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Research Article
Copy Number Variation of Agouti Signaling Protein (ASIP) Fragment and its Relationship with Coat Color in Indonesian Goat Breeds

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Abstract

Background: The coat color is important for the production of wool, for heat tolerance in hot climates, cultural value and for the identification of the breed. Goat has very important roles in the agricultural economies of Indonesia. The goat is not only to produce meat, milk, wool and compost, but also for cultural and religious events. Batak tribe in North Sumatra has a unique tradition of customary celebration by using goat. Goat that has completely white coat should be used, so that the white coat has economic value much higher than other colors. **Materials and Methods:** This study used blood from each individual. Four subpopulations of domestic goat breeds in Indonesia, namely Samosir goat, Kacang goat, Gembrong goat and Peranakan Etawah goat. The agouti signaling protein (ASIP) gene fragments amplified and sequenced. The length of the ASIP gene amplification products was 610 bp for every individual and analyzed to detect variations on every Indonesian local goat. **Results:** From all samples of Indonesian goats, there is only one difference on Samosir goat 1 c.158 G-T. This polymorphism only foundin Samosir goat 1 while the other Indonesian local goats and other organisms were the similar. This variation would change to the order of amino acids in goat Samosir 1. **Conclusion:** There is no variation in the ASIP fragment of Indonesian goats that can be used to investigate the relationship between exon 4 of ASIP and coat color expression of Indonesia local goats. The exon 4 of the ASIP cannot be made target to investigate different coat color in some Indonesian goat breeds.

Key words: Agouti signaling protein, *Capra hircus*, coat color, SNP

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In goat (*Capra hircus*), coat color is important for wool production, for heat tolerance in hot climates and for breed identification[1,2]. Goat has very important roles for the agricultural economies of Indonesia. The goat is not only to produce meat, milk, wool and compost, but also for cultural and religious events. Batak culture in North Sumatra, goat is white color that is necessary for the implementation of the ceremonies. Therefore, the economic value of white goats is much higher than other colored goats. For that, very important to know the factors that affect the determinants of coat color[3,4].

A large number of coat color phenotypes have been described in different mammalian species. Due to this diversity, distribution and biochemical activity of melanocytes in which two types of melanin pigment (eumelanins and pheomelanins, which produce the colors black/brown and yellow/red, respectively) are synthesized. The extension and agouti are the main locus that affect the relative amounts of eumelanin and pheomelanin production in these cells[5]. In classical genetic studies of the goats, reported a large number of alleles at the agouti locus with an effect on the color of the feathers and the distribution patterns. From initial study, dominant A (Wt) (white/tan) alleles are advised to cause the white color of the Saanen goat. The binding of melanocortin 1 receptor to proopiomelanocortin (MC1R) is leading that activates the synthesis of eumelanin via the transcription and post translational. Agouti signaling protein (ASIP) is a soluble factor, secreted by the dermal papilla cells in a hair bulbs, the antagonistic effect of proopiomelanocortin. In this way, ASIP increases the synthesis of phaeomelanin, a yellow-redpigment[6].

Analyzing the DNA of anonymous markers in genetic diversity studies is possible to get information about the structure of the population and the origin of the population, but usually these alerts can not be directly observable phenotypic difference between explaining and the inside of the population[7]. Coat color is one of the primary phenotype traits that distinguish the breeds of livestock. In sheep, the classic genetic studies have identified several loci that affect these properties and comparative analysis has determined genetic homology of entire species of other mammals[5,8]. In goats, a large number of alleles at the agouti locus, noted for the wide variability in plumage, it has been predicted by classical crossover studies in some breeds.

The agouti signaling protein (ASIP) gene has been investigated to be associated with coat color production in animals; previous studies mapped the coat color locus on a chromosome 13 for foxes[9,10] mice, cats, horse[11-13] cattle, pigs, dogs[14-16] sheep and goats[17,18]. Kanetsky *et al.*[19] investigating 147 healthy people as controls, 176 people with Dysplastic Nevi (DN) and 423 positive melanoma patients with or without a diagnosis of Dysplastic Nevi (DN) concluded that the ASIP gene did not show a direct relationship with melanoma, still makes sense biologically interactive effects that the possibility of the ASIP has a combination with MC1R variant or one of many other genes involved in the pigmentation pathway.

