Association of Gonadotropin-Releasing Hormone Receptor (GnRHR) gene (c.18085 T>G) and litter size in Ettawa crossbreed goat

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Abstract. In the present study, the association of Gonadotropin-Releasing Hormone Receptor gene (GnRHR) and litter size were investigated using the PCR-RFLP method as a genetic marker candidate for litter size in 35 individuals from Ettawa crossbreed goats. This research has two steps conducted in the laboratory and in the field. Data on litter size and blood samples were collected from the field. In the laboratory was carried out DNA isolation, amplification, and restriction. Molecular analysis of DNA isolation were precise, the enzyme Msp1 (C*CGG) with a product length of 706 bp was used for amplification and restriction analysis, releaved TT genotype in GnRHR gene fragment. In the exon 3 of chromosome 6, the single nucleotide polymorphism (SNP) c.18085 T>G was found. Ettawa crossbreed goat in calculations using the hardy-weinberg law, insignificant results are obtained because the value is more than > 0.05 (db; 1) = 0.50. The genotypes and allele frequencies found in the Ettawa Crossbreed Goat GnRHR genes are monomorphic and the GnRHR gene did not associate with litter size. The conclusion is that GnRHR exon 3 gene (SNP) c.18085 T>G did not associate with litter size and can not be used as a genetic marker on Ettawa crossbred goats.

Keyword: Ettawa crossbreed goat, Litter size, GnRHR gene, Msp1 enzyme, PCR-RFLP

1 Introduction

Goats were reared for several reasons, including their abilities to adapt in diverse ecosystems (frigid, harsh, and arid zones), adaptive under low input environments, tolerance to heat stresses, diseases, and parasites, and their abilities to provide socioeconomics, cultures, and religious benefits, as well as cash readily for immediate needs and meaningful income among poor households. Indigenous goats have been part of rustic subsistence in many developing countries [1].

Etawah-crossbreed goats are one of the most important types of dual-purpose goats in tropical areas, especially Indonesia [2]. It is a crossbreed between an Etawah goat and an Indonesian local goat [3]. Ettawa Crossbreed goat is known as dairy production. These goats

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are often crossbreed with other local goats of Indonesia through reproductive technology, such as artificial insemination (AI) or natural mating. goats have been maintained for a long time, so that they are considered to be local goats with better genetic quality than other local goats. Milk production of ECDG can reach 1.5-3 liters per day [4]. Goat's milk fat and protein is quite high at 6.08% and 4.48% [5]. The average litter size of Ettawa crossbreed goats and birth weight (kg) were 1.89±0.66 and 3.84±0.73 respectively.

Litter size has a significant impact on farmers' economic gains, which can determine the availability of the number of goats in a livestock business. Goats can have several kids at same time, which is known as the litter size. Each goat's litter size is impacted differently by genetic changes that accelerate ovulation and increase the number of offspring produced per pregnancy [6]. The genetics of litter size emphasizes the crucial of major fecundity genes in goats. There needs to be an effort to increase the number of litter sizes in ettawa crossbreed goats to produce optimal litter size. Development of Ettawa Crossbreed goats which have the goal to enhance the potential goat that exists in the country. One of the most important factors to increase the number of litter sizes is the selection of goats.

The last decade has seen an incredible increase in the use of application of molecular genetics for identification of gene loci and chromosomal areas containing single nucleotide polymorphisms (SNPs) that affect commercially significant traits in the selection of livestock [7]. The use of marker assisted selection technology enables precise selection of advantageous sequence variants that have been demonstrated to improve performance, hence removing the majority of the uncertainty involved in traditional phenotypic selection [8]. Gonadotropin-Releasing Hormone Receptor (GnRHR) is one of the candidate genes for goat litter size under marker-assisted selection.

Gonadotropin Releasing Hormone Receptor (GnRHR) is a crucial regulator of the reproductive system, which causes the pituitary gland to produce and release the luteinizing hormones (LH) and follicle-stimulating hormones (FSH) [9]. GnRHR is responsible for stimulating the synthesis and to release of pituitary gonadotropins (leutenizing and follicle stimulating hormones). These gonadotropins in turn control ovulation and the development of gonadal steroids because of its critical role in regulating the activities of hypothalamo-pituitary-gonadal axis.

Etawa crossbreed dairy goats have been maintained for a long time, so that they are considered to be local goats with better genetic quality than other local goats. Etawa crossbred Dairy Goat (ECDG) is a dual-purpose goat that can produce milk such as Etawa goat and produce meat such as Kacang goats [5]. Milk production of ECDG can reach 1.5-3 liters per day [6]. Goat's milk fat and protein is quite high at 6.08% and 4.48% [7]

There has not been any information about the association of GnRHR genetic variations with litter size on ettawa crossbred goats. Given that the GnRHR gene is linked to luteinizing hormone and follicle-stimulating hormone release, it may be a candidate gene for litter size on goats. The objectives of this study were to identify polymorphisms GnRHR gene and evaluate the associations between GnRHR gene and litter size on ettawa crossbreed goats.

