FSH Exon 9 (English)

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IDENTIFICATION OF *SINGLE NUCLEOTIDE POLYMORPHISM* (SNP) IN EXON 9 AND INTRON 9 OF *FOLLICLE STIMULATING HORMONE RECEPTOR* (FSHR) GENE IN PESISIR CATTLE

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ABSTRACT

This study aims to *identify single ncreotide polymorphism* (SNP) in exon 9 and intron 9 of *follicle stimulating hormone receptor* (FSHR) gene in Pesisir cattle. The samples used in this study were 70 blood samples of Pesisir cattle maintained at BPTU Padang Mengatas, Payakumbuh, West Sumatera. Blood sample were isolated using the DNA Purification Kit *genomic protocol* by promega. The DNA isolation results was then amplified using a pair of primer primer F : 5′-TTA GGC CCT GTG ACT GTG AG-3′ and R : 5′-CTG GCA AAG AGG GAA CAA GAG -3′ which produce FSHR gene fragments with 962 bp long. Then the amplified products were sequenced using the services of 1st Base Singapore. The result showed that the population of Pesisir cattle in this study was polymorphic. There were 5 polymorphisms in the exon 9 and intron 9 regions of 70 samples of Pesisir cattle that were sequenced, which were located at the 18 A>G, 213 T>C, +49 A>T, +232 A>C, and +252 G>A. There were 3 trantition type mutations at position (8, 213 and +252) and 2 transversion type mutation at position (+49 and +232). Based on the result of this study, it can be stated that the genotype frequency of Pesisir cattle population was not in the Hardy-Weinberg equilibrium.

Key words: FSHR gene, Pesisir cattle, *single nucleotide polymorphism*

Introductions

Pesisir cattle is one of the Indonesian germplasm from West Sumatra which has a smaller body size compared to other local cattle. Pesisir cattle have a significant contribution to the fulfillment of meat needs for people in West Sumatra. As a local cattle, Pesisir cattle have several advantages namely being able to survive in not good environmental conditions and have high reproductive efficiency (Sarbaini, 2004).

The potential genetic resources of local Indonesian cattle were very diverse and have not been used optimally. The growing cattle population in Indonesia has produced diverse genetic resources. The diverse genetic resources will make a population more resistant to live in the long term and higher adaptable to environmental changes (Frankham et al., 2002).

The effort to improve the genetic quality of livestock productivity can be done through selection at the molecular level. The implementation of selection at the molecular level on genetic resources has not been widely used. This utilization is carried out as an effort to obtain superior local cattle that have high productivity in increasing the production and reproduction capabilities of Indonesian local cattle (Nasution, 2013).

Nasution (2013) also stated that improving the genetic quality of livestock on production values, especially in reproduction to produce better livestock phenotypes and genotypes, is strongly influenced by the presence of genes in it. One of the essential genes in influencing reproduction in local cattle is the Follicle Stimulating Hormone (FSH) gene. FSH can stimulate the gonads in females. FSH can not work directly into the cell so it requires the help of FSHR. FSHR works directly into cells which can help increase the production of ovum cells in females.

Follicle Stimulating Hormone Receptor is a transmembrane receptor that interacts with follicle-stimulating hormone. The FSHR gene is required to carry the FSH gene to the target tissue so that it can be translated into protein to become the FSH hormone. The FSHR gene is located on chromosome 11 and consists of 10 exons and 9 introns with a length of 194885 bp (Houde et al., 1994). One of the functions of FSHR is to support the activation of specific receptors in stimulating follicular growth in the ovaries.

The target DNA that was amplified in this study was the FSHR gene fragment located at exon 9. The FSHR gene was found on chromosome 11 consisting of 10 exon 9 introns with length 194885 (NCBI GenBank access code NC-037338). Exon 9 has a length of 185 bp. In exon 9 there are DNA sequences that affect reproduction. Identification of diversity based on nucleotides can be done using the SNP (Single Nucleotide Polymorphism) technique using a PCR (Polymerase Chain Reaction) product. The SNP technique has the advantage that it is easy to apply and read the data.

