Variation of prolactin and β-Lactoglobulin genes in the Indonesian FH Cattle

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Abstract. Prolactin is a polypeptide hormone, encoded by the prolactin (PRL) gene, synthesized and secreted by anterior pituitary, and affecting milk yield and composition. β-Lactoglobulin (BLG) is the major whey protein in the milk of ruminants. This study was conducted to identify the PRL and LGB genes polymorphism in the Indonesian FH cattle. A total of 139 individual cattle blood samples from West Java were used to obtain DNA samples through the DNA extraction process. Identification of the PRL and LGB genes was performed using PCR-RFLP method with RsaI (PRL gene) and HaeIII (BLG gene) restriction enzymes. The PRL gene was amplified using forward primer 5'-ccaaatccactgaattatgctt-3' and reverse primer 5'-acagaaatcacctctctcattca-3'. The BLG gene was amplified using forward primer 5'-tgctggacaccgactacaaaaag-3' and reverse primer 5'-gctcccggtatatgaccaccctct-3'. The PRL gene was amplified using forward primer 5'-acagaatccacctctctcactca-3'. The BLG gene was amplified using forward primer 5'-tgtgctggacaccgactacaaaaag-3' and reverse primer 5'-getcccggtatatgaccaccctct-3'. The PRL and BLG genes in the Indonesia FH cattle were polymorphic based on the PCR-RFLP analysis but the heterozygosity value was low. There were two alleles (G and A) and three genotypes (GG, GA, and AA) identified in the PRL gene of the Indonesian FH cattle with genotype frequencies were 0.914, 0.079, and 0.007 for GG, GA, and AA genotypes respectively. There were two genotypes (CC and CG) identified in the BLG gene with genotype frequencies were 0.91 (CC), and 0.09 (CG). Information about the PRL and BLG genes polymorphism in this study can be considered for further study to analyse its association with milk yield trait.

Keywords: PRL, BLG, polymorphism, FH, Indonesia

1. Introduction
The Friesian Holstein (FH) cattle breed is the most famous dairy cattle breed. According to the database of the Ministry of Agriculture of the Republic of Indonesia [1], 550,000 FH cattle were scattered in Indonesia in 2018, mainly on the island of Java, especially West Java, Central Java and East Java Provinces. The production of milk and protein yield were substantial characteristics in the dairy industry. Based on various studies, milk production of the FH cattle in Indonesia still low [2, 3, 4] and affected...
the amount of imported milk in Indonesia. A selection program that assisted by molecular selection based on specific genes that highly associated with economic characteristics can provide the population of FH cattle which has a good quality in milk production [5].

Prolactin (PRL) is one of a polypeptide hormone that encoded by the PRL gene and secreted by the anterior pituitary gland. This gene has five exons and four introns that encode 199 amino acids [6]. PRL plays a role in mammmogenesis, lactogenesis, maintenance of milk secretion, and galactopoiesis which affecting milk yield and composition [7]. Prolactin is also involved in a variety of biological functions related to immune function, tegument growth, synergy with steroids, osmotic regulation, and reproduction [8]. Nowadays, molecular genetic technology has been able to promote effective selection and breeding strategies through application of certain DNA markers that were associated with various economic traits. Based on several reports, polymorphism of the PRL gene has a significant impact on milk production and also fat content [9, 10, 11, 12]. Therefore, the PRL gene can be considered as a powerful DNA marker candidate to increase dairy cattle productivity [13].

Polymorphism information about targeted genetic marker candidates was very important to design a strategy of cattle breeding program. Polymorphism of the PRL gene in the Indonesian FH cattle has been reported in Wulandari et al. [14] but limited in variation of the PRL gene exon 3. Investigation on other exons still needed to fulfill information about the PRL gene. This study was conducted to identify variation of the PRL gene exon 4 and β-Lactoglobulin (BLG) exon 4 gene in the Indonesian FH cattle using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.

2. Materials and methods

2.1. Blood samples and DNA extraction
A total of 139 blood samples of FH cattle were used in this study. The blood samples of FH cattle were collected from West Java Province, Indonesia. About 3-5 mL of blood were taken from coccygeal vein and collected in anticoagulant-added tubes. The Genomic DNA Mini kit (Geneaid Biotech Ltd., Taiwan) were used in this study to obtain DNA samples from whole blood samples.

