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Detection of the effect of the *KISS1* gene on reproductive parameters in Saanen goats

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Abstract

The *KISS1* gene, which activates G protein-coupled receptor-54 (GPR54) and plays a crucial role in the neuroendocrine regulation of GnRH secretion, is known to encode a family of neuropeptides called kisspeptins. Therefore, the *KISS1* gene is thought to be associated with sexual maturity, offspring development, and estrus. The g.2124T>A polymorphism of the *KISS1* gene, is believed to affect the reproductive system, has been studied in Indonesian goat breeds, Damascus, Zarabi, Baladi, Gaddi, Kaligesing, Guanzong, Xinong Saanen and Boer goat breeds until now. These studies are generally related determining polymorphism frequencies or the effect of genotype on litter size. The study aims to determine the allele and genotype frequencies of the *KISS1* gene g.2124T>A SNP by the PCR-RFLP method and define the relationship between the genotypic structures and reproductive parameters such as luteal growth, maximum corpus luteum diameter, and mating rate in Saanen goats (n=30). According to the results, the genotype frequencies in Saanen goats for the *KISS1* gene g.2124T>A were 10.35%, 55.17%, and 34.48% for AA, TT and AT, respectively. Moreover, the genotypic structure did not have a statistically significant effect on the investigated fertility characteristics. The average values of expected heterozygosity (He), observed heterozygosity (Ho), the effective number of alleles (Ne), fixation index values (FIS), and the polymorphism information content (PIC) were 0.3996, 0.3448, 1.6655, 0.8629 and 0.855, respectively. The Hardy-Weinberg chi-square ($\chi 2$) value was found to be 0.5435. In conclusion, it was found necessary to study the *KISS1* - g.2124T>A polymorphism in larger herds with different gene pools as a reason for the existence of genotypic variation and the narrow population size.

Keywords: Fertility, Goat, *KISS1*, Saanen, Reproduction

Introduction

The reproductive behaviors in goats are seasonally dependent, and alterations related to this condition are regulated by annual light periods and environmental factors. The seasonal nature of reproductive activity is the primary factor limiting year-round breeding (1). Throughout the breeding season, goats undergo various consecutive hormonal cycles, the boundaries of which, varying in length according to breeds, determine the interval between two ovulations (2). To understand the hormonal and physiological events controlling the reproductive cycle, reproductive methods such as estrus synchronization and embryo transfer need to be employed (3). In order to overcome this physiological challenge, certain exogenous hormone protocols have been developed to extend reproductive activity throughout the year (4). These methods employed for estrus synchronization in goats are easily applicable, exhibit a high success rate, and are relatively cost-effective approaches (5). In the post-ovulatory period, granulosa and theca cells undergo

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proliferation and differentiation, transforming into luteal cells and forming the corpus luteum (CL) structure. The CL persists throughout pregnancy, secreting progesterone (6). It is known that high progesterone secretion is associated with the total number and size of CLs (7). In cases where fertilization does not occur, the CL structure undergoes regression, paving the way for the initiation of a new estrous cycle. Therefore, the presence of CL is crucial for the continuity of pregnancy (8). The quantity of offspring obtained in a single gestation is of paramount importance in goat farming for economic reasons, as in all animal species. Ensuring the continuation of pregnancy not only directly influences reproductive performance but also holds significance in terms of the number of offspring in a single gestation for small ruminant breeding. (9). Beyond environmental interventions such as hormonal applications and synchronization, the impact of the genotypic structure on reproductive performance should also be considered. Various genetic studies have been conducted to determine candidate genes and their effects on the number of offspring in a single gestation which is a significant parameter of reproductive performance. Recent investigations suggest that the *KISS1* gene may have a high degree of influence on multiple births (10, 11). Kisspeptin activity has been detected in the theca cells of growing follicles, the surface epithelium of the ovary, and corpus luteum structures in recent studies (12, 13).

