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Research Article

Novel g.2055A>C and g.2064T>A Polymorphisms of KISS1 Gene and Its Association with Reproductive Traits in Local Indonesian Goats

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Abstract: Kisspeptins are known as neuropeptides encoded by the KISS1 gene, which potentiates GnRH neuron excitability. Unfortunately, the role of the KISS1 gene in reproductive traits remains unclear in Indonesian native goat breeds. The current study purposed to detect the genetic variation of the KISS1 gene and investigate the association with reproductive traits in goats to acquire marker-assisted selection (MAS) for the breeding program. Further, ninety blood samples from randomly selected animals were used for DNA isolation and SNP genotyping. The blood serum was collected from sixteen treated goat does to assess FSH levels using ELISA method. The differences between studied parameters were determined using independent samples T-test from SAS Software. Two SNPs, g.2055A>C (SNP1) and g.2064T>A (SNP2) were discovered in intron 1 of KISS1 gene in Indonesian native goat breeds. Two genotypes were identified from each SNPs: AA and AC at SNP1 and TT and TA at SNP2. The allele A (0.96) at SNP1 and allele T (0.93) at SNP2 were discovered as dominant alleles in the population. Furthermore, chi-square test showed that both SNPs were under Hardy-Weinberg equilibrium. The variants of KISS1 gene have no effect on litter size ($p>0.05$). Moreover, AA genotype of SNP1 had a higher FSH level than that observed in AC genotype ($p<0.0001$), especially in the follicular phase. SNP2 does not correlate with FSH level relatively ($p=0.23$). Furthermore, using of AA genotype at SNP1 of KISS1 gene as MAS for the breeding selection program could escalate the reproductive traits in goats.

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1. Introduction

In many circumstances, most of goats are reared in extensive/semi-extensive low-input systems with a relatively slight environmental influence (Peacocka and Sherman, 2010) leading low productivity (Bingöl and Bingöl, 2018). Reproduction, i.e., ovulation rate (OR) and litter size (LS), is critical to a profitable sheep or goat business. Further, animal selection based on genetic potency for reproductive traits could be a prominent strategy to increase animal performance (Notter, 2008; Li et al., 2009; Redden and Thorne, 2020). Unfortunately, the heritability for reproductive efficiency is low, causing hard fast genetic advancements (Sarma et al., 2019).

High fecundity in livestock species usually equals greater economic efficiency. Fecundity is the capability of animals to deliver live offspring (Notter, 2008). Fertility traits are complex quantitative traits managed by a few major genes and many minor genes (Wang et al., 2022). The KISS1 gene is recognized as an essential regulatory task in reproduction (Pinilla et al., 2012). KISS1 gene in caprine was discovered in chromosome 16, consisting of two coding regions (exons) and one non-coding region (intron). The length of the transcript is 408 bp and encodes 135 amino acids. This gene attains around 2.62 kilobases (ENSCHIT00000037363.1). Mulyono et al. (2019) and Hardyta et al. (2021) reported that there was no relationship among SNPs of the KISS1 gene with reproductive traits in local Etawah Grade (EG). Febriana et al. (2022) found g.2055A>C (SNP1) and g.2064T>A (SNP2) as novel SNPs of the KISS1 gene in Indonesian native goat breeds. Unfortunately, the correlation between these two SNPs with reproductive traits remains unclear.

Kisspeptins are neuropeptides encoded by the KISS1 gene (Pinilla et al., 2012). Nowadays, Kisspeptin–G-protein-coupled receptor 54 (GPR54) is a signaling pathway required for normal fertility, primarily located in GnRH neurons (Irwig, et al., 2004; Herbison et al., 2010; Mayer and Boehm, 2011; Kirilov et al., 2013). Kisspeptin has potent effects on GnRH neuron excitability (Han et al., 2005; Zhang et al., 2008; Dumalska et al., 2008; Pielecka-Fortuna and Moenter, 2008; Liu et al., 2008), indicating that this is a location for kisspeptin's actions within the HPG axis. The secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) out of the anterior pituitary was induced by the GnRH, which is needed to initiate puberty and maintain reproductive function (de Roux et al., 2003; Funes et al., 2003).

