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Analysis of CSN2 Exon 7 gene diversity and its association with Sapera goat milk composition

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ABSTRACT

Sapera goats, a crossbreed between Saanen and Etawa Grade goats, are known for their milk production and adaptability to the Indonesian climate. Genetic selection is necessary to improve the Sapera goat's milk quality and quantity. CSN2 gene is one of the genes that can affect the composition of goat milk. Therefore, this study aimed to identify the mutation points (SNPs) at the CSN2 exon 7 gene and its association with Sapera goat's milk composition. This study used sixty-six blood and milk samples of Sapera goats collected from the Livestock Research Center, Ciawi. This study found two mutation points (SNPs) at g.8946C>T and g.8956G>A. The association analysis showed that SNP g.8946C>T was significantly associated with the lactose and salt content of Sapera goat's milk. In conclusion, SNP g.8946C>T can be used as a genetic marker to improve the composition of Sapera goat milk with high lactose and salt content.

Keywords: *CSN2 Exon 7 gene, Milk composition, Sapera goat, SNP*

INTRODUCTION

The crossbreeding program is designed to combine different breed characteristics. Saanen goats are known for their high milk production, while Etawa Grade goats are known for their adaptability to Indonesia's tropical climate. The benefits of Saanen and Etawa Grade goats are considered as one option to increase local goat genetic potential through crossbreeding, known as Sapera goat. Sapera goat's advantages include milk production reaching 2-4 L/day (Pramono *et al*., 2023) and adapting to the Indonesian climate (Pamungkas *et al*., 2020).

Goat milk is a source of animal protein that can be an alternative substitute for cow's milk to fulfill people's nutritional needs. Goat milk has excellent quality, so it is often associated with various benefits for body health, including as an antioxidant, anti-inflammatory, and preventative for cardiovascular disease and atherosclerosis (Rahmatalla *et al.,* 2021). In addition, goat milk

is easier to digest and safe for lactose intolerant sufferers because it has a smaller fat globule size and fatty acid chain when compared to cow's milk (Verruck *et al.,* 2019).

The quality of goat milk may vary depending on genetic traits, physiological factors, environmental factors, and maintenance management. Protein is one of the essential ingredients in the quality of milk. Besides that, protein is also controlled by significant genes, divided into casein and whey. Casein is encoded by four genes, namely CSN1S1 (αS1-CN), CSN1S2 (αS2 -CN), CSN2 (β-CN), CSN3 (κ-CN), while whey is encoded by two genes, namely LALBA (α-lactalbumin) and LGB (β-lactoglobulin). Casein content can reach 80% of the total protein in goat milk and other ruminants. Besides that, casein has a moderate heritability value, so it can be used as a marker to improve the quality of goat milk (Amills *et al.,* 2012).

β-CN is the most casein composition, ranging from 50% to 64% of the total casein content in goat milk (Biadała and Konieczny, 2018). Furthermore, β-CN produces the bioactive peptide which inhibits the ACE-i (Angiotensin Converting Enzyme Inhibitor) enzyme's role in increasing blood pressure or hypertension (Verruck *et al.,* 2019). The CSN2 gene is located on chromosome 6, which has nine exons and is about 10.7 kb long. Exon 7 has a length of 492 bp and is the most extended exon in the CSN2 gene. Exon 7 also encodes 82% of mature proteins. Several mutation points were found that can affect the protein composition of goat milk (Li *et al.,* 2022). A previous study revealed that there were 12 variations of the CSN2 gene allele in goats (Li *et al.,* 2022), one of which was a "null" allele found in exon 7, which was associated with the loss of β-CN content in milk (Martin *et al.,* 2013).

Anggraeni *et al*. (2021) conducted molecular research on CSN2 gene diversity using the PCR-RFLP technique at locus g.8913 C>A. However, the study found no polymorphism and was not associated with milk composition. Therefore, this study aims to identify the diversity of the CSN2 exon 7 gene in Sapera goats using the Sanger Sequencing method and its association with milk composition. The results of this study can be used as a DNA marker candidate to get dairy goats with better milk composition.

