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Association of g.331T>C and g.1909T>C locus in KISS1 gene with fecundity traits of Kacang and Boerka goats

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Abstract. *Maskur, Jan R, Lestari, Rozi T, Muhsinin M. 2023. Association of g.331T>C and g.1909T>C locus in KISS1 gene with fecundity traits of Kacang and Boerka goats. Biodiversitas 24: 4870-4876.* The neuropeptide kisspeptin encodes the KISS1 gene and is crucial for reproductive system signals. This study aimed to identify polymorphisms of fecundity genes by observing candidate genes for prolific traits, namely the KISS1 gene (exon 1 and intron 1), and their relationship with litter size in Kacang and Boerka goats (*Capra aegagrus hircus*). The total sample used was 211 female goats. The stages of the research included DNA extraction, PCR amplification and genotyping. The association of KISS1 gene genotypes with litter size traits in Kacang goats and Boerka was analyzed using the SAS 9.1.3 software with General Linear Model (GLM) procedure. The results showed that the g.331T>C locus of the KISS1 exon 1 gene was monomorphic in all Kacang and Boerka goats populations. The locus g.1909T>C of the KISS1 intron 1 gene was polymorphic in all Kacang and Boerka goats populations. The locus g.1909T>C of the KISS1 intron 1 gene found three genotypes (TT, AT, AA). The frequency of allele T (0.775 and 0.815) was higher than allele A (0.225 and 0.185) in all Kacang and Boerka goats populations. The association of TT and AT genotypes with litter size significantly differed from that of the AA genotype in the Kacang goat. Meanwhile, in the Boerka goat, the TT genotype significantly differed from the AT and AA genotypes. The goat KISS1 gene polymorphism is critical to reproductive function and may be a reproductive characteristic marker.

Keywords: Indonesian goats, kisspeptin, livestock reproduction, prolific characteristics

INTRODUCTION

Local goats in Indonesia are generally Kacang goats as native Indonesian goats and goats resulting from crosses between local goats and import goats (*Capra aegagrus hircus*), such as the Boer goat. Out of a total goat population of around 19 million (BPS 2022), Kacang goats have the largest population (83%). This type of goat has a low live weight and growth capacity and is more of a more prolific type of goat (Depison et al. 2020). Kacang goat is a meat-producing goat with an average litter size of 1.56-1.98 kids per birth (Mudawamah et al. 2021). One of the results of crossing Kacang with the Boer goat is the Boerka goat (Solehudin et al. 2022). The Boerka goat is a new genotype resulting from a cross between a Kacang goat and a Boer goat, which has reproducibility, high meat weight capacity, and is easily adaptable to tropical conditions in Indonesia. The average litter size of Boerka goats is 1.45-1.54 kids per birth (Adhianto et al. 2017). The litter size data shows that Kacang and Boerka goats have prolific characteristics.

Currently, the genetic research on the characteristics of the goat is lagging, and the limited information on litter size traits in goats is mostly derived from reports of sheep (*Ovis aries*) that major in BMPR1B, BMP15, and GDF9 (An et al. 2013; Ahlawat et al. 2015). Besides, there were few association analyses of a single gene, for example, the Single Nucleotide Polymorphisms (SNP) of POU class 1 homeobox 1 (POU1F1) and KISS1 genes, insertion/deletion (indel) variants of lysine demethylase 6A (KDM6A) and β-Type platelet-derived growth factor receptor (PDGFRB), and transcript variants of catenin beta 1 (CTNNB1) had been reported to be associated with goat litter size trait (An et al. 2013; Cui et al. 2018; Zhang et al. 2018; Zhu et al. 2019). Several genetic markers are related to goat litter size. However, the key genes in caprine that control litter size have not been completely revealed. Numerous genes regulate litter size (Rahawy and Al-Mutar 2021).