2.2. Amplification DNA
The amplification of PRL and BLG genes were conducted in a Mastercycler® gradient (Eppendorf, Hamburg, Germany). The forward primer 5’-ccaatcctgaattatgctt-3’ and reverse primer 5’-acagaaatcacctctctcattca-3’ were used to amplify the PRL gene exon 4 that recommended by Lu et al. [15]. The primer that used to amplify the BLG gene exon 4 was recommended by Doosti et al. [16] i.e. forward primer 5’-ctgtgtgtacaccaagacagaaag-3’ and reverse primer 5’-ccctccgtatatagccacct-3’. The amplified size of the DNA fragment were 294 bp (PRL gene) and 247 bp (BLG gene). The PCR reagents composed of: 1 μL DNA samples (5-50 ng/μL), 6.25 μL Taq polymerase mixture (KAPA2G Fast Ready Mix PCR Kit 1X), 0.5 μL (0.2 μM) forward and reverse primers, and H2O up to 12.5 μL final volume. The optimum condition for PCR programme was as follows: initial denaturation at 94°C (5 minutes); followed by 35 cycles of 94°C (30 seconds), 56°C (PRL gene) and 55°C (BLG gene) for 45 seconds, 72°C (45 seconds); and terminated by a final 72°C (5 minutes). The gel electrophoresis were conducted in 1% agarose gel followed by SyBr® staining and visualized on G:Box UV transilluminator.

2.3. Data analysis
The RFLP method using Rsal and HaeIII restriction enzymes (Biolabs Inc., New England) were used to identify the PRL and BLG genes polymorphism in the Indonesian FH respectively. The restriction site of Rsal and HaeIII were 5’...GT//AC...3’ and 5’...GG//CC...3’ respectively (temperature and incubation time following the producer’s guidelines). The genotypes information were determined based on the gel electrophoresis result (visualized of the DNA fragment). The equations (based on Nei and Kumar [17]) to analyse the genotype frequency (equation 1 and 2), allele frequency (equation 3), observed heterozygosity (equation 4), and expected heterozygosity (equation 5) were as follows:
\[\chi_{ii} = \frac{n_{ii}}{N}\]
\[\chi_{ij} = \frac{n_{ij}}{N}\]
\[\chi_{i} = \frac{2n_{ii} + \sum n_{ij}}{2N}\]
\[H_{o} = \frac{n_{ij}}{N}\]
\[H_{e} = 1 - \sum p_{i}^{2}\]

where: \(\chi_{ii}\) = frequency of \(AiAi\) genotype (homozygote); \(\chi_{ij}\) = frequency of \(AiAj\) genotype (heterozygote); \(\chi_{i}\) = frequency of \(i\)th allele; \(n_{ii}\) = number of individuals with \(AiAi\) genotype; \(n_{ij}\) = number of individuals with \(AiAj\) genotype; \(H_{o}\) = observed heterozygosity; \(H_{e}\) = expected heterozygosity; \(N\) = number of samples. The equation based on Botstein et al. [18] was used to analyse the polymorphism information content (PIC): PIC = 1 - \(\sum_{i=1}^{n} p_{i}^{2}\) - \(\sum_{j=1}^{n-1} \sum_{i=j+1}^{n} 2p_{i}^{2}p_{j}^{2}\) where: \(p_{i}\) = allele frequency of \(Ai\); \(p_{j}\) = allele frequency of \(Aj\).

3. Results and discussion

The PRL and BLG genes were successfully amplified using the PCR method. The visualized DNA fragment of PRL gene indicated the same size as reported in [15] i.e. 294 base pair (bp). In addition, the visualized DNA fragment of BLG genes also indicated the same size as reported in [16] i.e. 247 bp. Based on the result of PCR-RFLP analysis, the PRL/RsaI gene in the Indonesian FH cattle were polymorphic with three genotypes (GG, GA, and AA). In addition, the BLG/HaeIII gene was also polymorphic with two genotypes (CC and CG). The differences in size and the numbers of bands (RFLP product) that appear in the visualization process were used to identify the genotype of PRL gene (Figure 1) and BLG gene (Figure 2).

**Figure 1.** Visualization of Gel Electrophoresis of the PRL Gene genotypes (M=100 bp Ladder Size Standard; 1-25=samples; GG Genotype=samples 1-12, 15, 17-21, 23-24; GA Genotype=samples 13-14, 16, 18, 25; AA Genotype=sample 22).

**Figure 2.** Visualization of Gel Electrophoresis of the BLG Gene Genotypes (M=25 bp Ladder Size Standard; 1-9=samples; CC Genotype=samples 1-7, and 9; CG Genotype=sample 8).
The allele and genotype frequencies in PRL and BLG genes were presented in Table 1. In PRL gene, the highest genotype frequency in the Indonesian FH cattle was GG (0.87) and followed by AG (0.11) and AA (0.02). In BLG gene, the genotype frequency of CC (0.91) was higher than CG (0.09). In general, the $H_e$ value of the PRL gene was lower than the $H_e$ value (0.11<0.13), indicating that the population of the Indonesian FH cattle in this study was not in Hardy-Weinberg equilibrium and the genetic diversity is quite low. The $H_e$ value of BLG gene was similar to $H_e$ value and indicating the medium value (moderate) of genetic diversity in the Indonesian FH cattle BLG gene. In addition, diversity level of the PRL and BLG genes in this study was also low (based on the PIC value was 0.12).