MATERIALS AND METHODS

Animals and Samples Collection

Sixty-six blood and milk samples of Sapera goats were collected from National Livestock Research Center, Ciawi, Bogor Regency. The Sapera goat utilized in this study was not exposed to various management interventions to mitigate the impact of environmental factors. The management protocols employed encompassed feeding (comprising forage, concentrates, and mineral blocks), comprehensive facilities and infrastructure, sanitation, conversion of waste into compost, and measures to prevent and control dairy goat diseases.

The blood and milk samples were obtained from lactating Sapera goats. The milk samples test usedLactoscan Milk Analyzer to analyze the fat, solid non-fat (SNF), density, lactose, salt, protein, and total solid. Goat milk samples were then classified based on the TAS 6006-2008 (Thailand Agricultural Standard 6006-2008) for the fresh goat milk category (Table 1). TAS 6006 -2008 is a standard that guides ensuring goat's milk's excellent quality (Pramono *et al*., 2023). Blood samples were taken in the jugular vein and then stored in a 10 mL vacutainer tube containing anticoagulants (EDTA). Blood samples were then extracted for DNA using a modified Geneaid DNA Kit procedure.

PCR Amplification and Sequencing

CSN2 exon 7 gene PCR products were amplified using AB system machines. PCR volume of 25 µl were consisting of 2 µl of DNA, 6.1 µl of nuclease-free water, 0.3 µl of primer F: 5' – GGC ACA GTC TCT AGT CTA TC -3' and 0.3 µl primer R: 5' – CCT TTC TGC TGT ACC AGG AG -3', 16 µl MyTaq HS Redmix. The primer of the CSN2 gene (AJ011018.3) was self-designed using the Molecular Evolutionary Genetic Analysis (MEGA11) program and Primer

Table 1. Fresh Goat Milk Category

Parameters	Thai Agricultural Standard 6006-2008		
	Premium	Good	Standard
Fat (%)	>4	>3.5 - 4	3.25 – 3.5
Protein (%)	>3.7	>3.4 - 3.7	3.1 – 3.4
Total Solid (%)	>13	>12 - 13	11.7 - 12

Stats with a product length of 418 bp. The MEGA was used for aligning the CSN2 gene sequence, while Primer Stats was used to evaluate the potential PCR primers. The amplification process started with predenaturation at 95° for 5 minutes, denaturation at 95° for 15 seconds, annealing at 60° for 15 seconds, extension at 72° for 10 seconds, and final extension at 72° for 1 minute. PCR products were sequenced using the Sanger Sequencing method from 1st Base, Selangor, Malaysia.

Statistical Analysis

PCR sequencing results were analyzed using the Finch TV and MEGA11 programs. The sequencing results wer analyzed and visualized using Finch TV, and the MEGA11 program was to align and to compare the sequencing results with the CSN2 exon 7 primers. In addition, exon 7's CSN2 gene diversity was analyzed by calculating allele and genotype frequencies, heterozygosity values, Hardy-Weinberg equilibrium, and PIC (Polymorphism Information Content) using the PopGene program. PopGene was used to calculate genetic diversity and the Hardy-Weinberg equilibrium.

Milk composition data were corrected to the traits in the lactation phase and further corrected to the traits in the parity by the formula:

$$Lphase = \frac{\bar{X}\ traits\ in\ 2nd\ lactation\ phase}{\bar{X}\ traits\ in\ N\ lactation\ phase} \times observed\ traits$$

$$Parity = \frac{\bar{X}\ traits\ in\ 3rd\ parity}{\bar{X}\ traits\ in\ N\ parity} \times observed\ traits$$

Where:
Lphase = lactation phase; parity; = average of traits; N = effect of lactatiphase / parity on N (1,2, and n).
Afterward, the data were analyzed to determine the relationship between CSN2 exon 7 genotype and milk composition using General Linear Model (GLM) on the SAS 9.4 program.

