Genotyping and Chi-Square Analysis of 967 bp Leptin Gene in Bligon Goat

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Abstract. Leptin gene has contribution to affect the economical function in livestock such as growth rate and reproduction. The aim of the study was to identify the genetic marker related to growth and reproduction base on single nucleotide polymorphism in Leptin gene as a basic data for genotyping in Bligon goat. Thirty blood sample of Bligon goat have been extracted for DNA analysis. The target sequence of Leptin gene was amplified by polymerase chain reaction method. Four single nucleotide polymorphisms were detected at position g.758G/A, g.864C/T, g.1171G/A, dan g.1454G/A. Three out of four SNP can be used for genotyping. Two genotypes at those loci were found in this study. Genotype CC at locus g.864C/T have greater amount than genotype CT. The same result was also for genotype GG at both locus g.1171G/A and g.1454G/A have higher number than the genotype AG. Frequency of allele C (0.97) and allele G (0.97) at Bligon goat population was higher than allele T (0.03) and allele A (0.03). The chi-square analysis shows that the population of Bligon goat still in genetics equilibrium with the value X²=0,849. This study provided the basic information for genotyping of local goat in Indonesia based on Leptin gene. The association of the genotype in Leptin gene with growth and reproduction trait can be the next agenda research in Bligon goat.

1. Introduction

Leptin gene in Capra hircus is located on chromosome 4 and composed by 3 exons. Based on GenBank accession no. NC_030811.1, Leptin gene in Capra hircus was consist of 16370 bp sequence[1]. Whole leptin gene sequence of 4.8 kb of indigenous goat in India has been identified with a total 22 variation [2]. The mutation in Exon 2 of Leptin gene in seven breed of Indian goat have been reported. The single nucleotide polymorphism (SNP) at g. 1029C/T in Exon 2 change the amino acid Valine to alanine. Six out of seven SNPs were located at intronic region (intron 1 and intron 2). Other study identified Six novel SNPs (g.117T > C, g.1642G > A, g.2883G > A, g.3053T > C, g.3190G > A, and g.3314T > C) in five Chinese goat. All of the six SNPs of the Leptin gene were associated with growth traits [3]. GG genotype at g.1642G > A and g.3190G > A loci showed higher birth weight and two months of age. Bakhtiar et al. [4] reported two SNPs g.170G>A and g.332G>A in exon 3 and the relationships with the characteristics of sperm quality and testicular dimensions.

The study on Leptin gene of local goat in Indonesia was very limited. In our knowledge, this is the first study on Bligon goat. Bligon goat was obtained from the crossing between Kacang goat (the native
goat of Indonesia) and Ettawah grade goat. The Ettawah grade goat was the upgrading between Kacang goat and Jamnapari goat imported from India. The phenotypic of Ettawah grade goat was close to the original breed from India, and then the phenotypic of Bligon goat was in between of those in Kacang goat and Ettawah grade goat. The previous molecular study on Bligon goat was identification of SNPs in MC4R gen which addressed for two targets sequence [5,6]. At first study with the target sequence at nucleotide number 183 to 569 based on Genbank Acc. No MN_001285591, there were no SNPs within Bligon goat, but we found two novel polymorphisms (SNP g286G>C and SNP g303C>T) compare to the genbank [6]. At the second study was found the two SNPs in MC4R gene of Bligon goat. Two SNPs of MC4R gene in Bligon were g.998A>G and g.1079C>T which located in nucleotide number 924 to 1562. Based on Chi-square analysis, both SNPs g.998A/G and g.1079C/T were in Hardy-Weinberg Equilibrium [5]. There were no study on sequence and chi-square analysis of Leptin gene in Bligon goat. Therefore, the aim of this study was to perform genotyping and chi-square analysis of Leptin gene in Bligon goat of Indonesia. The basic information of marker molecular identification will be useful for studying the relationship of the genotype with the economical traits.

2. Materials and methods

2.1. Data collection and isolation DNA
Genomic DNAs were obtained from blood DNA extraction of 30 samples. A total number 30 local goat (Bligon) for the research materials were reared in Banyusoco village, Gunungkidul-Indonesia. The blood samples were collected from jugular vein into K<sub>2</sub>EDTA tubes. Genomic DNA was isolated using SYNCTM DNA Extraction Kit (Geneaid, Taiwan).