The coat color of mammals is mainly determined by the distribution of 2 types of melanin, eumelanin (black/brown pigment) and pheomelanin (red/yellow pigment), where the relative number of productions and mainly controlled by the agouti locus and extension[5]. In some mammals, the extension locus shows the effect of epistatik on the agouti locus. A dominant allele at the extension locus is responsible for the color of black coat, while the recessive allele produces red/yellow pigmentation. On the other hand, the dominant allele at the agouti locus produces pigmentation pheomelanic recessive allele, whereas the cause of black coat is uniform. Genetic studies in several species, including goats showed the presence of alleles at a locus affecting agouti color pattern distribution of body coverings[5,20,21].

The purpose of this research is to investigate the ASIP gene polymorphism in some Indonesian goat which ASIP gene plays a role in determining of the coat color of the organism. Some Indonesian local goat breeds have the coat color difference unequivocally[22]. This study uses only one part of the ASIP gene, namely exon 4. The results of this study will provide information about relationship of the ASIP gene (exon 4 polymorphisms) with the coat color Indonesian goat breeds. The agouti gene plays an important role in the synthesis of pigment in domestic animals. Therefore, more attention has been focused on the study of Single Nucleotide Polymorphism (SNP) identification, analysis and association with color variations in different subpopulations of animals.

MATERIALS AND METHODS

Animals and DNA sample: This study used blood from every individual. Four subpopulations of domestic goat breeds in Indonesia (Fig. 1). The samples of goat with purposive sampling method, the first to determine the district native goat production centers. The sample was selected from several local goat breeds with different phenotypes (Table 1). Ten Samosir goats (SAM), 5 Kacang goats (KAC), 5 Gembrong goats (GEM) and 3 Peranakan Etawah (PE) goats.

Fig. 1(a-d): Phenotype of Indonesian local goats, (a) Samosir goat, (b) Kacang goat, (c) Peranakan Etawah (PE) goat and (d) Gembrong goat

Table 1: Coat color of Indonesian goat breeds and its origin

Breeds	Coat color	Location
Samosir goat	White	North Sumatera
Kacang goat	Brown-black	Central Java
Gembrong goat	White	Bali
Peranakan Etawah goat	White-black	Yogyakarta

DNA isolation and sequencing of ASIP fragments: Samples were obtained from blood, collected separately according to the type of goat. A blood sample was isolated and purified using gSYNC DNA mini kit (Geneaid). The primer was designed from primary bovine ASIP sequences, ASIP-F: 5'GGGACGTCTAGTCCGAGGAGT-3' and ASIP-R: 5'-CTGCCAGATCCAGAAAAGCG-3'. The area of complementary primer, designed according to the reference goat ASIP sequence (EF587236, except the ASIP-R-exon 3), which complements the bovine gene downstream region 30 ASIP (Ensemblentri ENSBTAT00000048322)[18]. The ASIP gene amplification, each using a primer pair has been designed for the exon 4. The composition of the PCR reaction was 3 µL (approximately 100 ng) genomic DNA, 25 µL Kapa 2G ready mix, 1 µL (10 pmol) each forward and reverse primer and ddH_2O in a total volume of 50 µL. The PCR amplification was conducted using a Infinigen Thermal cycler according to the program, an initial denaturation at 94°C for 5 min, followed by 35 cycles, each consisting of 30 sec denaturation at 94°C, 45 sec primers annealing at 48°C, 90 sec elongation at 72°C, then a final 6 min elongation at 72°C and storaged at -20°C. The PCR product was visualized using 1.5% concentration of agarose gel. Electrophoresis was run on 100 V condition for 40 min and the amplification result could be seen on the UV light. The purified PCR products were sequenced directly by 1st BASE sequencing INT (Singapore).

Data analysis: The length of the ASIP gene sequence amplification products was 610 bp for every individual and analyzed using the MEGA 6.0 software[23]. The ASIP gene sequence among of Indonesian local goats aligned along with ASIP sequences of other *Capra hircus* from GenBank using ClustalW in MEGA software. Analysis of the genetic variation seen based on the difference in ASIP gene sequence.

In addition, to provide further information, some ASIP fragments of several organisms downloaded from GenBank and used as comparisons for Indonesian goats. The difference in nucleotide showed single nucleotide polymorphism and genetic diversity. The number access code is as follows: *Aepyceros melampus* (KU564955), *Bos grunniens* (KJ630463), *Bos taurus* (NM_206843), *Canis lupus familiaris* (KF586870), *Capra hircus* (GU224268), *Ovis aries* (EU185099) and *Ovis aries* haplotype nonfunctional agouti signaling protein 2 (ASIP) (EU420023).