The *KISS1* gene, which activates the G protein-coupled receptor (GPR54) and plays a role in the neuroendocrine regulation of GnRH secretion, is known to encode a neuropeptide family called kisspeptins (14, 15). The *KISS1* gene is reported to be associated with sexual maturity, offspring development, and estrus response (16, 17). The *KISS1* gene, believed to impact the reproductive system, has been studied in various goat breeds such as Baladi, Barki, Zaraibi (Egypt), Kacang, Kejobong, Sendura (Indonesia), Beetal, Black Bengal, Osmanabadi, Malabari, Gajam, Sirohi, Jakhrana, Ganjam, Sangamneri (India), as well as Guanzong, Boer, Damaskus, Xining Grey, Kaligesing, Cyprus, Irak Ethiopia, Gondar, Woyto Guji, and Xinong Saanen (9-11, 18-22). Febriana et al., (2022) found polymorphism in the *KISS1* g.2124T>A in Indonesian goat breeds (Kacang, Kejobong, Senduro); however, it was determined that this gene did not affect the number of offspring in a single gestation. In another study conducted on Damascus, Zarabi, and Baladi goat breeds, animals with the TT genotype for the g.2124T>A polymorphism were found to have higher levels of 17β-estradiol in the estrus phase compared to individuals with the TA genotype (22). Additionally, in the same study, goats with the TT genotype were found to have higher levels of progesterone in the mid-luteal phase. These results indicate that the *KISS1* gene may have an impact on reproductive performance. Similar results were obtained by Sankhyan et al., (2019) in Gaddi goats, with a correlation to the findings of El-Tarabany et al., (2017). The study revealed polymorphism in T125A (g.2124T>A), and TT genotype goats had a higher number of offspring in a single gestation (1.73 ± 0.15) compared to AT and AA genotypes. In contrast, in Kaligesing goats, a local breed in Indonesia, the TA genotype was higher (59.57%) compared to others; however, the investigated SNP (T125A) was reported not to cause any changes in 17β-estradiol and progesterone levels in the luteal or follicular phases (24). Furthermore, the expression of the *KISS1* gene in prolific goat ovaries such as Barbari and Jamunapari was determined, suggesting that the increased expression level might be a cause for the high number of offspring in these breeds. Likewise, in Xinong Saanen, Guanzhong, and Boer goat breeds, the TA genotype frequency was found to be high, similar to the Kaligesing breed (10). Additionally, in the same study, it was found that the combination (C1) of g.2124T>A and g.2270C>T SNPs had significant effects on the number of offspring in a single gestation. The conducted studies generally focus on determining polymorphism frequencies or detecting the impact of genotypes on the number of offspring in a single gestation. Notably, there is a lack of research on the effect of the *KISS1* gene on luteal structures. Furthermore, studies on the *KISS1* gene in Saanen breed goats are observed to be insufficient or absent in the literature. Saanen goat, which is widely reared in Türkiye as a dairy breed, makes a significant contribution to small ruminant breeding with a high milk yield (700-800 liters/average 280 days of lactation) and reproductive performance (25). Literature information regarding the SNP planned to be studied is limited for Saanen breed goats (only Xinong Saanen), and detailed studies are lacking.

For this purpose, in the planned study, the genotype of Saanen breed goats for the *KISS1* - g.2124T>A polymorphism will be determined, and the impact of this genotype on the luteal structures will be assessed using data obtained from ultrasonic examinations (detection and measurements of luteal structures). Thus, it is anticipated that the *KISS1* - g.2124T>A polymorphism will be elucidated in the target population, contributing to the literature, and the impact of this SNP on reproductive performance will be revealed.