2.2. Primer design and DNA Amplification
The target sequence of Leptin gene was identified followed the instruction which described by Hartatik[7]. Primers were designed using oligoprimer primer3 (free online at http://primer3.ut.ee/), and Capra hircus/AM114397.2 as reference of sequence. A pair of primer: forward (5’-AGCGGTATGGGATATGC-3’) and reverse (5’-AATGCCCAAGAGACACTGA-3’) was used in this study. Polymerase Chain Reaction (PCR) amplifications were carried out for 30 cycles in a 30 µl total volume mixture of reaction mixture containing 1.5 µl genomic DNA (approximately 100 ng), 1.5 µl (10 µM) forward primer, 1.5 µl (10 µM) reverse primer, 15 µl PCR KIT (KAPA BIOSYSTEMS) and 10.5 µl aquabidest.

2.3. Sequencing and SNPs detection
The PCR products were sequenced using sanger dideoxy sequencing method. The sequence products were then aligned using Bioedit (version 7.2.5). A total 30 sample sequences were compare to the sequence from GenBank Acc. No. AM114397.2 as template. The SNPs were recognized when the single letter does not match with the template sequence. The result of electrophoregram at the certain position of SNPs were checked to clarify the individual genotype. The similarity of Leptin gene sequence in in Bligon goat was compared to the other goat by the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Genbank data acc.No. AM114397.2 (Italian goat); NC_030811.1 (San Clemente goat, Amarica), GU944974.2 (Indian goat); JQ739232.1 (Indian goat) and JQ739233.1 (Indian goat) were used for comparison with Leptin gene sequence of Bligon goat. Haplotype combinations for the three SNPs were constructed the Haploview 4.2 application[8].

2.4. Genotyping and allele distribution
The genotype of each individual sample can be identified from electrophoregram. All information was collected with BioEdit program tools. Allele frequencies were calculated from all the SNPs in this study. Genotype and allele frequency of all samples were tested to Hardy-Weinberg equilibrium[9]. The following model analysis was:
\[ X^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i} \]

Where: \( X^2 \) = chi-square value, \( O_i \) = observed frequency, \( E_i \) = expected frequency and \( n \) = number of possible outcomes of each event.

3. Result and discussion

3.1. Detection of SNP MC4R gene in Bligon goat

Sequence product size 967 bp of Leptin gene from 30 Bligon goats was preceded for multiple sequence alignment using Bioedit program with parameter 1000 bootstraps. Identification of SNP position and the possible genotype was described in Fig.1. Four SNPs of Leptin gene were identified SNP g.758G>A, SNP g.864C>T, SNP g.1171G>A, and SNP g.1454G>A. The numbering of SNP was related to the GenBank acc. No. AM114397.2. Four SNPs in this study compared to the references from GenBank acc. No. AM114397.2 and NC_030811.1, those SNP's were located at intron 1 dan intron 2. There are four genotype variations of Bligon goats in this study (CGG=BG1, YGG=BG2, CRG=BG3, CGR=BG4). The representative of the individual variation was presented at Figure 1B. These variations have been submitted into GenBank Acc. No. MN 635653 to MN 635656 (ID2278133). Four variation of Bligon goat (AC, GT, RY, AT) was detected in the present study [5]. This variations occur related to SNP g.998A>G and g.1079C>T of MC4R gene in Bligon goat. The partial sequences of MC4R gene of Bligon goat have been submitted into GenBank Acc. No. MN 635657 to MN 635660 (ID2278163). This data will provide the contribution for the new information about genetic diversity of local goat in Indonesia.

Compare to the GU 944974.2 (Indian goat), in this study was found two extra SNPs with substitution (SNP g.655T>C) and insertion/deletion (SNP g.1489T/). The mutations were located at upstream and downstream of the previous SNPs detected in this study (Figure 1). Based on BLAST analysis, Bligon Leptin gene showed homology of 99.90% with AM114397.2 (Italian goat, exotic breed), and 99.87% with GU944974.2 (Indian goat, breed Sirohi); JQ739232.1 (Indian goat, breed Baradi) and JQ739233.1 (Indian goat, Breed Beetal). Haplotype combinations for the three SNPs (SNP g.864C>T, SNP g.1171G>A, and SNP g.1454G>A) were constructed the Haploview 4.2 (Figure 2). Linkage disequilibrium (LD) for Bligon goat in this study presented the low value of LD since the figure show gray colour. The haplotype of the individual heterozygote sample (YGG, CRG, CGR) was only 0.017% compare to the common haplotype (CGG). The result from haploview of common haplotype was 0.800, and the other haplotype TGG, CAG and CGA was 0.067. This data determine the diversity of Bligon goat, however the heterozygote individual in the population was very rare.
Figure 1. Identification of four SNPs of Leptin gene in Bligon goat. (A) Electrophoregram and (B) Multiple alignment sequence