RESULTS AND DISCUSSION

Identification of polymorphism in ASIP gene: Primer PCR was designed on bovine ASIP sequence that is expected to amplify ASIP gene of Indonesian local goats. The primary

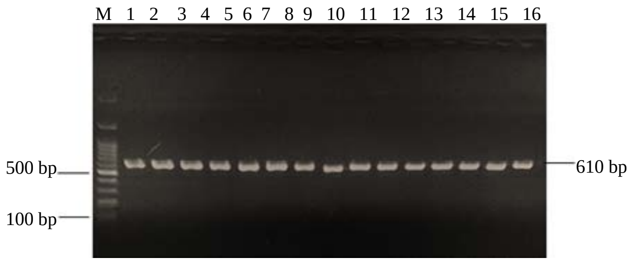

Fig. 2: Agarose gel electrophoresis of the amplification product in accordance with the goat ASIP gene, M: DNA marker 100 bp

Table 2: Differences amino acid at a Indonesian local goat with some organisms that are downloaded from GenBank based on ASIP fragment sequences

Breeds	1	4	11	24	28	31	39	50	53	55	57
Samosir 1	R	P	T	D	P	P	F	S	G	N	T
Samosir 2	.	.	.	.	.	.	.	.	V	.	.
Samosir 3	.	.	.	.	.	.	.	.	V	.	.
Samosir 4	.	.	.	.	.	.	.	.	V	.	.
Samosir 5	.	.	.	.	.	.	.	.	V	.	.
Samosir 6	.	.	.	.	.	.	.	.	V	.	.
Samosir 7	.	.	.	.	.	.	.	.	V	.	.
Samosir 8	.	.	.	.	.	.	.	.	V	.	.
Samosir 9	.	.	.	.	.	.	.	.	V	.	.
Samosir 10	.	.	.	.	.	.	.	.	V	.	.
Kacang 1	.	.	.	.	.	.	.	.	V	.	.
Kacang 2	.	.	.	.	.	.	.	.	V	.	.
Kacang 3	.	.	.	.	.	.	.	.	V	.	.
Kacang 4	.	.	.	.	.	.	.	.	V	.	.
Kacang 5	.	.	.	.	.	.	.	.	V	.	.
Gembrong 1	.	.	.	.	.	.	.	.	V	.	.
Gembrong 2	.	.	.	.	.	.	.	.	V	.	.
Gembrong 3	.	.	.	.	.	.	.	.	V	.	.
Gembrong 4	.	.	.	.	.	.	.	.	V	.	.
Gembrong 5	.	.	.	.	.	.	.	.	V	.	.
PE 1	.	.	.	.	.	.	.	.	V	.	.
PE 2	.	.	.	.	.	.	.	.	V	.	.
PE 3	.	.	.	.	.	.	.	.	V	.	.
Capra hircus	.	.	.	.	.	.	.	.	V	.	.
Aepyceros melampus	.	.	.	.	.	.	.	.	V	.	.
Bos grunniens	.	.	.	.	.	S	.	.	V	.	.
Bos taurus	.	.	.	.	.	.	.	.	V	.	.
Canis lupus	K	S	P	N	S	.	S	T	V	S	R
Ovis aries	.	.	.	.	.	.	.	.	V	.	.
Ovis aries 2	.	.	.	.	.	.	.	.	V	.	.

amplified exon 4 of the ASIP gene. All samples in this study could be amplified with a length of 610 bp (Fig. 2). The results of the amplification carried sequencing to obtain the nucleotide composition of the target gene. Sequencing results analyzed by doing alignment on all samples. Intron parts are removed so that the remaining parts are only exon parts. Identification of all samples, to show the differences from the nucleotide composition of ASIP fragments. From all samples of Indonesian local goats, there is only one difference on Samosir goat 1 c.158 G-T (Fig. 3). This polymorphism occurs only on Samosir goat 1 while the other Indonesian local goats are similar. This change will result in changes to the order of amino acids in Samosir goat 1, so different from the other goats (Gly53Val).

All samplesof Indonesian local goats have 100% amino acid similarity with the *Capra hircus*, *Aepyceros melampus*, *Bos taurus* and *Ovis aries* obtained from GenBank except Samosir goat 1. If compared to a Indonesian local goat with *Canis lupus* based on the arrangement of amino acids, there are 9 different amino acids (Table 2).

Polymorphism in a population occurs because of the difference in the order of nucleotides. Samosir goat 1 experienced a heterozygous polymorphism. It can be seen by sequencing of genomic DNA samples (Fig. 4).

