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The Association of SNP (t.18085 T>G) of GNRHR Gene with *Litter Size* as a Candidate Genetic Marker in Senduro Goat

Nur Hidayah[a], Tri Eko Susilorini[a], Ari Ardiantoro[a], Guruh Prasetyo[a], Heri Damayanti[a], Suyadi[a].
[a]Animal Science Faculty, Brawijaya University, Malang, Indonesia
*Corresponding author: suyadi@ub.ac.id

Abstract
GNRHR gene is a candidate gene for litter size due to its essential role in regulating the activities of the hypothalamus-pituitary-gonadal axis, which synthesizes and releases gonadotropins. This study aimed to investigate the association of *GNRHR* gene with litter size as a candidate genetic marker on 76 Senduro goats using RFLP method. The analysis of DNA isolation was precise. Amplification and restriction analysis used enzyme Msp1 (C*CGG) with a product length of 706 bp, released TT genotype and T allele in GNRHR gene fragment. Single nucleotide polymorphisms (SNPs) t.18085 T>G was found in exon 3. The Hardy-Weinberg law calculation obtained insignificant results because the value is more than > 0.05 (db; 1) = 0.50. Allele frequencies and genotypes obtained are monomorphic. Therefore, the GNRHR gene in exon 3 was not associated with Senduro goats' litter size.
Keywords: Senduro goat; litter size; PCR-RFLP; GNRHR gene; Msp1 enzyme.

Introduction

Goats are essential for rural communities in Indonesia, where rural people's livelihoods include farmers. Raising livestock is a side business to increase the income for farmers (Suyadi et al., 2020). Goat farming provides several benefits, including the animals' propensity to adapt to their surroundings, low capital requirements, and simple maintenance needs. Senduro goats are indigenous goats. These goats are regarded as a potential genetic resource for producing milk and meat. In the Lumajang Regency region of East Java, Senduro goats are raised extensively.

Senduro goat has been produced from crossbreeding of Indian Ettawa goats, Kacang goats, and Jawarandu goats for over a century. These goats are advantageous since they produce 1.3 L of milk per day on average (Wardani et al., 2022). This goat is often referred to as a local goat because it has adapted to the environment in Indonesia (Susilorini et al., 2017). Senduro goats are indigenous livestock in Indonesia, particularly dairy goats, and they are among the goats that produce the most goat milk (Arief et al., 2020).

Litter size is an important economic trait and is closely related to the economic benefits of livestock breeding (Liang et al., 2021). Each goat's litter size is impacted differently by genetic changes that speed up ovulation and enhance the number of kids produced per pregnancy (Abuzahra et al., 2023). The genetics of litter size emphasize the importance of significant fecundity genes in goats. There needs to be an effort to increase the number of litter sizes in Senduro goats to produce optimal litter size. The development of Senduro goats aims to enhance the potential goat that exists in the country. One of the most critical factors in increasing the litter size is the selection of goats.

Recently, selection programs used in several countries have begun implementing DNA marker-based selection programs or Marker Assisted Selection (MAS). Marker-Assisted Selection (MAS) is a selection method utilizing candidate genes. MAS has an advantage for farmers because this selection is carried out early, which can reduce maintenance costs, and selection can be done early so that culling of goats with poor genetic potential can be done immediately (Maylinda et al.,2015). Gonadotropin-Releasing Hormone Receptor (GNRHR) is one of the candidate genes for goat litter size under marker-assisted selection. This selection (MAS) using the GNRHR gene needs to be done due to a crucial decision to get good genetic potential in goat's early time.

GNRHR is a crucial regulator of the reproductive system, which causes the pituitary gland to produce and release luteinizing hormones (LH) and follicle-stimulating hormones (FSH) (Yang et al., 2011). The synthesis and release of the gonadotropic hormones LH and FSH control the development of gametes and gonadal hormones (Naor et al., 2009). Follicles cannot progress through the early antral stage without enough FSH or LH, preventing ovulation (Li et al., 2011). An essential process in the endocrine control of reproduction is the interaction between GnRH and its receptor. The pituitary, the gonads, and the hypothalamus all express the GNRHR.

There has not been any information about the association of GNRHR genetic variations with litter size in Senduro goats. Given that the GNRHR gene is linked to luteinizing hormone and follicle-stimulating hormone release (Li at al., 2011), it may be a candidate gene for litter size in goats. This study aimed to identify polymorphisms in the GNRHR gene and evaluate the associations between GNRHR gene and litter size.