However, due to these traits' poor heritability and sex-limited nature, a traditional selection based on polygenic quantitative approaches for increasing reproductive traits in small ruminants becomes problematic (Janssens et al., 2004). Molecular tools combined with the traditional breeding technique are necessary to reach better results in animal breeding programs (Olsen et al., 1999). Further, breeding strategies based on molecular techniques and marker-assisted selection (MAS) as genetic information are critical for enhancing reproductive efficiency (El-Tarabany et al., 2017). The current study purposed to detect the genetic variation of the KISS1 gene, investigate the association with goat reproductive traits, and acquire marker-assisted selection for a breeding program.

2. Material and Methods

2.1. Experimental animal and phenotypic data

All experiment procedures implicating the animals and samples were under supervision by the Animal Ethics Committee of the Faculty of Animal and Agricultural Sciences, Diponegoro University, Indonesia, numbered 57-06/A-4/KEP-FPP, where the research was established. This research was carried out from August – November 2020. The animals in this research were selected randomly from three different regions. Blood samples (3 mL each) were taken from ninety uncorrelated does (thirty each from Kacang (KC) goats, Kejobong (KJ) goats, and Senduro (SD) goats) and stored in EDTA vacutainers. The goat data recording was collected, including litter size, parity (first to fifth parities), doe age, and the origin farms.

2.2. Polymerase Chain Reaction (PCR) and DNA sequencing of KISS1 gene

DNA isolation and PCR were used to amplify the particular fragment. The genomic DNA was extracted from whole-blood samples using a GeneJET Genomic DNA Purification Kit (Thermo

Scientific, USA). The quantity and purity of genomic DNA extraction were determined by 1% agarose gel electrophoresis and visualised under ultraviolet light in a gel documentation system.

A 227 bp fragment of the KISS1 gene at intron 1 was amplified by PCR using the following set of primers (F: 5'-GGACTCCACGACAAGAGGAG-3'; R: 5'-TCCCTTACCCAGAAAGAGCA-3') designed with Primer3 Plus web program based on the GenBank sequences of goat KISS1 gene (GU142847.1) The total volume of PCR amplification is 50 µL consisting of 4 µL DNA extraction containing 20-30 ng/µL, 1 µL for each primer (10 pmol/µL), 19 µL ddH2O and 25 µL of MyTaq Red Mix Bioline (1st BASE). The protocol for PCR was 5 min at 95°C for initial denaturation, followed by 35 cycles of denaturation at 94°C at 30 s, annealing at 59°C at 30 s, extension at 72 °C at 30 s, and final extension at 72 °C at 7 min. The amplicons were then sequenced in forward and reverse direction. The chromatograph could be used to detect the SNPs and heterozygosity.

2.3. Hormonal assay

Sixteen goat does with various LS were administered with 0.30 mg medroxyprogesterone acetate (MAP) intravaginal sponges during 14 days. The blood serum was obtained five times (0, 3, 6, 9, and 12 hours) after the intravaginal sponge was taken. Three milliliters of whole blood were stored in plain vacutainer tubes. The blood samples were then centrifuged (3000 rpm/5 min) to collect blood serum. The enzyme-linked immunosorbent assay (ELISA) method was used to assess FSH levels.

2.4. Statistical and phenotypic association

2.4.1. SNP genotyping

The MEGA X software (version 10.1) was used to align multiple sequences with the sequence from the GenBank (GU142847.1) to screen the presence of gene variants.

2.4.2. Linkage disequilibrium, genotype frequencies and allele frequencies.

Linkage disequilibrium (D') was estimated using DnaSP 6th version. Genotype frequencies were calculated using the direct counting methods (Nei and Kumar, 2000).

$$X_{ii} = \frac{n_{ii}}{n} \quad (1)$$

The allele frequencies were calculated according to the following formula (Nei and Kumar, 2000).

$$X_i = \frac{\left(2n_{ii} + \sum_{j \neq 1} n_{ij}\right)}{2n} \quad (2)$$

Where X_i is allele frequency of the i^{th} allele, X_{ii} is genotype frequency of the ii^{th} genotype, n_{ii} is the total number of samples with genotype ii, n_{ij} is the total number of samples with genotype ij and n is the number of total samples.

2.4.3. Hardy–Weinberg equilibrium, heterozygosity and association analysis

The Arlequin Software Package version 3.5. was used to count observed heterozygosity (Ho), expected heterozygosity (He), and Hardy–Weinberg Equilibrium (HWE) test (Excoffier and Lischer, 2010). The differences between studied parameters were determined using independent samples T-test from SAS Software (SAS® University Edition, 2018).