Materials and Methods

Animals and Synchronization

The goats involved in the research were housed at Bursa

Uludag University, Faculty of Veterinary Medicine Animal Health and Production, Research, and Application Center (latitude 40° 11' N, longitude 29° 04' E, altitude 155 m). The goats were housed in pens with sand/hay flooring, offering access to an outdoor sheltered paddock under natural photoperiod and temperature conditions. Their diet consisted of dry grain wheat hay (1500 g/doe/day) supplemented with commercial pellets (18% crude protein; 800 g/doe/day, 2800 Kcal). Throughout the study, no additional food was provided to the goats. Animals had ad libitum access to clean drinking water and mineralized salt, with no alterations made to their nutrition during the experimental period. The does and bucks shared the same enclosure, but physical contact between them was prevented. The study was conducted during the transition period to the breeding season (June). Goats aged >10 months, live body weight >25 kg, and body condition score >3 (on a scale of 1-5) were utilized. All methodologies and management procedures employed in this study were scrutinized and approved by the Animal Experiments Local Ethics Committee of Bursa Uludag University (Approval number: 2023-03/01). Intravaginal sponges containing 60 mg medroxyprogesterone acetate (MAP) were applied to the goats for 6 days for estrus synchronization. Twenty-four hours before the sponge removal, 300 IU equine chorionic gonadotropin (eCG) and 125 µg d-cloprostenol (PGF2α) were injected intramuscularly. After the removal of the sponges in all goats, estrus signs were monitored using teaser males every 12 hours (08:00 and 20:00) from 24 to 120 hours, with each observation period lasting at least 30 minutes. All goats underwent examinations using a B-mode real-time ultrasound device equipped with a 7.5 MHz transrectal linear probe (model UST-660, Prosound 2, Hitachi Aloka Medical, Ltd., Tokyo, Japan). Corpus luteum (CL) structures on the ovaries were identified and measured approximately on the fifth day of the estrous cycle. During the examination, goats were held in a standing position, and after applying water-soluble contact gel, the linear probe was gently directed towards the rectum. After passing the urinary bladder and uterine horns, lateral movements were applied to the ultrasound probe to visualize structures on the ovaries (4). The mating rate was obtained by the observation of the doe first standing to be mounted by the teaser buck.

DNA isolation

Blood samples (4 mL) were aseptically collected from the Vena jugularis of goats into K3-EDTA tubes (2 tubes). Genomic DNA was isolated from the collected blood samples using the phenol-chloroform method (26). The purity and quantity of the DNA samples were assessed using a NanoDrop device. DNA samples, whose quantity and quality were ensured, were stored at -20°C until the implementation of the subsequent laboratory procedures.

SNP analyses

The PCR-RFLP method was used to determine polymorphisms related to the *KISS1* gene. For the targeted *KISS1* g.2124T>A polymorphism, detailed information including the primers that were used, the size of the targeted region, PCR conditions, restriction enzyme, and the reference study is provided in the table below (Table 1) for polymorphism identification. For the examined g.2124T>A polymorphism, individuals with the AA genotype are expected to exhibit a single uncut band (377 bp), while individuals with the TT genotype are expected to present two bands (256 and 121 bp). The products obtained after PCR and RFLP procedures will be evaluated through agarose gel electrophoresis.

Table 1. The PCR conditions, restriction enzyme and primer sequences of the *KISS1* gene

Gene-SNP	Primers (5'-3')	Length	PCR Conditions	Restriction Enzyme	Reference
KISS1 g.2124T>A	F: CCC GCT GTA ACT AGA GAA AG R: CAT CCA GGG TGA GTG ATA CT	337 bp	94°C 5 min. (95°C 1 min.,56°C 1 min.,72°C 1 min.) x 35 cycle 72°C 5 min.	*XmnI*	*An et al., 2013; Othman et al., 2015*

Agarose Gel Electrophoresis

The products obtained after PCR and RFLP procedures were evaluated using agarose gel electrophoresis. For this purpose, the PCR product of the *KISS1* gene was assessed on a 1.5-2% agarose gel using electrophoresis at 85-90 volts. Enzyme-digested products were run on a 2-2.5% agarose gel at 80-85 volts for 50-100 minutes, proportionate to the product size. Subsequently, all obtained products were visualized and assessed using the DNr's MiniLumi bio-imaging system.

Statistical Analyses

The mean and standard errors (SEM) of data obtained from phenotypic information were analyzed using the SPSS software (Version 20). To assess the distribution of the obtained means, the Shapiro-Wilk test was applied, and subsequently, the statistical status between groups was evaluated using the Kruskal-Wallis method based on the results of this test. Individual comparisons between groups were performed using the Mann-Whitney U test as required by the method. For mating rate analyses, the Chi-square test was employed.