Kisspeptin protein is encoded by the KISS1 gene, located on the long arm of chromosome 16 in goats (Wolfe and Hussain 2018). The KISS1 gene contains two coding regions (exons) and a single non-coding region (intron), and its 408 bp transcript encodes 135 amino acids. This gene contains approximately 2.62 kilobases. (ENSCHIT00000037363.1). Kisspeptin is an essential upstream regulator of GnRH (gonadotropin-releasing hormone) neurons (Maitra et al. 2014). Kisspeptin is essential for brain sexual differentiation and early puberty, regulating gonadotropin secretion and controlling fertility metabolism in adult animals. As a result, the KISS1 gene is crucial for livestock reproduction. According to Maitra et al. (2014), KISS1 is considered a key mediator in mammals' molecular mechanisms of reproduction (puberty and proliferation). Gahete et al. (2016) stated that the KISS1 gene can control specific cell types and developmental status depending on the pituitary gland's hormonal secretion. Given its function in regulating

physiological reproductive status, the KISS1 hormone is essential for monitoring fertility (Mattam et al. 2021).

Various studies have been carried out by associating the KISS1 gene with litter size; the research of Abuzahra et al. (2023) showed that the KISS1 gene is a candidate gene that affects litter size. An et al. (2013) and El-Tarabany et al. (2017) discovered a polymorphism in the number of KISS1 gene loci correlating with litter size. The single nucleotide polymorphism (SNP) g893G>C was identified as being associated with litter size in Iraqi black goats (Rahawy and AL-Mutar 2021). Therefore, it was essential to investigate the interaction of several loci for the standardization model of goat farming (Ahlawat et al. 2015). This study aimed to identify and analyze the diversity of the KISS1 gene and its relationship with litter size in Kacang and Boerka goats. Previous studies related to litter size have the potential to be carried out on various breeds of goats. So it is also important to conduct similar studies on Kacang and Boer goats to provide scientific information for designing a set of DNA markers conceivably valuable for marker-assisted selection (MAS). Selection procedures could improve prolific traits, economic, and genetic profit using genetic information acquired through marker-assisted selection.

MATERIALS AND METHODS

Experimental animals and DNA isolation

The total sample used was the whole blood of 211 female goats to identify the diversity of the KISS1 gene. The sample consisted of 111 Boerka goats (Figure 1) and 100 Kacang goats (Figure 2). The number of kids for every birth and the average litter size was recorded. All samples were collected at PT. Sadhana Arif Nusa, East Lombok, West Nusa Tenggara, Indonesia (8°38'19.3"S 116°25'15.7"E). Each animal's blood was collected using a venoject tube with K2EDTA 0.5 M in the morning and stored at -25°C for several weeks. The genomic DNA extraction was performed using the Genomic DNA Mini Kit Geneid (ISO 9001:2008 QMS) procedure.

PCR amplification and genotyping

Based on the *Ovis aries* GenBank database (GenBank accession No. GU142847), reverse and forward intron 1 and exon 1 KISS1 gene primers were designed based on the caprine KISS1 gene published nucleotide sequence using Primer 3.0 software. The primers for the forward and reverse KISS1 gene amplification are presented in Table 1.

A total volume of 15 µL was used for DNA amplification, which included 1 µL DNA, 0.5 µL Primers, 6.25 µL MyTaq HS Red Mix, 2x and 7.25 µL Water (dH_2O). The reagent mixture was homogenized in 1.5 µL tubes before being distributed to each tube containing DNA samples and placed in the PCR machine. DNA amplification was performed in an Eppendorf Nexus Mastercycler PCR machine with pre-denaturation temperatures of 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 30 seconds. In one cycle, the final elongation stage was performed at 72°C for 5 minutes. In a 15 µL reaction mixture, 10 U of MwoI for intron 1 and 10 U of BsrI for exon 1 were used to separately digest 5 µL of each PCR product at 37°C overnight. The DNA fragments were separated using 2.5% agarose gel electrophoresis and then visualized using an AlphaImager EP Documentation and Analysis Systems (Alpha Innotech Corporation, USA).