$$Yij = \mu + Gi + eij$$

Where: Yij = milk composition traits (fat, solid non-fat (SNF), density, lactose, salt, protein, and total solid); μ = the population mean; Gi = genotype factor; eij = the residual error

RESULTS AND DISCUSSION

Quality of Sapera Goat Milk

In Indonesia, there are no quality standards for fresh goat milk. However, TAS 6006-2008 provides fresh goat milk quality criteria. The requirements of goat milk must be fit for consumption as normal, clean, and the color is white or cream. According to TAS 6006-2008 (Thai Agricultural Standard 6006-2008), quality grading can be categorized by fat, protein, and total solid. The quality grades of Sapera goat milk based on TAS 6006-2008 are classified as Premium, except for the protein in g.8946C>T with AA genotype as Good (Table 2). Environmental factors and livestock physiology factors can influence the nutritional content of goat milk. Getaneh *et al*. (2016) explained that the environmental factors were season and type of diet, while physiological factors included livestock age and multiple births.

Amplification and DNA Variants of CSN2 Exon 7 Gene

The CSN2 exon 7 gene in the Sapera goat was successfully amplified at 418 bp using 1% agarose gel (Figure 1). The amplification results were bright and unshaded bands.

Tortorici *et al.* (2014) reported, alleles char-

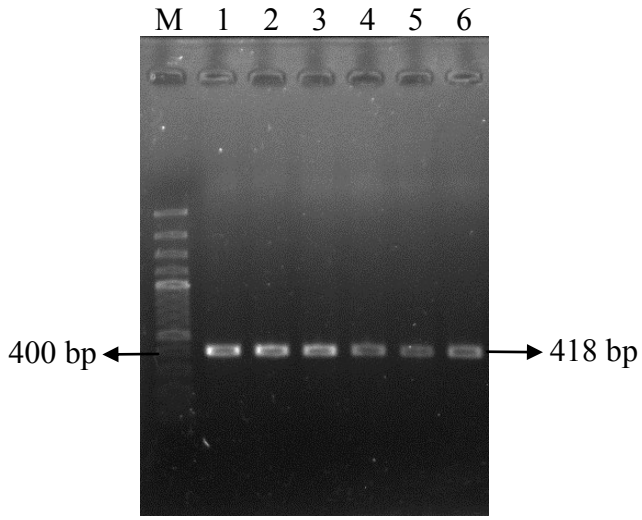

Figure 1. The amplification of CSN2 exon 7 gene. M = Marker 100 bp; 1-6 = Dairy goat samples; bp = base pair

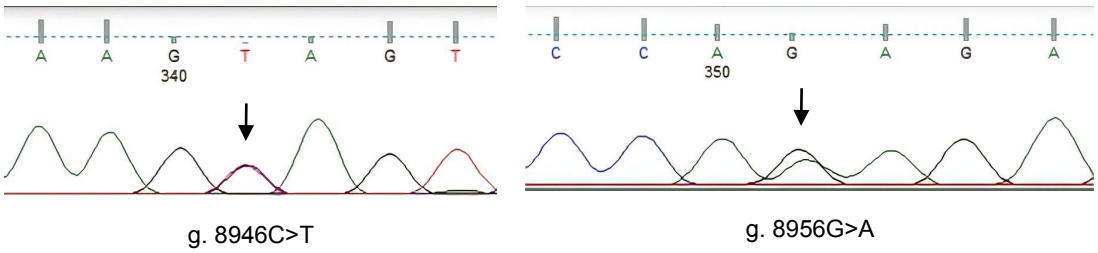

Figure 2. SNPs found in the CSN2 exon 7 gene. The arrow shows the mutation point. A = Adenine; T = Thymine; G = Guanine; C = Cytosine

acterization in the exon 7 CSN2 gene were the E allele at g.8913C>A, O' allele at g.8915C>T, and the C allele at g.8946C>T. The mutation can be either a transitional mutation or a transversion mutation. This study found two SNPs mutations, namely g.8946C>T and g.8956G>A (Figure 2). The mutation type of the two SNPs was a transitional mutation. Meanwhile, no mutations were found at g.8913C>A and g.8915C>T in the Sapera goat.