Regarding the genotypic data obtained from the herd, population genetic parameters such as genotype and allele frequencies, expected heterozygosity (He), observed heterozygosity (Ho), Hardy-Weinberg Equilibrium (HWE),

effective allele number (Ne), fixation index values (FIS) or polymorphism information content (PIC) were evaluated. The values of He, PIC, and Ne were calculated following the references of Nei and Roychoudhury (1974) and Botstein et al., (1980). Allele and genotype frequencies were assessed using the Hardy-Weinberg equilibrium chi-square test ($\chi 2$), and the PopGene32 program was utilized for this purpose (29).

Results

Genotypic parameters

The 377 bp PCR products were obtained after agarose gel electrophoresis and are shown in Figure 1. After the amplification, the PCR products were digested by the XmnI restriction enzyme. According to the gel electrophoresis results, the investigated Saanen goats were found polymorphic for *KISS1* g.2124T>A polymorphism (Figure 2). The polymorphism for TT genes was the most frequent genotype with 55,17% in Saanen goats. The other genotype frequencies, AT (377 and 256 bp) and AA (377 bp) were calculated at 34,48% and 10,35%, respectively. The allele frequencies were 0.276 and 0.724 for A and T, respectively. The assessment of population genetics parameters exhibits that the expected heterozygosity was 0.3996, the observed heterozygosity was 0.3448, the number of the effective alleles was 1.6655, the FIS value was 0.8629 and the polymorphic information content was 0.855. Based on HWE, the investigated flock had not deviated from the equilibrium (P=0.4609).

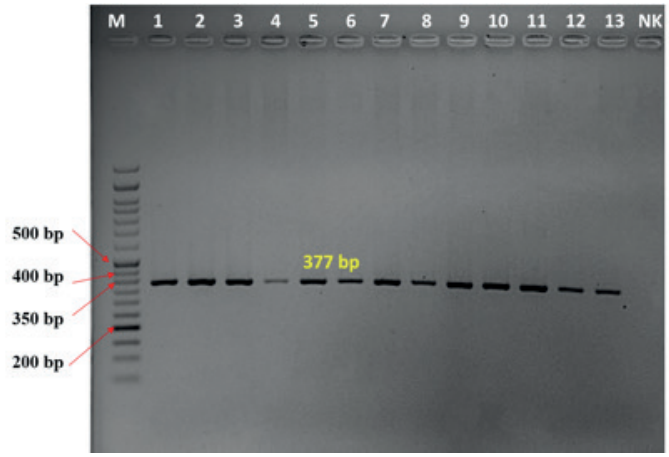

Figure 1. Agarose gel electrophoresis image showing PCR product of the KISS1 gene in Saanen goats

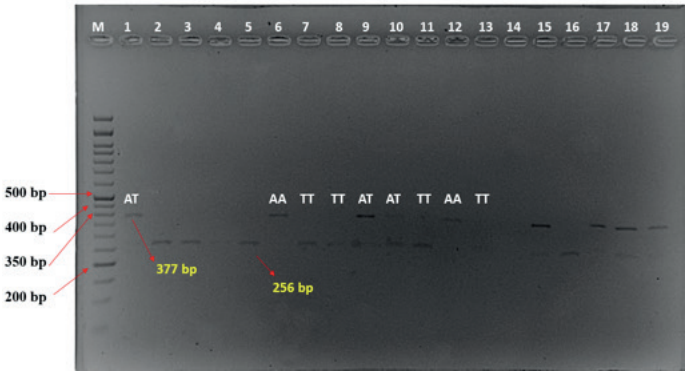

Figure 2. The enzyme products of KISS1 g.2124T>A polymorphism in Saanen goats

Reproductive parameters

A summary of data regarding the interaction between the polymorphism status of genes and luteal growth (mm/day), maximum corpus luteum diameter (mm), and mating rate (%) after the synchronization of estrus in the goats are shown in Table 3. In terms of luteal growth parameters, there were no statistical differences found in the study (P>0.05). The highest growth was measured as 1.72 mm/day in the animals that presented the TT genotype. Since there is a huge numerical gap amongst the results, the main reason for this phenomenon is the uneven distribution in the number of animals (n=3 with AA genotype). The corpus luteum structures are given in Figure 3. The mean maximum corpus luteum diameter of the study was measured as 8.38±0.64 in the study within the animals with TT genotype. The results of this parameter were also close to each other, yet any kind of difference with statistical importance was not found (P>0.05). In terms of mating rates among the different genotypes of the study, the animals with the AT genotype yielded the highest rate with 70%. However, the statistical difference was not there again and the results were pointed out in the same statistical manner as two previous findings of the study.