Table 1. KISS1 gene sequences and primers

Gene	Primer sequence 5'-3'	Amplicons	Restriction enzymes	Annealing temperature (°C)
KISS1 (exon 1)	F: TGCAAAGCCGAGTGTGCAGG R: TGAAGGCGGTGGCACAAAGG	594 bp	BsrI	60
KISS1 (intron 1)	F: CCCGCTGTAACTAGAGAAAG R: CATCCAGGGTGAGTGATACT	377 bp	MwoI	60

Note: F: forward, R: reverse

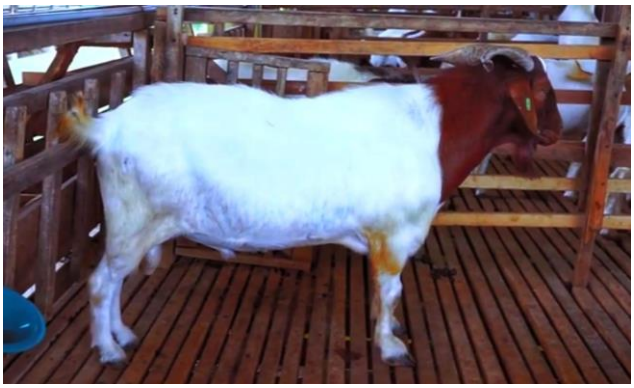

Figure 1. Boerka goat

Figure 2. Kacang goat

Data analysis

Using Popgene software version 1.31, the frequency of the genotype and alleles, heterozygosity, and Hardy-Weinberg equilibrium were examined. Gene Calc was used to investigate the polymorphism information content (PIC) (https://gene-calc.pl/pic). Using the Exon-Intron Graphic Maker (http://wormweb.org/exonintron), the KISS1 gene's structure was created. The Molecular Evolutionary Genetics Analysis (MEGA-X) program corrected SNP position determination to Ensembl's DNA sequence (ENSCHIG00000024611). Duncan's multiple range test was used to identify significant differences between genotypes and litter size traits. The association of KISS1 gene genotypes with litter size traits in Kacang goats and Boerka was analyzed using the SAS 9.1.3 software with GLM (General Linear Model) procedure, and the model applied:

$$Y_{iklm} = \mu + C_i + B_k + (BC)_{ik} + S_l + E_{iklm}$$

Where: Y_{iklm} is the trait measured on each of the iklmth animals; μ is the population mean; C_i is the fixed effect of the ith combined genotype; B_k is the fixed effect of the kth breed; $(BC)_{ik}$ is the interaction between the ith combined genotype and the kth breed; S_l is the fixed effect of the lth sire; and E_{iklm} is the random error.

RESULTS AND DISCUSSION

Gene structure and SNP position

The Kiss1 gene in goats is located on chromosome 16 (1,340,290-1,342,908), consisting of 3 exons and 2 introns with a length of 3819 bp (Accession No. AB433789/NC-022308.1). The SNP's position in the recent study is in exon 1 at a base of 331 bp and intron 1 at a base of 1.909 with base mutations of thymine to cytosine. The two SNP positions were corrected using MEGA-X and Ensembl software and presented in Figures 3 and 4.

In a previous study, some SNPs of the KISS1 gene have been found in sheep and goat breeds. An et al. (2013) had been detected eleven novel SNPs (g.384G>A, g.1147T>C, g.1417G>A, g.1428_1429delG, g.2124C>T, g.2270C>T, g.2489T>C, g.2510G>A, g.2540C>T, g.3864_3865delCA and g.3885_3886insACCCC) in Xinong, Saanen, and Guanzhong goat breeds. In Jining Grey goat, there are two mutations (G3433A and C3688A) in exon 3, three mutations (G296C, G454T, and T505A) in intron 1, and an 18 bp deletion/insertion (1960-1977) in intron 2 and no mutations in exon 2 (Cao et al. 2010). In nine Indian goat breeds, nine SNPs were identified in intron 1 (G296C, T455G, T505A, T693C, and T950C) and intron 2 (T1125C, A2510G, C2540T, and A2803G) (Maitra et al. 2014). Two SNPs, g2124T>A and g2270C>T, were found In Egyptian sheep and goat breeds (Othman et al. 2015). A novel polymorphism (G231C) was also identified in the promoter region of the KISS1 gene in Black Bengal, Ganjam, and Raighar goats (Sahoo et al. 2021). Several researchers have also reported that some SNPs significantly affect litter size in goat and sheep breeds (Zheng et al. 2018). Several researchers have also reported that some SNPs are associated with and significantly affect litter size in goat and sheep breeds.