Based on the ASIP fragment sequence, had a genetic distance is very close 0-0001, as well if the comparison between Indonesian goat and *Capra hircus* (Table 3). Organisms that have the most genetic distance away from Indonesian local goats based sequence ASIP fragments is *Canis lupus familiaris*.

Polymorphism of ASIP gene: Indonesia has several breeds goats that have a specific phenotype. Coat color is easily observable phenotype to distinguish each Indonesian local goats. Differences in every breed phenotype were caused by expression of some different genes. This study aims to investigate the differences of ASIP fragment sequences contained in some local goats. Based on the results obtained, it was not found differences in this fragment sequence among Indonesian local goats. From all samples of Indonesian local goats, there was only one difference on Samosir goat 1 c.158 G-T (Fig. 3). The coding region of the ASIP gene is determined by three exons (2, 3 and 4 according to the nomenclature in mice). The full-length (approximately 610 bp) sequence from exon 4 of ASIP was amplified and sequenced in this study. Missense mutations in ASIP gene have been previously reported in domestic sheep[16]. In sheep, the ASIP protein consists of 133 amino acids that comprise a signal peptide and functional amino acids. Previous studies showed ASIP mutations related to animal coat color but mainly

Breeds	2	10	27	30	31	45	48	66	70	82	84	87	91	102	108	116	129	148	158	159	164	165	170	171
Samosir 1	G	C	A	G	A	G	T	C	G	C	A	A	C	C	G	T	C	T	G	G	A	C	C	C
Samosir 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Samosir 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Samosir 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Samosir 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Samosir 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Samosir 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Samosir 8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Samosir 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Samosir 10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Kacang 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Kacang 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Kacang 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Kacang 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Kacang 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Gembrong 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Gembrong 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Gembrong 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Gembrong 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Gembrong 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
PE 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
PE 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
PE 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Capra hircus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Aepyceros melampus	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	T	.	.	.	.	.
Bos grunniens	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	T	.	.	.	.	.
Bos taurus	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.
Canis lupus	A	T	T	T	C	A	C	T	A	T	C	G	.	T	C	C	.	A	T	T	G	T	G	A
Ovis aries	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
Ovis aries 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.

Fig. 3: Amino acid differences in some Indonesian local goats based ASIP fragment sequence

Table 3: Genetic distance between Indonesian local goat and some organisms based ASIP fragment sequences

Breeds	1	2	3	4	5	6	7	8	9	10
Samosir										
Kacang	0.001									
Gembrong	0.001	0.000								
PE	0.001	0.000	0.000							
Aepyceros melampus	0.011	0.011	0.011	0.011						
Bos grunniens	0.022	0.022	0.022	0.022	0.022					
Bos taurus	0.011	0.011	0.011	0.011	0.011	0.011				
Canis lupus familiaris	0.124	0.123	0.123	0.123	0.130	0.144	0.130			
Capra hircus	0.001	0.000	0.000	0.000	0.011	0.022	0.011	0.123		
Ovis aries	0.001	0.000	0.000	0.000	0.011	0.022	0.011	0.123	0.000	
Ovis aries 2	0.001	0.000	0.000	0.000	0.011	0.022	0.011	0.123	0.000	0.000

addressed the ASIP functional-deletion mutation and missense mutations. Tang *et al.*[24] analyzed polymorphisms G-T, mapping in exon 4 of the goat ASIP gene, in the 12 descendants of China and found that most of them are in equilibrium, the exception is the Nanjiang brown (strain rapid growth) and a population of Guizhou.They interpreted as evidence of irregularities selective pressures acting on the ASIP gene of goats.

All samples of Indonesian local goats have 100% amino acid similarity with the *Capra hircus*, *Aepyceros melampus*, *Bos taurus* and *Ovis aries* obtained from GenBank except Samosir goat 1. Fontanesi *et al.*[17] studied the separation of the three polymorphisms ASIP gene (Ala96Gly, Cys126Gly and Val128Gly) in six descendants of goats and found that Ala96Gly and Cys126Gly are in equilibrium, but Val128Gly are in disequilibrium in Girgentana and Saanen goat population.