The selection or assortative mating [19] and few numbers of sires in the farms [20] might be several reasons that caused the low level of diversity of the PRL and BLG gene in this study. Moreover, the low of AA genotypes and A allele frequencies in PRL gene due to low of GG genotype and G allele frequencies in BLG gene can be an indication that farmer intervention through selection process in the Indonesian FH cattle population was carried out intensively and causes the random mating failure.

The A allele in the PRL/RsaI gene (exon 4) of FH cattle of Indonesia as the common allele and similar to Palestinian Holstein (0.71), Bali (0.95) and Vietnamese Holstein (0.82) cattle [6, 21, 22]. However, previous studies reported that G allele in the PRL/RsaI gene as the dominance allele in Jersey (0.89), Iranian FH (0.93), Chinese Holstein (0.89), Deoni (0.54), Fleckvieh (0.88), Sahiwal (0.81), Achai (0.56) and East Anatolian Red (0.76) cattle [9, 12, 13, 15, 23, 24]. Moreover, the GG genotype was associated with higher fat content in Jersey cattle [9]. Boleckova et al. [25] reported a higher fat and protein contents in Fleckvieh cattle that was associated with GG genotype. In contrast, Das et al. [23] obtained that no association between genotype in the PRL/RsaI gene to milk production in Deoni cattle.

| Gene | Genotype (Frequency) | Allele (Frequency) | $H_e$ | $H_o$ | PIC |
|------|----------------------|--------------------|------|------|-----|
| PRL  | AA (0.02)            | A (0.07)           | 0.11 | 0.13 | 0.12|
|      | AG (0.11)            | G (0.93)           |      |      |     |
|      | GG (0.87)            |                    |      |      |     |
| BLG  | CC(0.91)             | C (0.95)           | 0.10 | 0.10 | 0.09|
|      | CG (0.09)            | G (0.05)           |      |      |     |
|      | GG (0.00)            |                    |      |      |     |

$H_e$= expected heterozygosity; $H_o$=observed heterozygosity; PIC= polymorphism information content.

The genotypes of BLG gene in this study were not similar with Doosti et al. [16] or Nury and Anggraeni [26]. We found the CC genotype (Figure 2), which are characterized by only one band showed (247 bp). This genotype has never been reported before, therefore this genotype might be a candidate for a specific genotype in Indonesian FH cattle population. However, further investigation need to be conducted to ensure that the cut sites for the restriction enzyme (HaeIII) were not exist in the cattle with CC genotype.

Several previous studies suggest that the polymorphism of BLG gene was associated with quality of milk (higher fat content, increased cheese production and higher protein production). Based on genotypes that reported in Doosti et al. [16], the AA genotype has the highest average milk production compared to the BB and AB genotypes in Hissar cattle [27]. The BB genotype has a rich in fat and protein, very valuable in the process of cheese making, while the animals with AA genotype produces milk with a low percentage of fat [28]. Karimi et al.[29] reported that the milk yield and protein percentage were higher in AB genotype, but this difference was not significant. In addition, other studies suggest that the AA genotype of β-Lactoglobulin gene was closely related to higher milk and protein production, more yield of lactoglobulin, low level of casein and fat than the BB genotype [28, 30, 31, 32].

Identifying genes with important effects on characteristics incorporated with selection with molecular markers, such as Prolactin and β-Lactoglobulin, were considered as one of the most important
tools in animal improvement research [8]. In addition, there are two compulsory requirements to optimize the use of genetic markers in dairy cattle industry, i.e polymorphism information and highly associated with dairy cattle productivity traits (milk yield, protein content, fat content, etc). Validation process of the genetic marker candidates is the crucial step to apply the marker assisted selection (MAS) programme in Indonesia. Therefore, polymorphism information of the PRL and BLG genes in this study can be useful to conduct further investigation regarding to its association with productivity traits.

4. Conclusion
It can be concluded that the PRL and BLG genes in the Indonesian FH cattle was polymorphic condition but the heterozygosity value was low. Information about the PRL and BLG genes polymorphism in this study can be considered to conduct further study to analyse the genotypes association with productivity traits in the Indonesian FH cattle.

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