El-Tarabany et al. (2017) stated that the SNP of the KISS1 gene in intron 1 was significantly associated with litter size in Damascus and Zaribi goats. The research results by Rahawy and AL-Mutar (2021) stated that the diversity of the KISS1 gene was significantly associated with litter size in Cyprus and Iraqi black goats. High levels of KISS1 gene expression were found in the pituitary of productive goats and were associated with litter size in Jintang black goats (Zheng et al. 2018).

Figure 3. SNP position of the KISS1 gene (g.331T>C) on exon 1

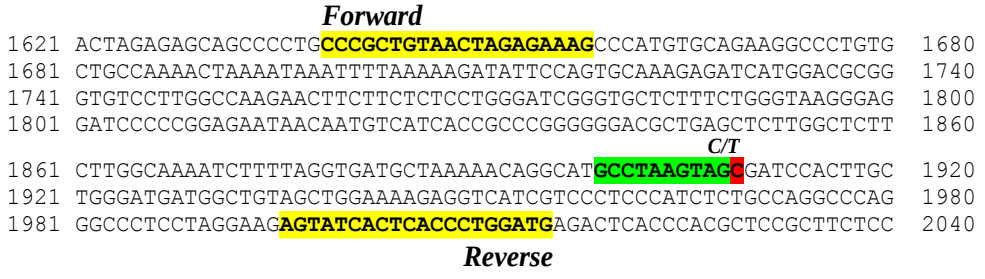

Figure 4. SNP position of the KISS1 gene (g.1909C>T) on intron 1

KISS1 gene (exon 1 and intron 1) polymorphism

The PCR amplification of exon 1 of the KISS1 gene produced an amplicon fragment with a length of 594 bp (Figure 5). The wild type of the Kiss1 gene exon 1 sequence has a cleavage site for the BsrI restriction enzyme (ACTGG|) at the 331 base position that produces alleles with sizes 321 and 273 bp (G allele). The result of the RFLP analysis using the BsrI restriction enzyme indicated that the KISS1 exon 1 gene in Kacang and Boerka goats was monomorphic because it only had the GG genotype (Figure 6). Like this study, Mulyono et al. (2019) reported that the KISS1 exon 1 gene in Ettawah crossbreed goats was also monomorphic. Feng et al. (2009) also showed that the KISS1 gene (exon 1) was monomorphic in Jining Gray, Mongolian Cashmere, Angora, and Boer goats through PCR-SSCP; there was no association between this locus and litter size.

In contrast to the results study, polymorphic sites in exon 1 of the Kiss1 gene have been published in goat and sheep breeds. Tarekegn et al. (2017) observed three SNPs (g.3416G>C, g.3811C>T, and g.3963T>C) that had a significant influence on litter size in Ethiopian Gondar and Woyto Guji goat breeds. Rahawy and AL-Mutar (2021) also found three different SNPs (g.893G>C, g.973C>A, and g.979T>G) in exon 1 of the Kiss1 gene in Iraqi black and Cyprus goat breeds, and one of them (g.893G >C) was significantly (P<0.05) associated with litter size. In sheep breeds, six mutations (C981T, C996T, T997C, C1034G, G1035A, and C1039T) and four genotypes (AA, AB, BB, and CC) were found in exon 1 of the KiSS-1 gene. Genotypes AA, AB, and BB were found in prolific Small Tail Han sheep, while genotypes AA and CC were found in prolific Hu sheep (Zergani et al. 2022).