This study was conducted to identify SNPs in the FSHR gene exon 9 and intron 9 and is expected to be a reference or basic information for further research to complete the molecular genetics framework to improve the genetic quality of livestock.

Materials and Methods

This study used 70 blood samples of female Pesisir cattle aged 2-5 years, which were taken at BPTUHPT Padang Mengatas, Payakumbuh, West Sumatra.

DNA Isolations

DNA isolation of blood samples from Pesisir cattle was carried out using the DNA Purification Kit genomic protocol by Promega. The isolated DNA was then electrophoresed to see DNA on 1% agarose gel and stored at -20^{o}C until it was ready to use.

FSHR Gene Amplification

Gene amplification using the PCR machine with a pair of primers F: 5'-TTA GGC CCT GTG ACT GTG AG-3' and R: 5'-CTG GCA AAG AGG GAA CAA GAG-3' (Yurnalis, 2019). The gene amplification procedure used pure tag

ready-go PCR from GE Healthcare (USA) with a predenaturation program at 95°C for 5 minutes, denaturation at 95°C for 45 seconds, and annealing at 58°C for 45 seconds.

PCR was performed with 35 cycles. Electrophoresis with 1% agarose gel which was given Ethidium Bromide solution and evaluated using a UV transmulator to see the results of the amplification of the FSHR gene. Gene amplification was said to be successful if the agarose gel showed bands formed in each well-containing DNA samples of PCR products. The target DNA can be determined by comparing the position of the band formed with the position of the DNA ladder band. Then the results of the electrophoresis are documented with a camera.

Sequencing

The PCR product from the FSHR exon 9 gene was sequenced using the services of 1st Base Singapore.

Data Analytics

The diversity of the FSHR gene can be known by calculating the genotype frequency and allele frequency using the recommended formula from Nei and Kumar (2000).

1. Genotype frequency

$$\chi_i = \frac{\sum n_i}{N}$$

Description:

x_i = observed genotype

N = total number of individual samples

n_i = total number of individual samples with genotype i

2. Allele frequency

$$\chi_i = \frac{(2n_{ii} + \sum_{j \neq i} n_i)}{2n}$$

Description:

x_i = allele frequency i

n_{ii} = number of individuals for the genotype ii

n_{ij} = number of individuals for the genotype ij

n = number of samples

Hardy-Weinberg Equilibrium

The Hardy-Weinberg equilibrium test was carried out to determine whether the genotype and allele frequencies of the exon 9 FSHR gene in Pesisir cattle were in the balance p2 + 2pq + q2 = 1 then tested with the chi-square test using the following formula:

$$\chi^2 = \sum\sum \frac{(O_{ij} - E_{ij})}{E_{ij}}$$

Description:
χ^2 = chi-square test
O_{ij} = number of genotypic observations
E_{ij} = expected number of genotypes

Results and Discussion

FSHR Gene Amplification

The PCR results can be seen from the electrophoretic samples which were visualized using a UV-Transimulator using 1.0% agarose gel. The primer pair attachment sites were matched to the Coastal cattle FSHR gene sequence (Primer3 Output) accessed from NCBI Genbank No NC-037338 and the FSHR gene fragment was 962 bp long located in the exon 9 region. The amplification results in this study can be seen in Figure 1.

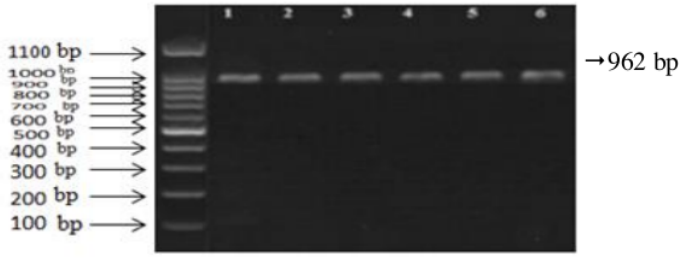


Figure 1. PCR product amplification of the FSHR exon 9 gene in Pesisir cattle
M = Marker

Based on Figure 1 above, it can be stated that the amplification of the FSHR gene using a pair of primers can be amplified properly. The amplification process during electrophoresis can be declared successful if in one gel block only one DNA band is visible, but not all samples show the same thickness of DNA band. This was due to differences in the concentration of extracted DNA in the template used for PCR as well as differences in the concentration of DNA that was successfully amplified (multiplied) (Sambrook and Russell, 2001).