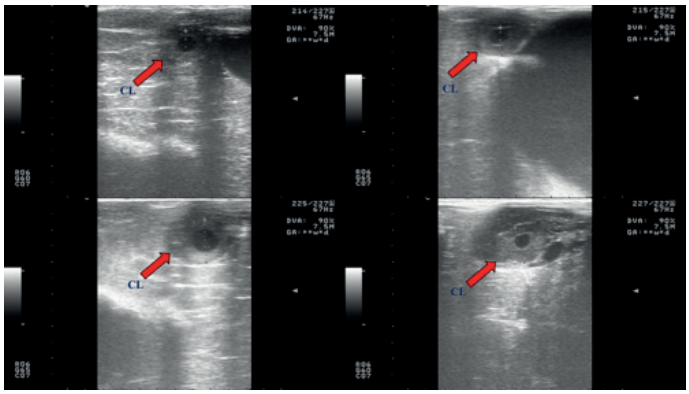

Figure 3. The ultrasound images of Corpus luteum structure in Saanen goats.

Table 3. Effect of *KISS1* gene g.2124T>A polymorphism on reproductive parameters in Saanen goats

Genotype		*Reproductive Parameters*			
	n	**Luteal Growth (mm/day)**	**Max CL Diameter (mm)**	**Age (Year)**	**Mating Rate**
AA	3	0.10±0.00	8.20±0.00	2.17±0.72	2/3 (66.67 %)
TT	16	1.72±0.22	8.38±0.64	3.29±0.29	7/16 (43.75%)
AT	10	1.11±0.39	8.32±0.73	2.93±0.28	7/10 (70.00 %)

Discussion

In ruminants, the hormone progesterone (P4) is synthesized by the corpus luteum and plays a pivotal role in both the initial establishment and subsequent sustenance of pregnancy (30). The structure of CL holds intactness and produces progesterone to keep the next generation alive (31). The CL is known to be formed from the remains of graffian follicles after ovulation mainly with the control of luteinizing hormone (LH) (32). The hormonal functions of the CL are controlled by the hypothalamus-pituitary axis (33), therefore GnRH plays a crucial role in this act. Kisspeptins that are encoded by the *KISS1* gene, have a massive effect on the regulation of GnRH and naturally the breeding functions (34). It is shown by the previous studies in ewes (35, 36) that the kisspeptins regulate the LH surge by stimulating the release of GnRH. In a current study, Mishra et al., 2019 indicated the characterization and immunolocalization of *KISS1* receptors in the CL structure of buffalo.

The success of animal breeding has closely been linked to the reproductive performance of the small ruminants as the other farm animals. For this purpose, lots of polymorphisms or mutations of candidate genes have been studied up to now like the *KISS1* gene. The *KISS1* g.2124T>A polymorphism that has strong evidence related to the reproductive system has been investigated by Balabadi, Barki, Zaraibi (11); Gaddi (23); Kaligesing (24); Xinong Saanen, Guanzhong, Boer, (10); Damascus, Baladi (22) goat breeds. A comparison table of allele and genotype frequencies of different breeds is presented in Table 4. According to the literature (10, 11, 22, 23, 24) the most frequent allele was found as T for the mentioned breeds except Kaligesing goats. The results obtained in the present investigation are in agreement with the findings reported by Othman et al., (2015) and El-Taranbany et al., (2017) in Baladi and Zaraibi goats. The allele frequencies were determined 28.57% and 22.22% for A, 71.43%, and 77.78% for the T allele in Baladi and Zaraibi goats by Othman et al., (2015). El-Tarabany et al., (2017) pointed out that A and T alleles frequencies were 0.24 and 0.76 in Zaraibi goats. Our results showed similarities as demonstrated in previous studies performed in Baladi and Zaraibi goats; the allele frequencies were 0.276 and 0.724 for the A and T allele of *KISS1* g.2124T>A polymorphism in the current study. Differing from Hardyta et al., (2020) who claimed the most frequent allele was A with a 70.21% percentage in Kaligesing goats, the most frequent allele was found in T in Saanen goats, as given in Table 2. According to the genotype frequencies, the most frequent genotype observed TT (55.17%) in the present study. The observations are compatible with the work of Othman et al., (2015) who declared the TT allele frequency was 55.56%. Contrary to results published by Othman et al., (2015) and El-Tarabany et al., (2017), the AA genotype was detected in Saanen goats (10.35%). An et al., (2013b) emphasized that the heterozygote genotype (AT) was most frequent in Xinong Saanen goats. Moreover, the least common genotype frequency belonging to AA was observed in that study (19.28%). In close agreement with An et al., (2013b), it was determined that the least frequent genotype was AA with 10.35%. Heterozygous genotype frequency (34.48%) was found to be lower compared with Baladi, Barki, Zaraibi (11), Gaddi (23), Kaligesing (24), Xinong Saanen, Guanzhong, Boer (10) and Damascus (22) goats.

