

Polymorphism of Lactoferrin Gene and Its Association with Milk Production and Milk Composition of Senduro Dairy Goat

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Abstract— This study aimed to identify the association of lactoferrin gene polymorphisms in Senduro goats with milk production and milk composition. A total of 42 lactating Senduro goats were taken milk samples, blood samples, and daily milk production data. Milk protein analysis was carried out using the Lactoscan Milk Analyzer, while the genotyping of the lactoferrin gene was carried out using the PCR-sequencing method. The analysis showed that there was a polymorphism of the lactoferrin gene in Senduro goat, namely SNP G4179A, which was classified as low polymorphic. Lactoferrin gene polymorphisms did not associate with milk production and milk composition.

Keywords— Lactoferrin gene, Indigenous goat, PCR-sequencing, Milk yield.

I. INTRODUCTION

Senduro goat is one of the native and indigenous goat breeds that developed in Indonesia and is designated as a wealth of Indonesian local Animal Genetic Resources (SDGT) based on the Decree of the Indonesian Minister of Agriculture No. 1055/Kpts/SR.120/10/2014. Senduro dairy goat is a dual-purpose goat, as meat-producing goat and milk-producing goat (dairy goat) (Susilorini and Kuswati, 2019). So far, in the area of origin, namely Senduro Subdistrict, Lumajang Regency, East Java Province, Indonesia, Senduro goat is more used as a dairy goat which can produce milk production of 0.8 to 1.8 liters/head/day (Badan Standarisasi Nasional, 2018). To obtain optimal milk production in Senduro goats, steps to improve genetic quality can be done through livestock selection. Currently, advances in technology are rapidly advancing, enabling selection efforts to be carried out at the DNA level, namely by analyzing milk quality control genes. Due to advances in DNA technologies, it can be used to reliably accurate and intense selection, short generation intervals, and to enable genetic improvement from locally adapted breeds (Aiello, Patel, and Lasagna, 2018). One of these genes is the lactoferrin (LF) gene. The lactoferrin gene acts as a lactoferrin coding gene (Asmarasari, Sumantri, Gunawan, Taufik, and Anggraeni, 2019). Lactoferrin is a multifunctional protein found in milk and colostrum. Besides, it is also found in neutrophils, kidneys, epithelial cells, and mucosal fluid (González-Chávez, Arévalo-Gallegos, and Rascón-Cruz, 2019; O'Halloran, Bahar, Buckley, O'Sullivan, Sweeney, and Giblin, 2010). Polymorphisms of the goat lactoferrin gene have been found in the exon, intron, and UTR areas (Kaminski, Oleński, and Brym, 2006). Meanwhile, the

association of the lactoferrin gene polymorphisms with milk production has been found in dairy goats in China and Iraq (Guo, Jiao, He, Wei, Chang, Yue, Lan, Chen, and Lei, 2010; Ali and Al-Samai, 2018; Hofmannova, Rychtarova, Sztankoova, Kyselova, Milerski, and Vostry, 2018). However, there has been no previous research regarding the polymorphism of the lactoferrin gene and its relation to milk quality in Senduro goats. Therefore this study aimed to identify single nucleotide polymorphisms (SNP) of lactoferrin gene in Senduro goats, interactions between SNPs, and its association with milk production.

II. RESEARCH MATERIALS AND METHODS

A. Sampling and Data Collection

Blood and milk samples were obtained from lactating Senduro goat (n = 42). Selected Senduro goats are based on special qualitative requirements (Badan Standarisasi Nasional, 2018). All the animals were reared at Burno Village and Kandang Tepus Village, Senduro District, Lumajang Regency, East Java. Blood samples were collected from the jugular vein of the goat using a venoject needle connected to a 10 ml GP vacuum tube EDTA. The blood samples that have been collected were put into an icebox that already contains blue ice and stored in the refrigerator (freezer) in a frozen state at 4° C. While 100 ml of milk samples were collected then put into 100 ml milk bottles and stored in an icebox for ± 4 hours and then transferred to the freezer to be frozen. Furthermore, data collection on milk production was carried out when the farmer carries out the milking process, i.e. in the morning from 05:30 to 08:00. Milking is done once a day. Milk production was measured using a Green Leaf measuring cup 1000 ml and 500 ml with an accuracy of 10 ml.