According to the findings of this study, the SNP g.331T>C of the KISS1 gene (exon 1), which only contains one genotype, could not be utilized as a marker-assisted selection for fecundity qualities in the Kacang and Boerka goats. The possible reasons for explaining this condition: i) the likelihood that intensive selection has resulted in the accumulation of the GG genotype and the loss of other genotypes, or ii) the potential that only GG ewes exist because the KISS1 gene's SNP g.331T>C has not undergone mutation (Table 2).

The PCR amplification of intron 1 of The KISS1 gene produces the amplicon fragment with a length of 377 bp (Figure 7). The cleavage of the KISS1 gene amplicon (intron 1) by the restriction enzyme MwoI (GCCTAAG|TAGC) occurs at the 267 base position. It produces two alleles: T and A, with three genotypes: TT (267 and 110 bp) and AT (377, 267, and 110 bp) genotypes (Figure 8), whereas the untruncated resulted in the AA genotype (377 bp). The allelic and genotypic frequency distributions indicate a highly contrasting difference (P<0.01). The frequency of the T allele was significantly higher than the A allele, and the homozygote TT genotype was significantly higher than AT and AA genotypes in the two goat breeds (Table 3). Like this study, Mulyono et al. (2019) found two alleles (T and A), with a frequency of 89.15% and 10.85%, respectively, in Etawah-Grade doe.

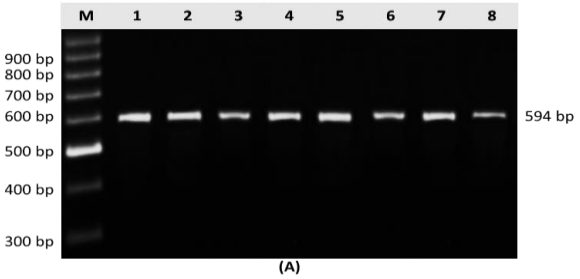

Figure 5. Exon 1 of Kiss1 gene amplicons (line 1 - 8); Marker 100 bp (M)

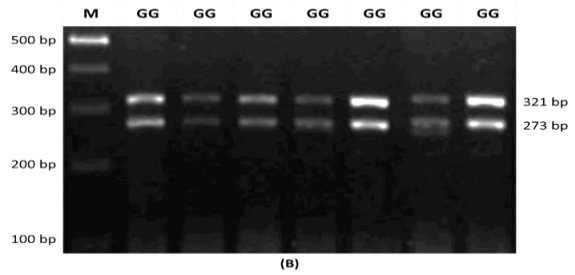

Figure 6. PCR-RFLP product of KISS1|BsrI gene (line 2 - 8); Marker 100 bp (M)

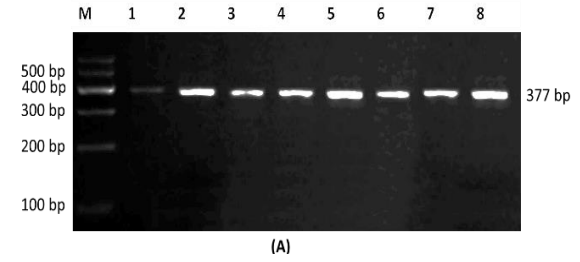

Figure 7. Intron 1 of KISS1 gene amplicons (line1 - 8); Marker 100bp (M)

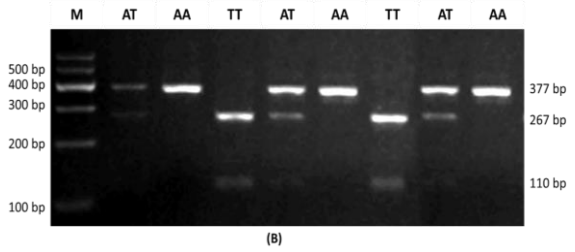

Figure 8. PCR-RFLP products of KISS1|MwoI gene (line 2 - 9); Marker 100 bp (M)

The T allele frequency of the KISS1 gene (intron 1) equally dominated the Kacang and Boerka goat populations. Previous studies in some goat breeds also discover an abundance of mutant T alleles compared to wild A alleles of intron 1 of the Kiss1 gene in Xmn1 cleavage sites, such as in prolific Egypt's small ruminant breeds (Othman et al. 2015), Baladi, Zaraibi, and Damascus goat breeds (El-Tarabany et al. 2017). In contrast, Sankhyan et al. (2019) found two alleles, T and A, in White Himalayan goats with no significant frequency differences, 0.57 and 0.43, respectively. However, they also reported the T allele in intron 1 of the KISS1 gene associated with greater litter size.