2 Materials and Methods

2.1 Materials

Materials in this study used 35 Ettawa Crossbreed goat and the polymorphism in the gonadotropin-releasing hormone receptor (GnRHR) gene was detected by PCR-RFLP. The characteristics of goats in this study were actively reproductive, given birth and healthy. The research was conducted from January 2023 to Mei 2023. This observation was carried out in different locations. For collecting blood samples and gathering information on litter size,

the location is a field at UPT PT and HMT Singosari in Malang. The other location is the laboratory at the Livestock Biotechnology Laboratory, Faculty of Animal Science, Brawijaya University, Malang for DNA extraction and analysis of blood samples of Ettawa crossbreed goats.

The first step on this study was collecting phenotypic data (litter size) and taking blood samples of each Ettawa Crossbreed goat in UPT PT HMT Singosari Malang. Ettawa Crossbreed goat was taken a blood sample in the neck, precisely in the jugular vein as much as 5 ml. The second step in the laboratory includes DNA analysis (DNA isolation, Amplification, and Restriction). DNA isolation to separate DNA from white blood cells. In the beginning, 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), nucleus Free Water (NFW) (Promega), Buffer tango, gel agarose, ethanol absolute, 1X Green Master Mix (Promega), Restriction enzyme MspI (BioLabs), DNA marker, TBE 0,5X, Loading blue dye, Nucleic Acid Dye Diamond.

The equipments were used in this study including hand glove, centrifuge, tube rack, hot plate (BIAB), beaker glass (IWAKI), tip (Axygen scientific), micropipette (Select BioProducts), tube (Axygen scientific), Eppendorfvortex tubes (Wiggens vortex3000), microcentrifuge (Force mini SBC-140-3), centrifuge (HETTICH Mikro 185), machine for PCR (Bio Rad T100 Thermal cycler), analytical balance ohaus (OHAUS Pioneer), spatula, microwave (Panasonic NN-SM32HM), blue light gel doc (Gite 965 GW), sprayer, spoon, electrophoresis tool (Bio-Rad mupit-ex), incubator (Memmert IN), nanodrop spectrophotometer, electrophoretic gel system, gel printer, well comb, electric heater.

This genetic characterization consists of several stages, including DNA isolation, DNA qualification, and PCR-RFLP. This study used genomic DNA Mini Kit (Geneaid) for the DNA isolation. Moreover, DNA qualification was carried out 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 and showed the thickness of the DNA band subjectively. Gene candidate amplification using primer pairs was shown in Table 1. below:

Table 1. Length, Location and Sequence of primary pairs

Gene	Length	Location	Sequence	GenBank
GnRHR	706	Exon 3	F: $^{5'}$CACCAGGTTGAATTACGAT$^{3'}$	CM004567.1
			R: $^{5'}$AGGTCTGTGTTTCTCAGTG$^{3'}$	

The amplification procedure was carried out in accordance with 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, and then 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

Stages	Gonadotropin-Releasing Hormone Receptor gene (GnRHR)		
	Temperature (^{0}C)	Time (Hour:minute:second)	Cycle
Pre-denaturation	94	00:05:00	1x
Denaturation	94	00:00:30	
Annealing	59	00:00:45	35x
Extention	72	00:01:00	
Final Extention	72	00:05:00	1x
Enzym restriction Mpsl	37	16:00:00	

2.2 Data Analysis

2.2.1 Genotype and allele frequency

A total of 200 eggs (50 eggs per storage duration) were used in this experiment. Eggs were stored in egg trays with the large end positioned upward. The temperature and relative humidity during storage were 24-26 °C and 60-70%, respectively. At the end of each storage period, eggs were subsequently transferred to a forced-draft incubator. The temperature and relative humidity for incubation were set at 37.7-37.9 °C and 50-60%, respectively, throughout the whole period. Regular hourly turning at a 90° angle occurred during the setter phase. At the 18th day of embryonic development, candling was performed to remove clear eggs, while those with viable embryos were transitioned from turning trays to hatching baskets and subsequently moved to the hatcher.

$$x_{ii} = \frac{nii}{N} \quad (1)$$

$$x_i = \frac{2n_{ii} + \Sigma_{i \neq j} n_{ij}}{2N} \quad (2)$$

Description :
x_{ii} = Genotype frequency
nii = Observed genotypes
N = Total population
xi = The allele frequency of the samples,
nij = Sample genotype ij,
N = Total sample.

3 Results and discussion

GnRHR genes identified using Msp1 cutting enzymes with CC↓GG cutting site are in Figure 1. The description three cut sites of 233 bp, 473 bp, and 706 bp were produced using the Gonadotropin-Releasing Hormone Receptor (GnRHR) gene and the Msp1 cutting enzyme in the Ettawa Crossbreed goat. To distinguish the differences between the individuals used, a DNA restriction pattern (cutting) is anticipated to be produced by the use of restriction enzymes.