B. Milk Composition Analysis

Milk composition analysis was carried out using Lactoscan Milk Analyzer serial 7035 made by Milkotronic Ltd., Bulgaria. The milk sample was homogenized first by stirring the milk using a stirring spoon. Next, put the milk sample into the backer glass as much as 25 ml. Insert a tube containing milk at the end of the needle which is part of a lactoscan tool. Pressing the OK button on the tool and the sample will be sucked into the tool indicating that the analysis is in progress. Furthermore, after the analysis process, the data will come out on the lactoscan screen in the form of fat, density, lactose (lactose), solid non fat (snf), solids, protein, and added water.

C. DNA Isolation and Amplification

DNA isolation from blood samples was carried out using the phenol-chloroform extraction method (Sambrook, Fritsch, and Maniatis, 1989) which was modified using the Genomic DNA Mini Kit for Blood Extraction Kit (Geneaid). Based on the goat lactoferrin gene sequence (Access No GenBank FJ609300) a pair of primers were designed, namely forward primer (5'-CCAAATGCTACCAATGGCAG-3') and reverse primer (5'-GATTCTTTACCGTCTGAGCC-3') amplifying the exon 2 of the lactoferrin gene. The volume of the DNA sample used was 1 µl. The total volume of the amplification reagent was 30 µl, consisting of 12 µl of go-taq green master mix, 0.3 µl of forward and reverse primers respectively, and 17.4 µl of nuclease-free water (NFW). Pre-denatured PCR amplification conditions for 5 minutes at 95° C, 35 denaturation cycles for 10 seconds at 95° C, annealing for 20 seconds at 58° C, extension for 5 minutes at 5 minutes, and final extension 10 minutes at 12° C. Furthermore, the electrophoresis of PCR products used a centrifuge (HETTICH Mikro 185) with 1.5% agarose gel (Promega) and followed by nucleic acid dye staining which would be captured in the GBOX documentation system (Syngene, Cambridge, UK).

D. Sequencing and Genotyping

The sequencing of PCR products was carried out at the 1st Base sequencing company, Selangor, Malaysia, with the PCR products first distributed into 22 µl of 96 well plates which were then closed using 8 cap strips and 200 µl of primers into Eppendorf tubes. Sequencing was carried out using a sequencer machine (ABI Prims 3100-Avant Genetic Analyzer) with the Sanger method. The sequencing results were analyzed using the BioEdit program [12] to observe the genotypes of the PCR sequencing products of the LF gene in Senduro goats. Furthermore, to determine whether there was a gene mutation in the LF gene fragment sequence in Senduro goats, an analysis was performed using the Molecular Evolutionary Genetic Analysis 5 (MEGA5) program.

E. Data Analysis

Based on the number of genotypes of the exon 2 lactoferrin gene that have been analyzed, statistical analysis was carried out based on previous research (Guo, Jiao, He, Wei, Chang, Yue, Lan, Chen, and Lei, 2010), including genotype frequency, allele frequency, observational heterozygosity, expectation heterozygosity, degree of polymorphism, and Hardy-Weinberg equilibrium (HW equilibrium). The association between SNPs of lactoferrin gene with milk production and milk composition (protein, fat, lactose, snf, and solid) were analyzed using the General Linear Model (GLM) model in the ANOVA program of the Windows IBM SPSS Version 26 (IBM Corporation, New York, USA). The model used is as follows.

$$Y_{ij} = \mu + t_i + \beta_{ij} + \epsilon_{ijk}$$

Note:

Y_{ij} = milk production and milk composition of each genotype

μ = population mean

t_i = the influence of the i-th genotype

$\beta_{(ij)}$ = constant

ϵ_{ijk} = error effect

III. RESULTS AND DISCUSSION

Amplification of Lactoferrin Gene

Amplification of the lactoferrin gene used a Thermal Cycler machine under annealing conditions of 60 ° C for 20 seconds and continued with electrophoresis using 1.5% agarose gel to obtain a PCR product with a length of 366 base pairs (bp). PCR conditions include a pre-denaturation process of 95 ° C for 3 minutes which functions to prepare before the denaturation process followed by a denaturation process with a temperature of 95 ° C for 10 seconds which aims to change the structure of double-stranded DNA into single-stranded DNA. Lactoferrin gene amplification was successful at 100% (46/46) for all samples studied. The results of 366 bp of the lactoferrin gene electrophoresis on 1.5% agarose gel are presented in Figure 1.

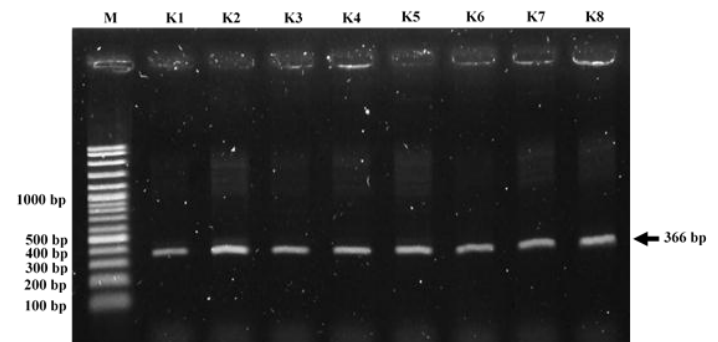


Figure 1. Electrophoresis results of Senduro goat lactoferrin gene using PCR (Note: M is 100 bp DNA marker, K1 - K9 = Senduro goats DNA samples, bp is base pair).

An important aspect of the success of PCR is the annealing because there is the attachment process of primer occurs in the DNA template. The annealing temperature of the primer ranges from 36° C to 72° C, but temperatures commonly used are 50 to 60° C (Muladno, 2010). The annealing temperature used in this study was different from the previous research which used a temperature of 56° C for 60 seconds, while another previous research used 46° C to 62° C (Ali and Al-Samai, 2018; Cosenza, Pauciullo, Illario, Gallo, Di-Berardino, Nicodemo, and Ramunno, 2005). However, this temperature difference did not affect the amplification results. The conditions for temperature differences and the length of annealing time were used because the mix composition used for gene amplification was different. The factors that led to the successful amplification of the LF gene included the primary attachment of genomic DNA (target genes), PCR reagents, and the condition of the PCR (thermal cycler) machine.

Genotyping of Lactoferrin Gene

Genotyping of Senduro goat lactoferrin gene used the sequencing method. The sequencing results were analyzed with the BioEdit program to observe the genotypes that emerged and continued with the application of Molecular Evolutionary Genetic Analysis 5 (MEGA5) to identify the presence or absence of gene mutations in Senduro goat lactoferrin gene. Based on the partial alignment of the results

of Senduro goat lactoferrin gene sequencing in the population with *C. hircus* (No Access GenBank FJ609300), the results are presented in Figure 2. Determination of position 1 starts from the start sequence of the ATG codon in exon 1. Based on the alignment results found one novel Single Nucleotide Polymorphism (SNP), namely G4179A. The G> A mutation, a transition mutation, is located at position 4179 of Senduro goat lactoferrin gene.

