SNPs detection in 5’-UTR region of the MC4R gene in Garut sheep

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Abstract. The melanocortin-4 receptor (MC4R) gene has been linked to controlling feeding behavior and body weight. The objective of this study was to detect the polymorphism within the 5’-UTR region of the MC4R gene in Garut sheep. A total of 36 blood samples were isolated and amplified using primers (forward: 5’-TTCGTTTGGGGCAAGTCAAG-3’ and reverse: 5’-GGAAACGCTCACCAACATGT-3’). Two SNPs, g.396C/T and g.399C/G, were discovered in the 5’UTR region based on sequence alignment (position number based on Genbank acc no. NC_040274). For both SNPs, only two genotypes were found in the samples. Both SNPs had identical allele and genotype frequencies. The C allele (86%, n=26) was higher than the T (g.396C/T) and G allele (g.399C/G) (14%, n=10). The homozygous CC genotype has a higher frequency (72%) in both SNP g.396C/T and g.399C/G, followed by CT and CG genotype (28%), respectively. The Hardy-Weinberg equilibrium analysis resulted in the sample population did not deviate ($\chi^2<5.59$). Further analysis could be suggested to provide an overview of this polymorphism effect in Garut sheep's growth traits.

Keywords: MC4R gene, single nucleotide polymorphism, Garut sheep

1. Introduction

Molecular genetics approaches using DNA markers are more beneficial strategies for more rapid genetic improvements in animal breeding programs [1]. DNA markers from genes that encode growth performance showed to be more accurate and effective as selection tools. For developing a selection marker, the exploration of single nucleotide polymorphisms (SNPs) is needed. SNPs combined with simple mutation models and analytical techniques could provide an excellent genome-wide scan of selectively neutral and adaptive variation [2].

Melanocortin-4 Receptor (MC4R) is a G trans-membrane receptor protein. Homeostatic capacity, feed intake, body weight, and lipid metabolism are all regulated by the MC4R gene [3,4]. The sheep’s MC4R gene is located on chromosome 23 based on the Genbank accession number NC_040274.1. This gene-sized 3869 bp consists of two exons [5]. In sheep, a mutation within the MC4R gene was revealed in Hu sheep [3,5,6], German Merino sheep [7], Chinese Merino sheep [8], and Tibetan sheep [9]. In Indonesia, especially in West Java, there is a local meat-type sheep called Garut sheep. The Garut sheep is well-known for having high litter size [10] and good body conformation [11]. The study of the MC4R gene in Garut sheep has never been studied before. Hence, the study aimed to analyze polymorphism within the MC4R gene's 5-UTR region in Garut sheep.
2. Materials and methods

2.1. Sample collection and DNA extraction
Thirty-six Garut sheep were used in this study. The animals were kept with standard management in PT. Agro Investama Malangbong, West Java. The blood samples were taken through the jugular vein of approximately 3 milliliters using an EDTA vacutainer. Samples were then carried to the laboratory at 4 °C for DNA isolation using gSYNC DNA Extraction Kit (Geneaid, New Taipei City, Taiwan).

2.2. DNA amplification and sequencing
DNA amplification using a pair of self-designed primers (F: 5’-TTCGTTTGGGGCAAGTCAAG-3’ and R: 5’-GGAAACGCTCACCAACATGT-3’) were done in Thermo Cycler to get the targeted PCR product. A 25 μL PCR reaction was formulated from 2 μL of genomic DNA, 12.5 μL of MyTaq HS Red Mix (Bioline, UK), 0.5 μL of each primer (forward and reverse) and 9.5 μL of double-distilled water (DDW). The amplification program was started from pre-denaturation 95 °C for 5 min, pursued by 35 cycles consisting of denaturation 94 °C for 30 s, annealing 59 °C for 30 s, extension 72 °C for 30 s, and then terminated with final extension 72 °C for 10 minutes. The 377 bp PCR product was visualized in 2% standard agarose gel. The PCR products were sent to Universitas Gadjah Mada Central Laboratory (LPPT-UGM) to genotype the SNP g.396C/T (reported by Wang et al. [12]) and g.399C/G (new SNP detected in this study) using one-direction (forward) sequencing. The sequencing results were then used for alignment in BioEdit program ver. 7.00 (Tom Hall, Ibis Therapeutics, California, USA).