Several studies on the diversity of CSN2 genes have been widely carried out in various dairy goat breeds. For example, research conducted by Anggraeni *et al*. (2021) showed that the SNP position g.8913C>A was monomorphic in Saanen, Etawa Grade, Sapera, and Saan-PE goats with a CC genotype, in contrast to the results of the study by Caroli *et al.* (2006) in Frisa goats with a CA genotype. Meanwhile, SNP position g.8915C>T was found polymorphic in Girgentana (Tortorici *et al.,* 2014) and Sarda goats (Vacca *et al.,* 2014). However, other results have not yet been found in the Sapera goat. According to Ellegren and Galtier (2016), genetic differences can be influenced by mutations, genetic drift, and natural selection, which cause variation within populations in dairy goats.

This study found that SNP at g.8946C>T was polymorphic with three genotypes in the Sapera goat. Previous studies have shown that changes in the position of g.8946C>T occurred in several goat breeds, including Banat's White, Carpathian, Girgentana, Sarda, Nubian, Desert, Nilotic, Taggar, Saanen, Bezoar ibex, and Nubi-

Table 2. Sapera Goat Milk Quality

Genotypes		TAS 6006-2008								
		Fat			Protein			Total Solid		
		Premium	Good	Standard	Premium	Good	Standard	Premium	Good	Standard
g.8946C>T	AA	√				√		√		
	AB	√			√			√		
	BB	√			√			√		
g.8956G>A	AA	√			√			√		
	AB	√			√			√		

Table 3. Genetic Diversity at g.8946C>T and g.8956G>A

Genotype	N	Frequency					Ho	He	X^2 test	PIC
		Genotype			Allele					
		AA	AB	BB	A	B				
g.8946C>T	66	0.197 (13)	0.439 (29)	0.364 (24)	0.417	0.583	0.439	0.486	0.711	0.368
g.8956G>A	66	0.636 (42)	0.364 (24)	0 (0)	0.818	0.182	0.364	0.298	3.105	0.253

N = the number of samples; (…) = the number of samples within genotypes; Ho = observed heterozygosity; He = expected heterozygosity; X^2 table = 3.84; PIC = polymorphism information content.

an ibex goats (Tortorici *et al.,* 2014; Vacca *et al.,* 2014; Kusza *et al.,* 2016; Rahmatalla *et al.,* 2021). SNP g.8946C>T changed the amino acid Alanine (GCA) to Valine (GTA). In addition, this study also found another polymorphic SNP at g.8956G>A with two genotypes. Position changes of SNP g.8956G>A need to be used in other dairy goats to generate more information and potentially become a significant point for selective breeding programs.

Genetic Diversity of CSN2 Exon 7 Gene

A population's genetic diversity can be determined by estimating genotype and allele frequencies, heterozygosity values (Ho and He), Hardy-Weinberg equilibrium, and PIC values. In this study, the SNP positions of g.8913C>A and g.8915C>T were not estimated because they were monomorphic. Allele and genotype annotation used were allele A and allele B. Based on the mutations at g.8946C>T and g.8956G>A, we obtained allele frequency, genotype frequency, observation heterozygosity, expectation heterozygosity, Hardy-Weinberg equilibrium values, and PIC (Table 3).