Figure 2. Haplotype analysis using haploview program

3.2. Genotyping and chi-square analysis
Four novel mutations were detected in the Bligon goat Leptin gene g.758G>A, g.864C>T, g.1171G>A, and g.1454G>A. The mutation was identified at intron 1 and intron 2. The mutation in exon 2 does not exist in this study. Three out of four mutations can be used for genotyping. From genotyping based on three loci in all sample was detected four variations of Bligon goats (CGG=BG1/GenBank Acc. No. MN635653, YGG=BG2/ GenBank Acc. No. MN635654, CRG=BG3/ GenBank Acc. No. MN635655, CGR=BG4/ GenBank Acc. No. MN635656). The heterozygote goat was clarified by checking the electrophoregram which shows the double peak (Y=C/T; R=G/A). Only out of 30 samples were heterozygotes (7%). Homozygote goat was dominant at all SNP loci. The frequency of homozygote goat
was 93% in this observation (g.864C/C, g.1171G/G and g.1454G/G). The genotype frequency and allele frequency of the three polymorphisms of the Leptin gene are showed in Table 1. The results of Pearson’s chi-square ($\chi^2$) test in all SNPs (SNP g.864C>T, SNP g.1171G>A, and SNP g.1454G>A) were 0.849 (Table 2). Chi-square ($\chi^2$) analysis showed that all of the three SNPs of Leptin gene were in Hardy–Weinberg equilibrium (HWE) in this study ($P<0.05$).

### Table 1. Genotype and allele frequency of Leptin gene in Bligon goat of Indonesia

| No | SNP     | Genotype | N | Genotype frequency | Allele frequency |
|----|---------|----------|---|--------------------|------------------|
| 1  | g.864C>T| CC (C)   | 28| 0.93               | C=0.97           |
|    |         | CT (Y)   | 0 | 0.07               | T=0.03           |
| 2  | g.1171G>A| GG (G)  | 28| 0.93               | G=0.97           |
|    |         | GA (R)   | 0 | 0.07               | A=0.03           |
| 3  | g.1454G>A| GG (G)  | 28| 0.93               | G=0.97           |
|    |         | GA (R)   | 0 | 0.07               | A=0.03           |

N= number of data

### Table 2. Chi square test of Leptin gene in Bligon goat of Indonesia

| No | SNP     | Genotype | Sum of total | X² |
|----|---------|----------|--------------|----|
|    |         | Observed | Expected     | D2/e |
| 1  | g.864C>T| CC (C)   | 28.23 0.23   | 0.002 0.849 |
|    |         | CT (Y)   | 1.16 0.84   | 0.82 |
|    |         | TT (T)   | 0 0.027 0.027 | 0.027 0.027 |
| 2  | g.1171G>A| GG (G)  | 28.23 0.23   | 0.002 0.849 |
|    |         | AG (R)   | 1.16 0.84   | 0.82 |
|    |         | AA (A)   | 0 0.027 0.027 | 0.027 0.027 |
| 3  | g.1454G>A| GG (G)  | 28.23 0.23   | 0.002 0.849 |
|    |         | AG (R)   | 1.16 0.84   | 0.82 |
|    |         | AA (A)   | 0 0.027 0.027 | 0.027 0.027 |

X² in the table Chi square test 0.05;2 = 5.99

4. Conclusion

Sequence analysis in this study revealed four SNPs in intron 1 and intron 2 (SNP g.758G>A, SNP g.864C>T, SNP g.1171G>A, and SNP g.1454G>A). Genotyping of Bligon goat in this study were shown by three SNPs (g.864C>T, SNP g.1170G>A and SNP g.1454G>A). The genotypes of Bligon goat in this study were CGG, YGG, CRG, and CGR. Heterozygote genotype was very limited number. The population in this study was in Hardy–Weinberg equilibrium (HWE) based on three SNPs. This finding provided the basic information for genotyping of local goat in Indonesia based on Leptin gene. A larger sample from heterozygote genotype of Leptin gene and number of local breed other than Bligon goat was needed for further work on association of the genotype with growth and reproduction trait.

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