Materials and methods

The Polymorphism of GNRHR gene was detected by the PCR-RFLP method on 76 Senduro female goats. These selected goats were healthy, actively reproductive, and had given birth at least one. The observation was conducted from January 2023 to Mei 2023. This research was carried out in different locations. The senduro goat was provided at Technical Implementation Unit Animal Breeding and Forage Providing, Singosari, Malang, East Java, for taking blood samples and litter size data. The DNA extraction and analysis of blood samples of Senduro goats was conducted at laboratory at the Livestock Biotechnology Laboratory, Faculty of Animal Science, Brawijaya University, Malang, .

A blood sample of senduro goat took in the neck, precisely in the jugular vein, as much as 5 ml. Moreover, the research laboratory includes DNA analysis (Isolation DNA, PCR, and RFLP). DNA isolation to separate DNA from white blood cells. Initially, research is carried out by taking blood samples of each goat as much as 5 mL with venoject in the area of the jugular vein in the neck region. Laboratory materials alkohol 70%, Genomic DNA Mini Kit (Geneaid), ethanol absolute, nucleus Free Water (NFW) (Promega), Buffer tango, gel agarose,1X Green Master Mix (Promega), TBE 0,5X, Loading blue dye, Nucleic Acid Dye Diamond, Restriction enzyme MspI (BioLabs), DNA marker.

We used a variety of necessary laboratory instruments and equipment in our investigation to make our research more accessible. We used hand gloves for safety and protection. A HETTICH Mikro 185 high-speed centrifuge was used to separate the samples effectively. Micropipettes from Select BioProducts allowed for precise measurements, and a tube rack organized our sample collection. Glass beakers from IWAKI were utilized for diverse solutions, and the hot plate (BIAB) permitted regulated heating procedures. During our investigation, pipette tips from Axygen Scientific and tubes made by the same company were used to transfer liquids accurately. Wiggens Vortex 3000 Eppendorf vortex tubes were essential for appropriate agitation and mixing. Using a Force mini-SBC-140-3 microcentrifuge, samples were centrifuged. The Bio-Rad T100 Thermal Cycler was used for DNA amplification. Spatulas, a Panasonic NN-SM32HM microwave, and Bio-Rad mupit-ex electrophoresis equipment were used to help with lab duties. Incubators were used to maintain the sample (Memmert IN). A blue light system called the Gite 965 GW was used for gel documentation.

This genetic characterization involves several stages, including DNA isolation, qualification, and PCR-RFLP. This study used a genomic DNA Mini Kit (Geneaid) for DNA isolation. Furthermore, DNA qualification was performed by electrophoresis using 1.5% agarose gel with diamond nucleic acid (Promega), with a voltage of 100 volts for 35 minutes.

Gel doc visualized the electrophoresis results. Gene candidate amplification using primer pairs is shown in Table 1. below:

Table 1: Length, location and sequence of primary pairs

Gene Bank	Length(bp)	Location	Sequence	Gene
GNRHR	706	Exon 3	F : $^{5'}$CACCAGGTTGAATTACGAT$^{3'}$ R : $^{5'}$AGGTCTGTGTTTCTCAGTG$^{3'}$	CM004567.1

Note : F = Forward R = Reverse

The amplification procedure was carried out following the PCR steps in Table 2. In the PCR reaction, the total volume of each sample was 15 µl, consisting of forward and reverse primers of 0.25 µl each, Nucleous Free Water (NFW) of 6.5 µl, Gotaqgreen of 7 µl, and DNA samples of 1 µl.
The results of the amplification were obtained by electrophoresis on 1.5% agarose gel stained with diamond nucleic acid (Promega) at 100 volts for 35 minutes. Then they used UV light from gel doc to visualize the result. The amplified band was cut with the Msp1 restriction enzyme from Thermoscientific brand according to the gene locus with the following composition of enzyme 0.4 µl, 10X buffer BSA (Buffer tango) volume 0.7 µl, 0.9 µl Free Nucleus Water, with 5 µl of PCR product DNA.

Agarose gel stained (2%) with diamond nucleic acid (Promega) was used for the electrophoresis, which took place at 100 volts for 45 minutes before being seen with a gel doc and UV light. DNA Ladder from Thermoscientific brand determined the genotype identification of each sample based on the size and pattern of 100 bp.