3. Result and Discussion

3.1. Detection of PCR amplicons and SNPs.

The KISS1 gene was amplified successfully; further, the PCR amplicons were separated using 2% agarose gels (Figure 1). It demonstrated that the length of amplified fragments and target DNA

fragments were consistent, and the specificity of the amplicon was apparent. Therefore, the amplicons could be directly analyzed by DNA sequencing (1st BASE Asia). The DNA sequences in Indonesian native goat breeds were 227 bp long and 99.8% identical to KISS1 gene of *Capra hircus*, which is registered on the GenBank database (GU142847.1). This condition indicated that the PCR products were targeted fragments of KISS1 gene.

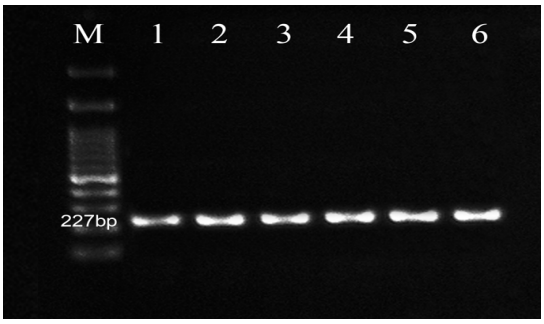
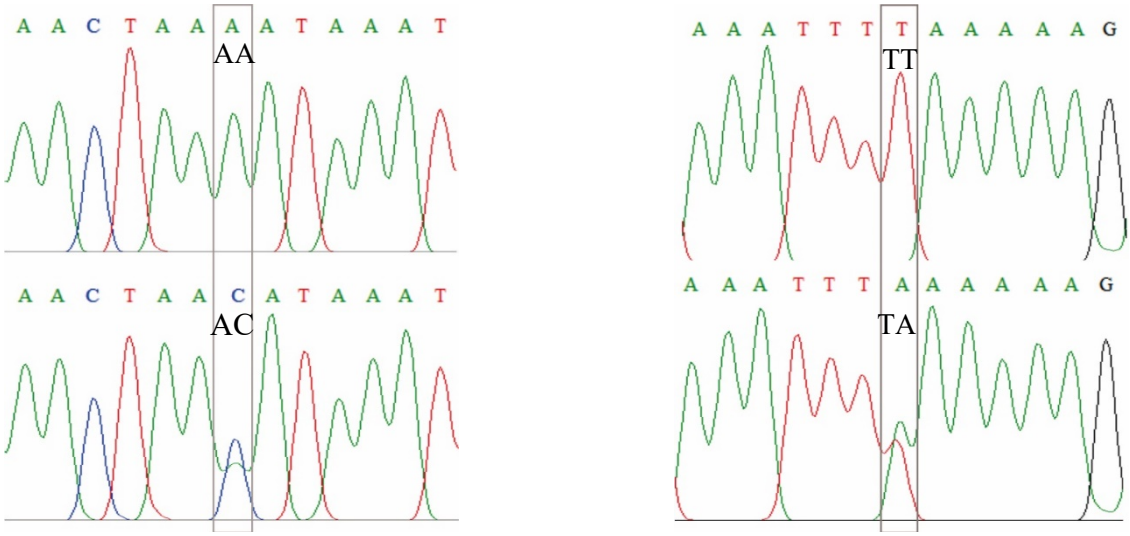


Figure 1. A 227 bp of KISS1 gene amplicon; M: Marker (DNA ladder 100 bp); Lane 1 and 2: Kacang goats; Lane 3 and 4: Kejobong goats; Lane 5 and 6: Senduro goats.

In the present study, two SNPs (SNP1 and SNP2) were discovered in intron 1 of KISS1 gene in Indonesian native goat breeds by screening the sample sequences and alignment with the database from GenBank (GU142847.1). Further, two genotypes were identified from each SNPs, namely AA and AC at SNP1 also TT and TA at SNP2 (Figure 2).



a) AA and AC genotype at SNP1 of KISS1 gene

b) TT and TA genotype at SNP2 of KISS1 gene

Figure 2. Chromatograms of SNP1 and SNP2 of KISS1 gene.