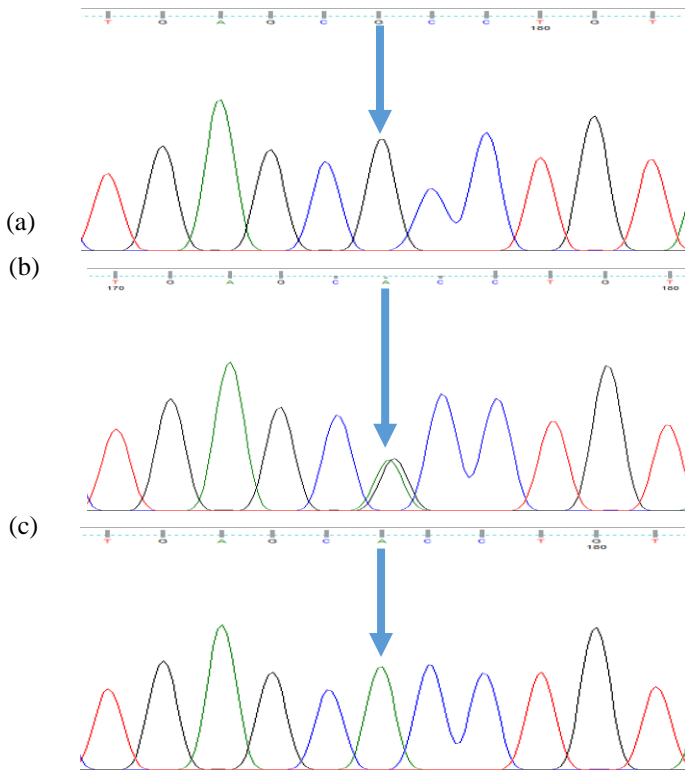


Figure 2. Sequencing maps of Senduro goat lactoferrin gene from different genotypes: (a) Genotype GG; (b) Genotype AG; (c) Genotype AA (Note: A is green; G is black; C is blue; T is red)

Based on the alignment results found one novel Single Nucleotide Polymorphism (SNP), namely G4179A, where guanine (G) turned into adenine (A), is located at position 4179 of the lactoferrin gene. The difference was found in dairy goats in China and Egypt, where one SNP was found, guanine turned into adenine at position 4066 (Guo, Jiao, He, Wei, Chang, Yue, Lan, Chen, and Lei, 2010; Nowier, Darwish, and Ramadhan, 2020). Local goats in China were found two SNPs, namely adenine (A) turned into guanine (G) at position 4120 and adenine (A) turned into cytosine (C) at position 4216 (Kang, Zheng, Zhou, Li, and Zhao, 2011). After validating the Ensembl, it was known that SNP G4179A had not been identified or had not been found at all so that it could be used as a candidate for the lactoferrin gene marker for Senduro goat.

Genotype Frequency, Allele Frequency, and Hardy-Weinberg Equilibrium

Analysis of genotype frequency and allele frequency can be used to determine genetic polymorphism. Statistical analysis of genotype frequency, allele frequency, and Hardy-

Weinberg equilibrium at SNP G4179A of Senduro goat lactoferrin gene is presented in Table 1. SNP G4179A found three genotypes, between AA with a genotype frequency of 0.02, AG with a genotype frequency of 0.05, and GG with a genotype frequency of 0.93. The frequency of allele A and allele G is 0.05 and 0.95, respectively. The allele frequency indicates that SNP G4179A is classified as polymorphic. The value of χ^2 indicates that SNP G4179A were in Hardy-Weinberg disequilibrium.

TABLE 1. Genotype frequency, allele frequency, and HW equilibrium

Genotype and genotype frequency			Alleles and allele frequencies		χ^2 test	χ^2 table
AA	AG	GG	A	G		
0.02 (1)	0.05 (2)	0.93 (39)	0.05	0.95	9.48	0.07

Note: χ^2 , chi-square.

Based on the observations of the SNP G4179A, this SNP is classified as polymorphic because there is more than one allele and the allele frequency is more than 0.01%. If in a population only one allele or character is found uniform then the allele is classified as a monomorphic allele. If an allele has a frequency equal to or less than 0.99 it is classified as polymorphic (Nei and Kumar, 2000). However, SNP G4179A is in Hardy-Weinberg disequilibrium because the chi-square test value is smaller than the chi-square table. Hardy-Weinberg disequilibrium can occur due to genotype errors or selection bias in the population (Llorca, Prieto-Salceda, Combarros, Dierssen-Sotos, and Berciano, 2005).