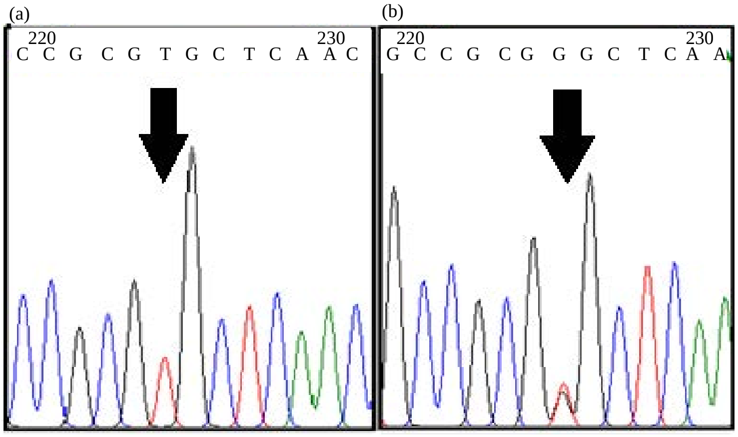

Fig. 4(a-b): Detection area and heterozygous polymorphisms with DNA sequencing was obtained, (a) TT and (b) GG c.224 T>G genotype ASIP

Lack Hardy Weinberg equilibrium in particular goat population can be explained by some technical (sample size) and biological (genetic drift, selection and inbreeding) factors. The cross is not expected to have a major influence on the ASIP genotype frequencies, because in general local goat breeds are managed extensively and the number of half-siblings derived from a given buck is relatively small.

In this study, we have analyzed four goat population and additional organisms from GenBank. In this study, there is no evidence that shows a correlation between ASIP gene (Exon 4) polymorphisms and coat color in Indonesian goats. This conclusion is not similar as previous studies by Fontanesi *et al.*[17] and Badaoui *et al.*[18] in the sense that polymorphisms were not found in ASIP exon 4 fragment of Indonesian local goat. This case could be concluded that the exon 4 of the ASIP cannot be made targets to investigate different coat color in some Indonesian goat breeds. The recent identification of copy number variation in ASIP locus of caprine describes complex legacy coat color in ruminant species[17]. In the future, additional gene structure characterization and assessment of their level of polymorphism in goat diverse population would be required to explain the genetic factors that modulate pigmentation and revealed the intricate network of interactions that they build to express this trait.

Relationship between ASIP gene and coat color of different goat breeds: The hypothesis of this study is that the SNP identified in this ASIP fragment can determine coat color differences in every Indonesian goat breed. Badaoui *et al.*[18] had amplified and sequenced 2.8 kb of goat ASIP gene from 48 individuals.There was a missense mutation in exon 4 ASIP gene are c.383 T>G, Val128Gly. This is in contrast with Indonesian local goats that four subpopulations were not found a variation on the ASIP gene fragment, despite amplifying across exon 4. Therefore, ASIP gene fragment cannot be used to investigate the relationship between the ASIP gene expression and coat colors in Indonesian goat. This is likely due to its short ASIP gene fragments were amplified and used different samples of goat with study of Badaoui *et al.*[18].

The high frequency of animals with genotype g.187GG in Murciano-Granadina breed (solid black or solid brown) could indicate the possible role of this site in affecting coat color in this breed, together with mutations in the MC1R gene[25]. In determining coat color expression is not only influenced by a single gene. There are several genes that play a role are interlocked to determine the expression of coat color. Several previous studies have shown there were several genes responsible for the coat color determination of an organism. The extension locus encodes the melanocyte-stimulating hormone receptor, also known as melanocortin receptor 1 (MC1R), the which belongs to the G protein-coupled receptor family[26]. The agouti signaling protein (ASIP) is a soluble factor, secreted by dermal papilla cells in hair bulbs, that antagonizes the effects of pro-opiomelanocortin. In this way, ASIP promotes the synthesis of phaeomelanin, a yellow-red pigment[6]. The TYRP1 gene is involved in maintenance of melanosome structure and affects melanocyte proliferation and melanocyte cell death[27].

CONCLUSION

Based on the sequences of the ASIP gene fragment of 610 bp amplified using ASIP-F primer: 5'-GGGACGTCTAGTCCGAGGAGT-3' and ASIP-R: 5'CTGCCAGATCCAGAAAAGCG-3', there was no variations that can be used to investigate the relationship between exon 4 of ASIP gene and coat color expression of Indonesia local goats. The exon 4 of ASIP gene cannot be made target to investigate different coat color in some Indonesian goat breeds. Identification of copy number variation in the ASIP locus is very complex to found its relationship with coat color inheritance in ruminant species. In the future, additional gene structure characterization and assessment of polymorphism in goat diverse population would be required to explain the genetic factors that modulate pigmentation.

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