3.2. Genotype distribution

Table 1 summarizes the genotype and allele frequencies in three Indonesian native goat breeds based on the nucleotide sequence of KISS1 gene intron 1. The genotypic frequencies of AA and AC at SNP1 were obtained to be 0.86 and 0.14 in KC goats, 0.87 and 0.13 in SD goats, and 0.91 and 0.09 in overall population, respectively. Furthermore, the respective genotypic frequencies of TT and TA at SNP2 were found to be 0.75 and 0.25 in KJ, 0.86 and 0.14 in SD goats, and 0.87 and 0.13 in the overall population. SNP1 in KJ goats and SNP2 in KC goats were monomorphic. The allele A (0,96) at SNP1 and allele T (0,93) at SNP2 were discovered as dominant alleles in Indonesian native goat breeds. The allelic frequencies and genotypic frequencies among three native goat breeds showed similar

distribution, except in monomorphic goat breeds. According to Singh et al. (2015), these discrepancies in allelic frequencies could be due to the fact that various breeds reared under dissimilar environmental conditions are subject to varying degrees of evolutionary forces. Furthermore, sampling variations also contributed to variances in allelic frequencies between breeds and populations.

Table 1. Allele and genotype frequency of KISS1 gene intron 1

Loci	Allele	Allele frequency				Geno type	Genotypic frequency			
		Population					Population			
		KC	KJ	SD	Overall		KC	KJ	SD	Overall
SNP1	A	0.93	1.00	0.94	0.96	AA	0.86 (26)	1.00 (30)	0.87 (26)	0.91 (82)
	C	0.07	0.00	0.06	0.04	AC	0.14 (4)	0.00 (0)	0.13 (4)	0.09 (8)
SNP2	T	1.00	0.88	0.94	0.93	TT	1.00 (30)	0.75 (23)	0.86 (26)	0.87 (78)
	A	0.00	0.12	0.06	0.07	TA	0.00 (0)	0.25 (7)	0.14 (4)	0.13 (12)

KC: Kacang goats; KJ: Kejobong goats; SD: Senduro goats; Overall: genotypic and allelic frequencies in all population. The number of genotyped individuals are represented in the brackets.

Furthermore, the current research showed that the chi-square test for both SNPs in the population was under Hardy-Weinberg equilibrium ($\chi^2 = 3.81$; $p>0.05$). Recent situations suggest that breeding selection for litter size had little effect on genotypic frequencies (Alim et al., 2019) and due to a deficiency of significant heterozygosity (El-Tarabany et al., 2017). The χ^2 value and heterozygosity are presented in Table 2.

Table 2. Heterozygosity and Chi Square of SNPs discovered at intron 1 of KISS1 gene

Loci	N	KC			KJ			SD			Overall		
		Ho	He	χ^2	Ho	He	χ^2	Ho	He	χ^2	Ho	He	χ^2
SNP1	90	0.14	0.13	0.04	0.00	0.00	-	0.13	0.18	0.04	0.09	0.08	0.05
SNP2		0.00	0.00	-	0.25	0.22	0.16	0.13	0.12	0.04	0.12	0.12	0.11

Ho: observed heterozygosity; He: expected heterozygosity; (-): the $\chi 2$ value could not be counted because the goat breed was monomorphic; χ^2 distribution table (0.05; df = 1) = 3,841; Overall: the value in all sample population; KC: Kacang goats; KJ: Kejobong goats; SD: Senduro goats; N: the number of sample.

3.3. Effects of KISS1 genotypes on litter size and FSH profile in goats

In the current research, Table 3 showed that the variants of KISS1 gene have no impact with litter size ($p>0.05$). In contrary, Budisatria et al. (2012) reported that Indonesian native goat breeds have high prolific traits. In addition, previous research reported that a polymorphism of KISS1 gene is associated with reproduction traits (Cao et al., 2010; Hou et al., 2011; An et al., 2013; El-Tarabany et al., 2017; Mekuriaw et al., 2017; Sahoo et al., 2019; Jeet et al., 2022), which in turn could escalate litter size. This finding indicated a presence of other SNPs, which affected large litter size in Indonesian native goat breeds. Febriana et al. (2022) reported that a SNPs at g.2425C>G, g.2436A>G, and g.2459G>A of KISS1 gene have an effect on larger litter size in Indonesian native goat breeds.