Table 2: Optimal temperature, time, and cycle of PCR stages

Gonadotropin-Releasing Hormone Receptor gene (GNRHR)			
Stages	**Temperature (^{0}C)**	**Time(hour:minute:second)**	**Cycle**
Pre-denaturation	94	00:05:00	1 x
Denaturation	94	00:00:30	35x
Annealing	59	00:00:45	35x
Extention	72	00:01:00	35x
Final Extention	72	00:05:00	1x

Data Analysis

Genotype and allele frequency

Genotype frequency, determined by dividing the total number of genotypes by the total number of samples, is the proportion or percentage of a specific genotype in a population.

$$x_{ii} = \frac{n_{ij}}{N}$$

Description :
x_{ii} = Genotype frequency
n_{ij} = Observed genotypes
N = Total population

The percentage of a specific allele in a population relative to all other alleles occupying the same locus from the PCR-RFLP characterization analysis is known as the allele frequency. Using the following formula from Nei and Kumar (2000), This study examined allele frequency:

$$x_i = \frac{2n_{ij} + \sum i \neq j \, n_{ij}}{2N}$$

Description :
xi = The allele frequency of the samples,
nii = Sample genotype ii,
nij = Sample genotype ij,
N = Total sample.

Results and discussion

Amplification of Gonadotropin-Releasing Hormone Receptor (GNRHR) Gene Fragment in Senduro Goat.

Polymerase chain reaction (PCR) amplifies specific fragments of DNA molecules and has been extensively applied in gene mutation detection (Mendenhall et al., 2012). The product of amplification of the Gonadotropin-Releasing Hormone Receptor (GNRHR) gene fragment obtained has a length of 706 bp. The amplification results were electrophoresis using 1.5% agarose visualized under UV light on gel doc presented in Figure 1 on Senduro goat.

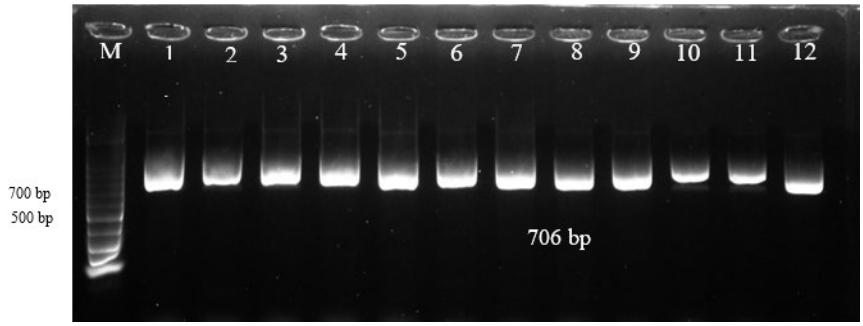

Figure 1 Electrophoresis of Gonadotropin-Releasing Hormone Receptor (GNRHR) PCR products in Senduro goat.

Figure 1 shows that the GNRHR gene in Senduro goat was amplified with a product length of 706 bp, and the GNRHR gene was located on chromosome 6 exon 3. The amplification process on the GNRHR gene was successful at an annealing temperature of 59ºC for 45 seconds with specific targets. The successful amplification product size was consistent with the target and had a good specificity (Febriana et al., 2022). PCR results showed that the GNRHR gene showed all DNA bands appearing in all samples. The optimal DNA band is a single, clean band, thick and on target.

The three steps of the fundamental PCR are heat denaturation of the target DNA (Denaturation), primer annealing of synthetic oligonucleotide primers (Annealing), and extension of the annealed primers by a DNA polymerase (Extension) (Ai et al., 2019). The annealing temperature and time depend on primer length, the length of the target base sequence, and base composition and concentration. The selection of temperature throughout the PCR process is crucial because the temperature, particularly the annealing temperature, is the most critical in determining the success of a PCR (Wijaya et al., 2018).

The base's composition, length, and primer concentration all affect the annealing temperature. The temperature at which the primer will bind to the DNA template is known as the annealing temperature. The annealing temperature based on each primer can be used to compute the melting temperature (Tm). It is crucial to look for the ideal annealing temperature conditions (Budi et al., 2023). After PCR amplification, gel electrophoresis is used for qualitative analysis, and the brightness of the band roughly estimates the initial DNA concentration.

Gonadotropin-releasing hormone Receptor (GNRHR) Gene Restriction Results

GNRHR gene identified using Msp1 cutting enzyme with CC↓GG cutting site are in Figure 2.