2.3. Statistical analysis
Analysis of genetic diversity, including allele and genotype frequency, heterozygosity (Ho and He), and χ² test for Hardy-Weinberg’s equilibrium in Garut sheep population were analyzed based on genotyping results using the POPGENE Ver program. 32 (Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Canada) based on the method of Maharani et al. [1].

3. Result and discussion

3.1. Polymorphism of MC4R gene
A 377 bp nucleotide sequence of partial MC4R gene was successfully amplified from Garut sheep. The amplified products were examined in agarose gel electrophoresis, and the results were matched with the predicted size and had a high precision (Figure 1). The PCR product covered partial 5'-UTR sequence, exon 1, and partial intron sequence (nucleotide 299 – 675 based on GenBank acc no. NC_040274.1). Based on the sequence alignment, two SNPs were identified in this study, namely SNP g.396C/T and SNP g.399C/G. Those SNPs were located in a non-coding sequence (5'UTR region). Manual inspection of the electropherogram showed that the SNP g.396C/T and g.399C/G confirmed polymorphic in Garut sheep (Figure 2 and 3). The homozygous TT and GG were absent in the SNP g.396C/T and g.399C/G, respectively. The SNP g.396C/T was previously disclosed in Hu sheep by Wang et al. [12]. Leppke et al. [13] stated that the nucleotide sequence at position 5’UTR is actually part of an exon but does not contribute directly to the formation of a protein sequence because this part is only transcribed but not translated in the ribosome.

Several previous research results showed that the polymorphisms of the MC4R gene were found in the non-coding regions such as 3’UTR, intron, and 5’UTR. Shishay et al. [5] studied the promoter and 5’UTR regions of the MC4R gene and found that variation within those regions contributes to the transcription of the MC4R gene. Furthermore, the promoter region of the MC4R gene contains one start transcription site (TSS), and the minimal functional promoter region is close to the TATA consensus. The mutations that occur in the promoter region show a positive association with several economic traits in livestock. This proves that variations in the promoter region that change the action of cis-elements can affect the phenotype of an individual [14].
3.2. Genotyping of MC4R gene polymorphism

Each sample was genotyped using the direct sequencing method based on the SNPs g.396C/T and g.399C/G. The allele frequencies, genotype frequencies, and chi-square analysis were calculated using PopGen 32 software for each SNP. As a result, in Garut sheep, the allele and genotypes frequencies for each SNP were identical (Table 1). The C allele (86%, n=26) was found more frequently than the T (g.396C/T) and G (g.399C/G) alleles (14%, n=10). In both SNP g.396C/T and g.399C/G, the homozygous CC genotype has the highest frequency (72%) followed by CT (28%) and CG genotypes (28%), respectively. Shishay et al. [5] reported the genotype frequencies of SNP in the same 5′ UTR region (SNP g.-103C/T) were 0.59, 0.33, and 0.08 for CC, CT, and TT genotypes, respectively. According to the $\chi^2$ test, the sample population was in agreement with Hardy-Weinberg’s equilibrium ($\chi^2<5.59$). In an enormous population, a polymorphic site is considered as a SNP if it has less than 99 percent allele frequency or less than 95 percent in the small population [15]. Moreover, the genetic diversity based on SNPs within-population will be constant between generations as long as there is no mutation, migration, selection, and controlled mating.
Table 1. Allele and genotype frequencies, observed and expected heterozygosity, and chi-square analysis of SNP g.396C/T and g.399C/G in Garut sheep.

| Locus  | Genotype frequencies | Allele frequencies | Obs Het | Exp Het | $\chi^2$ |
|--------|----------------------|--------------------|---------|---------|---------|
| g.396C/T | CC  | TT  | CT   | C       | T       |         |
|        | 0.72 | -   | 0.28 | 0.86    | 0.14    | 0.28    | 0.24    | 0.83    |
| g.399C/G | CC  | GG  | CG   | C       | G       |         |
|        | 0.72 | -   | 0.28 | 0.86    | 0.14    | 0.28    | 0.24    | 0.83    |

4. Conclusion
Two-point mutations, SNPs g.396C/T and g.399C/G of MC4R gene were found in Garut sheep using the direct sequencing and alignment method. One genotype was absent in SNP g.396C/T (TT genotype) and g.399C/G (GG genotype). The CC genotype and C allele were the most frequent genotype and allele in both SNP. Based on chi-square analysis for all SNPs, the Garut sheep have not deviated from HWE. Further investigation is suggested to provide an overview of this polymorphism's effect on growth traits in Garut sheep.

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