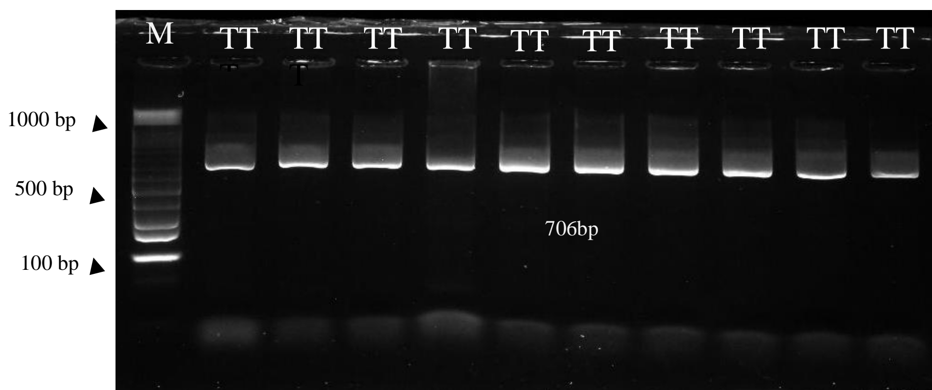

Fig. 1. Visualization of GnRHR|Msp1 RFLP results of Ettawa Crossbreed Goat

Table 3. Genotype and allele frequency of Ettawa Crossbreed Goat

Breed		n	Genotype Frequency			Allele Frequency	
			TT	TG	GG	T	G
Ettawa Goat	Crossbreed	35	1	0	0	1	0

The restriction enzyme will cut the DNA at a known location if there are a cutting site causing the DNA sequence to separate into DNA bands [16]. DNA strands will be cut in this investigation utilizing the restriction enzyme Msp1, which is 706 bp. Three fragments, 233, 473, and 706 will be added to the sequence if there is a mutation or alteration in the nucleotide sequence. The C*CGG nucleotide sequence will not be restricted if there is no mutation. It will produce one fragment, measuring 706 bp with the TT genotype.

According to Figure 1, the results of cutting the GnRHR|Msp1 fragment indicated one strip of bands (706 bp). This shows that there is no truncation and no mutation or no change in nucleotide sequence resulting in the TT genotype and the T allele. The GnRHR gene (exon 3) was studied using PCR-RFLP, but the results were monomorphic and could not be related to litter size. The results of this study are the same as Ahlawat, Harma, Maitra, Tantia, Roy and Mandakmale [17] found a single genotype (TT) in Malabari and Black Bengal goats. It was also added to the study of [18] that the band results on the visible 140 bp fragments showed that the enzyme did not recognize the restriction site. Therefore, the genotype of all 100 samples are ++. PCR-RFLP results obtained by amplicon sequence analysis, confirmed no polymorphism at the FecB locus in Markhoz goats. Research on the GnRHR gene in exon 3 with PCR-RFLP produced monomorphisms and could not be associated with litter size.

On the other hand, on Exon 1 which was not observed in this study found polymorphisms, based on [18] the polymorphism in the gonadotropin-releasing hormone receptor (GnRHR) gene was detected by PCR single-strand conformational (PCR-SSCP) and DNA sequencing methods in 224 Boer goats. In the exon I of the goat GnRHR gene, two single nucleotide polymorphisms (SNPs), G891T and G757A, were discovered. Their relationships with litter size in Boer goats were assessed. A study of associations found that the mutations G891T and G757A significantly impacted litter size. Furthermore, polymorphism was detected in exon 1 in West African Dwarf goats [19]. Moreover, according to [20] the gonadotropin-releasing hormone receptor (GnRHR) gene polymorphisms were examined in the current study as a potential genetic marker for litter size in 40 Iraqi goats. Additionally, analyses of the CC and CA genotype nucleotide

sequences revealed one mutation (A>T) in exon 2. The findings indicated that in the breeds of Iraqi goats, the CC genotype was related to larger litter sizes.

Table 3 demonstrates that in Ettawa Crossbreed goat in calculations using the hardy-weinberg law, insignificant results are obtained because the value is more than > 0.05 (db; 1) = 0.50. This suggests that in the sample that was used, the GnRHR gene cannot be used as a candidate gene marker.The goat Ettawa Crossbreed's genotype frequency for the GnRHR|Msp1 gene only one genotype (TT) and one allele (T) are present. The frequency of the T allele is one. A monomorphic allele, according to [21], has an allele frequency of 0.99 (99%) or less. The results of this study do not indicate diversity because there were very few samples and only came from one population. Limited genetic diversity in a livestock group may be caused by the selection process and the absence of new male introductions in a population [22].

4 Conclusion

Allele frequencies and genotypes of Ettawa crossbreed goats were monomorphic and could not be associated with litter size.

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