and make the Rsal enzyme digest partially.](image)

The AA genotype frequency (0.84) was higher than AB genotype, and the A allele (0.91) was higher than B allele (Table 3). The HWE analysis revealed that the IFH population in this study was equilibrium with P-value of 0.9319 (P>0.05). The BB genotype was not found in this study. The result of this study were similar with previous study in which the AA genotype frequency was higher than AB genotype; and the B allele frequency was lower than A allele [22–24]. The different result was reported in Jersey cow population, the highest genotype and allele frequency were AB genotype and B allele, respectively [23]. It could be due to the different breed that was used.

| Cattle | n  | Genotype | Allele | HWE Analysis |
|--------|----|----------|--------|--------------|
|        |    | AA       | AB     | A            | B            | χ²          | P-Value     |
| IFH    | 49 | 0.84     | 0.16   | 0.91         | 0.09         | 0.0073      | 0.9319      |

**Table 3. Genotype and Allele Frequencies and HWE analysis in bPRL|Rsal IFH Cattle**

| Trait          | Genotype | P-Value |
|----------------|----------|---------|
| Milk production| AA       | 3314.32 ± 1153 |
|                | AB       | 4016.63 ± 1344 |

**Table 4. Association Milk production Trait with Genotype bPRL|Rsal**

Table 4 shows the t-test result for the association of milk production trait with genotype bPRL|Rsal. The AB genotype had higher milk production than AA genotype with 4016.63±1344 kg/lactation. The association analysis by t-test revealed there was no association between milk production trait and
genotype bPRL|RsaI (P>0.05). The unassociated result between milk production trait and genotype in bPRL|RsaI has been reported in the Friesian Holstein [12,25]. Different results were also reported in Red Pied, Jersey and Chinese Hostein populations, revealing an association between milk production and genotype bPRL|RsaI [10,23,26].

4. Conclusion
This study concluded that the IFH cattle in the studied population had two genotypes and alleles, AA and AB genotypes, as well as A and B alleles. The population was in HWE equilibrium state. The AB genotype had higher milk production than AA genotype, but there was no association between milk production trait and genotype in bPRL|RsaI.

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