Table 3 showed that SNPs g.8946C>T and g.8956G>A were polymorphic with a significant allele frequency value was less than 0.99. SNP was polymorphic when it has a significant allele frequency of less than 0.95 or 0.99, and 0.99 was generally used when the number of samples exceeds 50 (Allendorf *et al*., 2012). The Hardy-Weinberg equilibrium test showed that all SNPs were in the Hardy-Weinberg equilibrium state (X^2 test < X^2 table). The observed heterozygosity (Ho) of g.8946C>T was lower than the expected heterozygosity (He) value. Meanwhile, the Ho value of g.8956G>A wasgreater than the He value. According to Sharma *et al.* (2015), the population's high observed heterozygosity value may indicate the population's diversity and contribute to adaptability to environmental changes. Fur-

Table 4. Association of SNP g.8946C>T and g.8956G>A with Milk Composition

Parameters	g.8946C>T				g.8956G>A		
	AA (13)	AB (29)	BB (24)	Pr	AA (42)	AB (24)	Pr
% Fat	6.18 ± 0.32	6.18 ± 0.21	6.84 ± 0.24	0.094	6.38 ± 0.18	6.49 ± 0.24	0.724
% SNF	7.76 ± 0.12	7.99 ± 0.08	8.07 ± 0.09	0.118	7.95 ± 0.07	8.02 ± 0.09	0.547
Density	25.21 ± 0.86	24.08 ± 0.58	25.48 ± 0.64	0.244	24.60 ± 0.49	25.17 ± 0.64	0.481
% Lactose	3.53 ± 0.06^{b}	3.69 ± 0.04^{a}	3.69 ± 0.04^{ab}	0.05*	3.65 ± 0.03	3.68 ± 0.04	0.593
% Salt	0.60 ± 0.01^{b}	0.64 ± 0.01^{a}	0.64 ± 0.01^{a}	0.033*	0.63 ± 0.01	0.64 ± 0.01	0.644
% Protein	3.70 ± 0.05	3.83 ± 0.03	3.84 ± 0.04	0.094	3.79 ± 0.03	3.83 ± 0.04	0.529
Total solid	14.02 ± 0.35	14.28 ± 0.24	14.94 ± 0.26	0.070	14.41 ± 0.20	14.57 ± 0.27	0.616

(…) = the number of samples within genotypes; ^{ab} = superscripts with different letter show significantly different;
* = significant value P<0.05

thermore, the results of PIC calculation were informative in both SNPs. The maximum value of PIC with two alleles were 0.5 (Chesnokov and Artemyeva, 2015).

Association between CSN2 Exon 7 Gene and Milk Composition

Association between g.8946C>T and g.8956G>A genotypes with Sapera goat milk composition showed that g.8946C>T was significantly associated with lactose and salt content with P-value < 0.05 (Table 4). Lactose is the primary source of carbohydrates, while salt provides essential minerals such as calcium, phosphorus, potassium, and magnesium (Bijl et al., 2013). On the other hand, SNP g.8956G>A was not associated with milk composition. Changes in encoded amino acids can affect the differences between g.8946C>T and g.8956G>A. Amino acid changes in g.8946C>T was from Alanine (GCA_{177}) to Valine (GTA_{177}). In contrast, g.8956G>A did not cause changes in the amino acid Glutamine. According to Khaldi *et al*. (2014), variations in amino acid composition can affect changes in milk nutrient content, including digestibility rate, hydrophobicity, calcium binding, and other nutrient content.

This study found that g.8946C>T with AB and BB genotypes had a higher lactose and salt content than AA genotypes. However, the results of this study were different from previous research by Sztankóová *et al*. (2013) and Vacca *et al*. (2014) in Czech and Italian dairy goat breeds, explaining that SNP g.8946C>T was associated with protein content (P<0.05). The composition of milk can vary and be influenced by genetic and non-genetic factors. Non-genetic factors that can affect the nutritional content of milk includingdiet, breed, individuals within the breed, environmental conditions, region, and lactation period (Park, 2017).

CONCLUSION

This study showed that Sapera goat milk hadmet the standard quality of milk composition, especially in protein, fat, and total solid content. Genetic diversity revealed that SNP position g.8913C>A and g.8915C>T were monomorphic, while the SNP g.8946C>T and g.8956G>A were polymorphic. The association between the CSN2 exon 7 gene and milk composition found that SNP g.8946C>T was significantly associated with lactose and salt content. The results of this study can be used for further and potential research in the development of breeding Sapera goats with desirable traits.

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