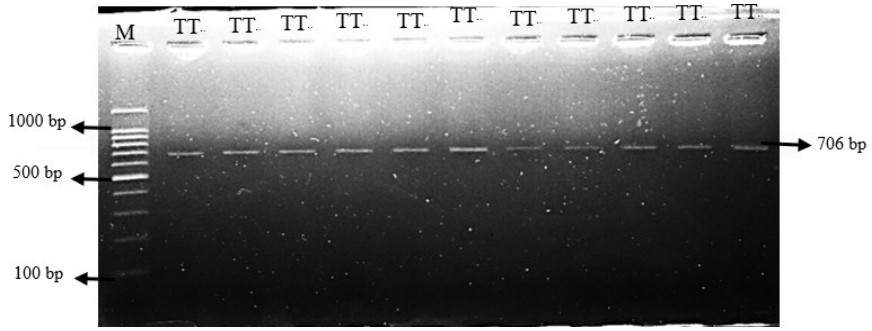

Figure 2 Visualization of GNRHR|Msp1 RFLP results of Senduro Goat

Table 3. Genotype and Allele Frequency of Senduro Goat

Breed	n	Genotype Frequency			Allele Frequency	
		TT	TC	CC	T	G
Senduro Goat	76	1	0	0	1	0

The characterization of Senduro goat using Gonadotropin-Releasing Hormone Receptor (GNRHR) gene with the Msp1 cutting enzyme obtained three cut points of 233 bp, 473 bp, and 706 bp. The use of restriction enzymes is expected to produce a DNA restriction pattern (cutting) to determine the differences between the individuals used. If there is a cutting site, the restriction enzyme will cut the DNA at a known position, causing the DNA sequence to split into DNA bands (Spetiawan et al., 2017).

This study used the Msp1 enzyme applied in this investigation to cut DNA strands on 706 bp. The following fragments (233, 473, and 706) will be added to the sequence If there is a mutation or alteration in the nucleotide sequence. However, this study resulted in one fragment (706 bp) due to no mutation and produced TT Genotype.

Based on Figure 2, the GNRHR|Msp1 fragment cutting results showed one strip of bands (706 bp). This shows no truncation, mutation, or change in nucleotide sequence, resulting in the TT genotype and the T allele. The research on the GNRHR gene (exon 3) with PCR-RFLP resulted in monomorphic and could not be associated with litter size. On the other hand, previous studies have found polymorphs in the exon 1 segment of the GNRHR gene that were not observed in this study (An et al, 2009).

Based on (An et al., 2009) using PCR-SSCP and DNA sequencing techniques, polymorphisms in the goat GnRHR gene exon 1 were found in 786 individuals from two goat breeds. Five amino acid substitutions were caused by two haplotypes (A and B), two observed genotypes (AA and AB), two single nucleotide polymorphisms (SNPs), and two haplotypes (A and B). Additionally, 224 Boer goats were used to test the Polymorphism in the gonadotropin-releasing hormone receptor (GnRHR) gene using PCR single-strand conformational (PCR-SSCP) and DNA sequencing techniques. In the exon 1 of the goat GnRHR gene, two single nucleotide polymorphisms (SNPs), G891T and G757A, were found (Yang et al., 2011). Polymorphism was detected d in exon 1 in West African Dwarf goats (Bemji et al., 2018). Table 3 shows that in Senduro goat in calculations using the Hardy-Weinberg law, insignificant results are obtained because the value is more than > 0.05 (db; 1) = 0.50. It means that the GNRHR gene cannot be used as a candidate gene marker in the sample used. The genotype frequency of the GNRHR|Msp1 gene Senduro Goat. There is only one Genotype (TT) and one allele (T). The T allele has an allele frequency of one. According to (Nei, 1987), an allele is monomorphic if its allele frequency is 0.99 (99%) or less. The findings of this study are because the number of samples is relatively small, and the samples only come from one population, so that diversity is not found. The selection process and the lack of new male introductions in a population might lead to limited genetic diversity in a livestock group (Agung et al., 2017).

Conclusion

In the results of this study, we found one Genotype (TT) and one allele (T) in GNRHR gene exon 3 using the Msp1 enzyme. Allele frequencies and genotypes of Senduro Goat were monomorphic and could not be associated with litter size. Collectively, these results help us to inform the genetic basis of goat reproductive traits that can be used in further studies.

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