Genetic diversity within the goat populations

Table 4 shows information about genetic diversity measures in Kacang and Boerka goat populations. The genetic diversity was described by observed heterozygosity (Ho), expected heterozygosity (He), polymorphism information content (PIC), and Hardy-Weinberg equilibrium (HWE). The distribution of allele frequency of the KISS1 gene (intron 1) is consistent with Hardy Weinberg's law at the significance level of 0.05. The observed heterozygosity was lower than expected, which was a departure from the Hardy-Weinberg equilibrium. The occurrence of heterozygosity deficits is caused by selection pressure at certain loci and inbreeding (Sharma et al. 2016) in Kacang and Boerka goats at PT. Sadana East Lombok. Serrote et al. (2020) stated that the PIC value has high information when the PIC value is 0.50, while the value is 0.25 < PIC < 0.50, and low when the PIC value is 0.25. Overall, the populations of Kacang and Boerka goats showed moderate genetic diversity.

Association of The KISS1 gene (g.1909T>C) with litter size

The results of the association analysis of the KISS gene for Kacang and Boerka goats based on the genotypes obtained in the intron 1 region with litter size are presented in Table 5. The analysis showed that the three genotypes of the KISS1 gene were significantly associated (P<0.05) with litter size in Kacang and Boerka goats. The TT and AT genotypes have significantly larger litter sizes than the AA genotype in Kacang goats. Meanwhile, in Boerka goats, the TT genotype had a significantly larger litter size than the AT and AA genotypes. The coefficient of diversity for all genotypes of the KISS1 gene ranged from 24.38-40.40%. This value indicates that all genotypes of the KISS1 gene have high diversity. Brito et al. (2017) state that diversity is said to be low if the coefficient of diversity is below 5%. If the coefficient of diversity is between 6-14%, it is said to be moderate, and high if the coefficient of diversity is above 15%.

The present study found that the T allele was associated with larger litter sizes in Kacang and Boerka goats. The significant association of the T allele with superior litter size in Kacang and Boerka goats was also consistent with the previous results in Gaddi (Sankhyan et al. 2019), Jining Grey (Cao et al. 2010), Boer (An et al. 2013) and some Indian goat breeds (Maitra et al. 2014). Some previous studies also demonstrated that the polymorphism of intron 1 of the KISS1 gene was found to have a relationship with high prolific and sexual precocity in goat breeds, including Barki, Baladi, and Zaraibi, and in sheep breeds; Barki, Rahmani, and Ossimi (Othman et al. 2015). SNPs g.2124T>A and g.2270C>T were significantly related to litter size in Xinong Saanen, Guanzhong, and Boer goats (An et al. 2013). El-Tarabany et al. (2017) showed that TA and TT at the same locus had a high association with the litter size of Egyptian dairy goats. Othman et al. (2015) also stated that the TT and TA genotypes had the highest frequency in Baladi, Barki, and Zaraibi goats and were associated with litter size.

Given the importance of the KiSS1 gene as a regulator of puberty onset, the current study supports the hypothesis that KiSS1 gene polymorphisms have some relationship with fertility in small ruminants. The significant correlation between the TT genotype and larger litter size in the Kacang and Boerka breeds (P<0.05) is in line with earlier findings by An et al. (2013), El-Tarabany et al. (2017), and Abuzahra et al. (2023). Furthermore, Cao et al. (2010) also discovered a link between allele C of the 296 loci and allele deletion of the 1960-1977 locus in the caprine KiSS1 gene and large litter size in the Jining Grey breed. Rahawy and Al-Mutar (2021) stated that a mutation in g.893G>C was identified as an SNP polymorphism associated with litter size in Cyprus and Iraqi black goats. In Indian goat breeds, polymorphism of SNPs G296C at intron 1 was associated with litter size (Maitra et al. 2014).

