Identification of restriction enzyme in the FSHR gene of Indonesian local cattle

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Abstract. The restriction enzyme is important for genotyping using the PCR-RFLP technique. Therefore, this study aims to identify the restriction enzyme mapping in the partial sequence of the follicle-stimulating hormone receptor (FSHR) gene in Indonesian local cattle. A total of 29 samples sized 306 bp, were aligned with Genbank sequence acc no. NC_032660, resulting three polymorphic sites, namely g.193G>C, g.227T>C, and g.275A>C. Furthermore, the restriction mapping analysis using the NEBcutter program V2.0 showed that no enzyme recognized the SNP g.275A>C, while the SNP g.193G>C and g.227T>C were identified by the AluI and MscI enzymes, respectively. The AluI enzyme cuts at two positions (193 bp and 243 bp) in the G allele sample producing three fragments namely 50 bp, 63 bp, and 193 bp, meanwhile, in the C allele, the AluI cuts only in position 243 bp, hence, the fragment products are 63 bp and 243 bp. In contrast, the MscI enzyme was only recognized in the T allele, producing fragments sized 77 bp and 229 bp but failed to identify the restriction site along with the PCR products in the C allele. Based on the results, the SNPs (g.193G>C and g.227T>C) and restriction enzymes (AluI and MscI) are applicable for genotyping local Indonesian cattle using the PCR-RFLP technique in future studies.

Keywords: FSHR gene, restriction enzyme, Indonesian local cattle

1. Introduction

DNA analysis and several types of identification assays, particularly PCR-based methods have been developed [1]. The combination of PCR and restriction fragment length polymorphism (RFLP) assays has certain advantages such as increasing simplicity, specificity, and sensitivity for genotyping animals compared to other PCR methods [2]. This method is based on enzymes' ability to cleave DNA by recognizing specific sequences and structures. When SNPs are located in an enzyme recognition site and affect its activity, differences between alleles are easily identified [3]. However, since cleavage inhibition due to methylation occurs in plasmids and genomic DNA, the possibility of digested PCR fragments needs to be considered [4].

FSH is a glycoprotein hormone needed for gonadal development and maturation during puberty, as well as gamete production in mammals. The FSHR gene is located in chromosome 11 and consists of...
10 exons and 11 introns, the first 9 exons enclose the extracellular domain while exon 10 enclose the transmembrane domain [5]. Furthermore, the follicle-stimulating hormone receptor (FSHR) gene plays a major role in normal reproductive function and oocyte maturation through its growth-promoting and steroidogenic effects in cattle [6]. The entire coding sequence for the FSHR gene in cattle has been cloned, and several SNPs have been discovered. Moreover, previous studies on the polymorphism of the FSHR gene using the PCR-RFLP method have been reported in dairy cattle in China [6], indigenous Sudanese cattle [7], Holstein cattle [8], Brown Swiss, Nelore, and Indo-Brazilian [5], and Bali cattle [9]. However, the identification of SNPs within the FSHR gene have not been studied in several Indonesian cattle breeds, especially Sragen (Figure 1) and Jabres (Figure 2) cattle. Therefore, this study aims to determine the polymorphic sites and the possible restriction enzyme in the PCR-RFLP method for genotyping the Indonesian cattle based on the FSHR gene.

2. Materials and methods

2.1. Sample collection and DNA amplification

A total of 29 samples consisting of Sragen (n = 10) and Jabres (n = 19) cattle were collected from Sragen and Brebes Regency in Central Java Province. Blood samples were taken from the jugular vein up to three milliliter using venoject and EDTA vacutainer tube. The samples were then transported to the laboratory for DNA extraction, using gSYNC™ Kit (Geneaid, New Taipei City, Taiwan). Furthermore, amplification of the FSHR gene was conducted in a 25 µl volume containing 2 µl genomic DNA, 0.5 µl of forward and reverse primers [5] (Table 1), 9.5 µl ddH₂O, and 12.5 µl of MyTaq HS Red Mix (Bioline, UK). Each amplicon was sequenced using an automated DNA sequencer (Applied Biosystems) at the Universitas Gadjah Mada Central Laboratory (LPPT-UGM).

| Primer sequence (5’ - … - 3’) | Target size | Annealing temperature |
|-------------------------------|-------------|-----------------------|
| F : CTGCTCCCTCAAGGTGCCTCTC   | 306 bp      | 60 °C                 |
| R : AGTTCTTGTCAAATGTCTTAGGGG   |             |                       |
2.2. Sequence analysis
To detect the SNP(s) present, all samples of FSHR sequences were aligned using BioEdit software version 7.0. SNP is defined as a different nucleotide that appears in a sequence alignment. Manual detection of the electropherogram was used to confirm the mutation within the samples.