Table 4. Frequencies of homozygous and heterozygous genotypes reported in studies on various goat populations for g.2124T>A polymorphism of *KISS1* genes.

Breeds	*Genotype frequencies*			*Allele frequencies*		*References*
	AA	**AT**	**TT**	**A**	**T**	
Baladi	-	57,14	42,86	28.57	71.43	*Othman et al., 2015*
Barki	-	62,50	37,50	31.25	68.75	
Zaraibi	-	44,44	55,56	22.22	77.78	
Gaddi	0,17	0,52	0,31	0.43	0.57	*Sankhyan et al., 2019*
Kaligesing	40,43	59,57	-	70.21	29.79	*Hardyta et al., 2020*
Xianong Saanen*	19,28	42,16	38,56	0.40	0.60	*An et. al., 2013b*
Guanzhong*	20,36	41,18	38,46	0.41	0.59	
Boer*	24,49	41,84	33,67	0.45	0.55	
Damascus	-	0,75	0,25	0.37	0.63	*El-Tarabany et al., 2017*
Zaribi	-	0,48	0,52	0.24	0.76	
Baladi	-	0,82	0,18	0.41	0.59	
Saanen	**10,35**	**34,48**	**55,17**	**0.276**	**0.724**	*Current study*

**Genotypic frequencies were calculated according to the number of genotypes that were given in the literature.*

In terms of population genetic diversity parameters, the PIC value was determined to be 0.855 in the Saanen population. It was known that a PIC value close to 1 indicates that it is highly informative. Thus, the obtained value from the study could be considered a highly informative polymorphism, and, the diversity of alleles is high in the examined locus. It was observed that the expected (0.3996) and observed (0.3448) heterozygosities were close to each other. To evaluate the HWE, the differences between the observed and expected frequencies of the genotypes were tested using a $\chi 2$ test with a significance level of $p<0.05$. the assessment of genotype distribution within the studied population was in Hardy–Weinberg equilibrium for both *KISS1* g.2124T>A polymorphism.

Table 2. The allelic and genotypic frequencies of *KISS1* gene polymorphism and population genetic diversities including Hardy-Weinberg equilibrium in Saanen goats.

GENOTYPE	**AA**	**AT**	**TT**
n	3	10	16
Frequencies (%)	10,35	34,48	55,17
ALLEL	**A**		**T**
Frequencies	0.276		0.724
He	0.3996		
Ho	0.3448		
Ne	1.6655		
PIC	0.855		
F_{IS}	0.8629		
χ^2(HWE)	0.5435		
P*	0.4609		

χ2 (HWE): Hardy–Weinberg equilibrium χ2 value; He: gene heterozygosity; Ne: number of effective alleles, PIC: polymorphism information content, *p >0.05; consistent with HWE.

Conclusion

In conclusion, the investigated Saanen breed was determined polymorphic for *KISS1* g.2124T>A polymorphism. Thus, it was found necessary to study the *KISS1* - g.2124T>A polymorphism in larger herds with different gene pools as a reason for the existence of genotypic variation and the narrow population size.

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