Table 2. Genotypic and allelic frequencies of g.331T>C SNP in Exon 1 of the KISS1 gene

SNP position	Goat population	N	Genotype frequency			Allele frequency	
			GG	GC	CC	G	C
g.331T>C	Kacang	100	1.000	0.000	0.000	1.000	0.000
	Boerka	111	1.000	0.000	0.000	1.000	0.000

Table 3. Genotypic and allelic frequencies of g.1909T>C SNP in Intron 1 of the KISS1 gene

SNP position	Goat population	N	Genotype frequency			Allele frequency	
			TT	AT	AA	T	A
g.1909T>C	Kacang	100	0.630	0.290	0.080	0.775	0.225
	Boerka	111	0.694	0.243	0.063	0.815	0.185

Table 4. Genetic diversity measures in Kacang and Boerka goat populations across the loci of the KISS1 gene (g.331T>C)

SNP position	Goat population	N	Ho	He	se^2	se	PIC	X^2(HWE)
g.331T>C	Kacang	100	0.290	0.349	0.0008	0.0275	0.290	2.837
	Boerka	111	0.243	0.301	0.0009	0.0307	0.256	4.104

Note: N: Sample size; Ho: Observe heterozygosity; He: Expected heterozygosity; PIC: polymorphic information content; and HWE: Hardy-Weinberg equilibrium

Table 5. Genotype association of locus g.1909T>C KISS1 gene with litter size in Kacang and Boerka goats

Locus	Breed	Genotype	n	Average litter size	Coefficient of diversity (%)
g.1909T>C	Kacang	TT	63	1.80 ± 0.46^{a}	31.10
		AT	29	1.75 ± 0.59^{a}	33.85
		AA	8	1.14 ± 0.56^{b}	40.40
	Boerka	TT	77	1.75 ± 0.44^{a}	24.96
		AT	27	1.67 ± 0.41^{b}	24.38
		AA	7	1.59 ± 0.49^{b}	31.21

Note: ^{a,b}different superscripts within the same column in one breed represent a significantly different ($P<0.05$)

The KISS1 gene's product, kisspeptins, is crucial for regulating reproductive processes. They mainly work at the hypothalamic level, regulating the release of gonadotropin-releasing hormone (GnRH) from hypothalamic neurons to control the onset of puberty (An et al. 2013; Smith et al. 2014; Yarmolinskaya et al. 2016). The hypothesis that the KISS1 polymorphism is associated with high prolificity in small ruminants is based on KISS1's function as a regulator of puberty onset and is supported by current strong evidence. It also acts in peripheral reproductive tissues to directly influence ovarian function, embryo development, implantation, and pregnancy (D'Occhio et al. 2020).

The association between litter size and the Kiss1 gene polymorphisms due to mutations in the intron region must be explained, considering that the intron region is a non-coding sequence. Mutations in the intron region may not lead to changes in the amino acid sequence of Kisspeptin, but they could nonetheless result in a change in structural and functional properties (Fernández-Calero et al. 2016). Mutations in intron regions of a gene may also lead to changes in the protein's amino acid sequence if it occur at the initial site of the mRNA splicing after transcription. Thus, it can be concluded that the results of this study indicate that the g.331T>C locus of the KISS1 gene was monomorphic, and the g.1909T>C locus was polymorphic in the Kacang and Boerka goats. The polymorphic sites (g.1909T>C locus) significantly impact the litter size of the goat populations. This study found that the T allele significantly affected prolificacy in line with previous studies on Gaddi, Jining Gray, Boer, and some Indian goat breeds. The KISS1 gene polymorphism plays an essential role in the reproductive function of goats and has a potential role as a genetic marker related to reproductive traits.

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