2.3. Restriction enzyme analysis
For each group, SNP was subjected to restriction enzyme analysis using the NebCutter program. The appearance of a red line under the targeted SNP within the sequence indicates the presence of specific restriction enzymes. Furthermore, restriction sites identified by NebCutter were used to generate RFLP profiles in Microsoft Excel using a simple formula for each marker provided by Castro et al. [10], while gel agarose for RFLP method was simulated using DNASTAR software.

3. Result and discussion
3.1. SNP identification
Polymorphisms of bovine FSHR gene were detected by PCR (Figure 3) and DNA sequencing methods. The electroforeogram showed a clear band for PCR product (306 bp), meanwhile, the sample sequences were aligned with bovine FSHR gene reported in GenBank with acc no. NC_032660. Therefore, three mutations were identified within the samples, namely SNP g.193G>C (AAAAGCTC to AAAACCTC), g.227T>C (GAATGGC to GAACGGC), and g.275A>C (CACCCCTG to CACACTT). All three SNPs were located in intron 1 of the FSHR gene (based on Genbank acc no. NC_032660). The CC genotype in SNP g.193G>C and g.227T>C was absent in the samples (Figure 4 and 5). However, for SNP g.2775A>C, all three genotypes showed a clear peak in electropherogram (Figure 6). Although the SNPs found were non-coding sequences, the majority still affect the phenotype, since the non-coding region RNAs (intron) control transcriptional and post-transcriptional gene expression [11]. Yang et al. [6] also reported two SNPs in the non-coding region (5’UTR) namely SNP -320 A>T (GTCGAGT to GTCGAGT) and -278 G>A (AGGGACA to AGGAACA). Furthermore, the GG genotype in SNP -278 G>A consists of a significantly high number of degenerate embryos (NDE), number of transferable embryos (NTE), and total number of ova (TNO) in Chinese Holstein cattle. Meanwhile, in the Chinese Holstein bulls, the mutation A-234500T of the FSHR gene was reported to be significantly associated with semen volume per ejaculate (VOL) and sperm concentration (SCON) [12]. The FSHR AluI site (g.193G>C) is found in European Type Brown Swiss, Nelore, Indo-Brazilian, and Bos Taurus x Bos indicus crossbred cattle in Mexico [5], Holstein cattle in Turkie [13], and Bali cattle [9].

Figure 3. A 306 bp PCR product of FSHR gene.
3.2. Restriction enzyme analysis

According to the European standards in molecular microbiology [14] and detection of genetically modified organisms [15], it is important to verify the PCR products generated. Reliable methods used to verify PCR products include restriction analysis with at least two restriction endonucleases (REases), as well as probe hybridization, or DNA sequencing [16]. The restriction mapping analysis is used to analyze the possible restriction enzyme in the PCR-RFLP method based on SNP g.193G>C, g.227T>C, and g.275A>C.

Therefore, restriction mapping using the NEBcutter program V2.0 discovered no enzyme which recognized the SNP g.275A>C. The SNP g.193G>C and g.227T>C were identified by the AluI and MscI enzymes, respectively. The AluI enzyme cuts at two positions (193 bp and 243 bp) in the G allele sample thereby producing three fragments namely 50 bp, 63 bp, and 193 bp. However, in the C allele, the AluI cuts only in position 243 bp, hence, the fragment products are 63 bp and 243 bp. Meanwhile, the MscI enzyme was only recognized in the T allele, producing fragments sized 77 bp and 229 bp but failed to find the restriction site along with the PCR products in the C allele. Furthermore, the gel simulation for the RFLP method using AluI and MscI restriction enzyme is shown in Figure 7. The simulation of agarose gel provides images that are comparable to conventional gel electrophoresis, specifically for DNA fragments that are smaller than 50 bp [10], while the AluI enzyme in G allele produce small fragment which is close to each other and appear as one single band. Taheri et al. [14] stated that the selection of restriction enzyme for genome analysis needs to consider the following 1)
fragment size produced after digestion, 2) the presence and frequency of enzyme recognition sites within the target DNA sequence, and 3) methylation sensitivity of enzymes. The AluI endonucleases were used to differentiate the genotype of the FSHR gene in Madrasin [15], Angus, Friesian Holstein (FH), Limousin, Simmental and Brahman [16], and Indigenous Sudanese [7] cattle.

**Figure 7.** Agarose gel simulation for AluI and MscI restriction enzymes (MW = 100 bp molecular weight).

4. Conclusion
Based on the results, three polymorphic SNPs (g.193G>C, g.227T>C, and g.275A>C) were found in the Sragen and Jabres cattle sequence. The restriction mapping analysis showed that one endonuclease was recognized in SNP g.193G>C (AluI) and g.227T>C (MscI), but no enzyme was found in SNP g.275A>C. Furthermore, AluI and MscI enzymes produced specific RFLP patterns to differentiate each genotype. Therefore, it was concluded that the SNPs (g.193G>C and g.227T>C) and restriction enzymes (AluI and MscI) are applicable for genotyping Indonesian local cattle using the PCR-RFLP technique in future studies.

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