Heterozygosity and Degree of Polymorphism (PIC)

Heterozygosity values and degree of polymorphism (PIC) can be used to measure genetic diversity accurately. The results of the statistical analysis of heterozygosity and PIC are presented in Table 2. H_o and H_e values were 0.048 and 0.091. H_o values and the H_e values did not differ much indicated that the low variation of genes from Senduro goat population (Heterozygosity < 0.5). The PIC value on SNP G4179A was 0.0866 indicating that SNP G4179A is classified as low polymorphic (PIC < 0.25).

TABLE 2. Heterozygosity and degree of polymorphism (PIC)

H_o	H_e	PIC
0.048	0.091	0.0866

Note: H_o , observed heterozygosity; H_e , expected heterozygosity; PIC, degree of polymorphism (polymorphism information content)

If the observed heterozygosity (H_o) was lower than the expected heterozygosity (H_e), it could indicate a degree of endogamy (group marriage) as a result of an intensive selection process (Tambasco, Paz, Tambasco-Studart, Pereira, Alencor, Freitas, Coutinho, Packer, and Regitano, 2003). Furthermore, the heterozygosity value which is below 0.5 indicates the low variation of a gene in the population (Javanmard, Asadzadeh, Banabazi, and Tavakolian, 2005). The PIC values on SNP G4179A was 0.0866, indicating that the SNP G4179A were classified as low polymorphic. Generally, the PIC value is divided into three types, namely low polymorphism with the PIC value < 0.25; median polymorphic with the PIC value > 0.25 and < 0.50; and high

polymorphic with the PIC value > 0.5 (Guo et al., 2010).

Association between Lactoferrin Gene Polymorphism and Milk Production and Milk Composition

Based on the analysis of variance according to the General Linear Model (GLM) procedure, the association of the genotype diversity of the LF gene on goat milk production and milk composition is presented in Table 3. The results show that there is no association between the genotype of SNP G4179A and milk production and milk protein of Senduro goats.

TABLE 3. Association between genotypes with milk production and milk composition

Genotype (s)	Number of goat (s)	Parameter					
		Milk yield (liter/head/day)	P (%)	F (%)	L (%)	SnF (%)	S (%)
AA	1	838,6	4.2	8.1	5.35	0.87	9.19
AG	2	1.681	4.5	6.7	4.74	0.78	8.19
GG	39	967,3	4.5	6.7	4.74	0.78	8.18

Note: P, Protein; F, Fat; L, Lactose; SnF, Solid-non-fat; S, Solid.

The polymorphism of genotype obtained in this research was not associated with milk production and milk protein of Senduro goat. The association of the LF gene with milk protein was found in Chinese dairy goats (Guo, Jiao, He, Wei, Chang, Yue, Lan, Chen, and Lei, 2010). The same thing was found in Arab goats, Czech dairy goats, and Egyptian goats (Ali and Al-Samai, 2018; Hofmannova, Rychtarova, Sztankoova, Kyselova, Milerski, and Vostry, 2018; Nowier, Darwish, and Ramadhan, 2020). Milk production and milk composition are influenced by many factors, including livestock breed, lactation period, parity, and lactation period (Mioč, Prpić, Vnućec, Barać, Sušić, Samaržija, and Pavić, 2008). Besides, the composition of milk is also influenced by age, genotype, maintenance procedures, and other random environmental factors (Asmarasari, Sumantri, Gunawan, Taufik, and Anggraeni, 2019)

IV. CONCLUSION

There is the polymorphism of Senduro goat lactoferrin gene with the discovery of SNP G4179A, where G4179A is classified as low polymorphic. After further observations, it was found that there was no association between genotypes with milk production and milk composition.

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