Table 3. Litter size based on different genotype of KISS1 gene on Indonesian native goat breeds

SNPs	Genotype	N	Means ± SE	P value
SNP1	AA	82	2.36±0.13	0.67
	AC	8	2.50±0.17	
SNP2	TT	78	2.38±0.13	0.86
	TA	12	2.33±0.25	

SE: standard error; N: the quantity of sample.

Kisspeptins are known to play a vital role in determining reproductive activities in various species, especially at the hypothalamus level of the gonadotropic axis (Tu et al., 2014). In mammals, the follicle stimulating hormone (FSH) is a pituitary gonadotropin that leads to gonadal function and follicle growth (Bartlewski et al., 2009, Aerts and Bols, 2010). The FSH is in charge of the survival and

proliferation of follicular somatocyte, further the cyclic recruitment of ovarian follicles through maturation for the early antral stage until ovulation (McGee and Hsueh, 2000). The association of various genotype of KISS1 gene with FSH level in Indonesian native goat breeds were provided in Table 4.

Table 4. Means of FSH level on different estrus phase based on genotype of KISS1 gene intron 1

Loci	Genotype	N	Means ± SE		
			Overall	**Luteal phase**	**Follicular phase**
			----------mIU mL^{-1}----------		
SNP1	AA	82	3.16 ± 0.38^{b}	2.77±0.43	3.34 ± 0.64^{b}
	AC	8	1.35 ± 0.07^{a}	2.59±0.65	1.38 ± 0.02^{a}
p value			<0.0001	0.89	0.005
SNP2	TT	78	2.63±0.29	2.54±0.38	2.76±0.44
	TA	12	4.26±1.27	3.65±1.29	5.19±2.65
p value			0.23	0.40	0.43

Overall: means of FSH level in all population; Different superscript letters at the same column and SNP indicated substantial difference ($p<0.05$); SE: standard error; N: the amount of sample.

Analytical statistics revealed that AA genotype of SNP1 had a higher FSH level than that observed in AC genotype ($p<0.0001$), especially in the follicular phase ($p=0.005$). SNP2 has no correlation with FSH level relatively ($p=0.23$). Further statistical analysis showed there was no differences ($p> 0.05$) in FSH level between genotypes of SNP2, neither the luteal phase ($p=0.40$) nor the follicular phase ($p=0.43$). Fleming et al. (1996) reported that slight discrepancies in the FSH plasma concentrations between the genotypes in the present study could be led by small number of animals used that did not adequately represent the population's condition. In addition, SNP2 might be associated with other reproductive traits. For instance, SNP rs1213704663 C/G at 3'UTR region of KISS1 gene may be involved in estradiol hypersecretion, which may be responsible for deregulating the mechanism of GnRH secretion and stimulating LH hypersecretion with an elevated LH-FSH ratio (Ali, 2015; Zhu et al., 2019).

Previous research in Kaligesing goats in Indonesia showed that the polymorphism of KISS1 gene has no differences on both 17β-estradiol and progesterone levels (Hardyta et al., 2021). On the other hand, El-Tarabany et al. (2017) discovered that TT genotype at T121A of KISS1 gene in Egyptian goat breeds had higher 17β-estradiol and a greater amount of progesterone than TA genotype. According to Febriana et al. (2021) reported that goat breed, litter size, genotype, and haplotype have a significant association with FSH level.

Basically, prolificacy is governed primarily by the ovulation rate in small ruminants, which is determined by the development of preovulatory ovarian follicular (Nett et al., 2002). FSH concentrations are increased in livestock with larger litter size (Wikins et al., 1997). According to El-Tarabany et al. (2017), polymorphisms of KISS1 gene have a connection with fertility in small ruminants particularly; thus, the present research might lead to a crucial and applicable contribution to breeding selection.

Conclusion

The present study revealed polymorphisms of the KISS1 gene, leading to an association of the genotype of the KISS1 gene on the FSH level. As the sample number selected for the research was tiny, other researchers were required to determine the relationship between the SNPs of the KISS1 gene and FSH level in Indonesian goat breeds. AA genotype at SNP1 of the KISS1 gene is correlated with the best level of FSH in goats. Furthermore, the utilization of the AA genotype at SNP1 in the KISS1 gene for the breeding selection program could escalate the reproductive traits in goats.

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