The DNA amplification process using a PCR machine begins with denaturation, which is the separation of two double helix DNA strands into two single strands. After the denaturation process continued with annealing, namely the attachment of the primer to the DNA template as the initial formation of the paired nitrogenous base. After annealing, it was continued with the extension process, namely the extension of the formation of nitrogenous bases from the DNA template.

The annealing temperature determines the success of amplification because the DNA elongation process starts from the primer. If the annealing temperature is too high, it can degrade the primer so that mispriming can occur, while if the temperature is too low, the DNA formed has low specificity (Handoyo and Rudiretna, 2000). The annealing temperature used in this study was 58°C. This temperature range can be used if it produces the same amplification product.

According to Muladno (2002), the annealing temperature ranges from 45°C to 72°C, but usually the temperature used is between 50-60°C. A temperature that is too high will cause amplification failure because primer attachment does not occur, on the contrary if the temperature is too low it will cause the primer to attach to the other side of the genome, resulting in the DNA being formed having low specificity, for that it is very important the optimum temperature at amplification process (Rybicky, 1996).

FSHR Gene Diversity and Mutation Detection

70 samples of PCR products were sent to Singapore for sequencing using the services of 1st Base Singapore, this process takes approximately 2 weeks. All samples sent were successfully sequenced and analyzed using the SeqManTM version 4.00 DNASTAR program. The reference sequence used in this study is the FSHR Exon 9 gene sequence which is accessed from NCBI Genbank No. NC-037338. The results of the sequencing show that there is diversity in the exon 9 and some intron 9 regions, which can be seen in Table 1.

Table 1. Mutation detection FSHR gene of Pesisir Cattle

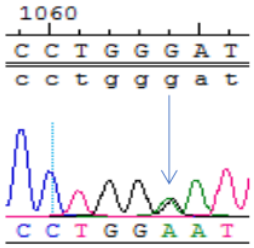
No	Mutation	Position	Mutation Type
1	A → G	8 (exon 9)	Transition
2	T → C	213 (exon 9)	Transition
3	A → T	+49 (intron 9)	Transversion
4	A → C	+232 (intron 9)	Transversion
5	G → A	+252 (intron 9)	Transition

Table 1 shows that 5 polymorphisms were found in the exon 9 to some intron 9 regions in the samples of Pesisir cattle. The results showed that all mutations occurred in the exon 9 region and some in intron 9. In the exon 9 regions, 2 types of mutations were found, while in the part of the intron 9 regions 3 types of mutations were found. The sequencing electropherogram showing the mutation of the exon 9 FSHR gene in Pesisir cattle is shown in Figure 2-4.

According to Asaf et al (2014) SNPs in the coding region can directly affect the protein, SNPs in the intron region can affect splicing, while SNPs in the promoter will affect gene expression. Introns are also regions of DNA sequences that are not transcribed into proteins, but now many research results have been found that show the function of introns, especially in eukaryotic cells, one of the important functions of introns in eukaryotic cells is increasing protein abundance. This indicates that although the intron region is not involved in protein synthesis, variations in introns can affect the translation process. The effect of introns was first observed in Simian Vacuolating viruses which produce undetectable protein products upon deletion of their intron sequences (Gruss et al., 1979 and Hamer et al., 1979).

Gambar 1. Transition A→G at position 8, transition T→C at position 213

Gambar 2. Transversion A→T at position +49, tranversion A→C at position +232



Gambar 3. Transition G →A at position +252

In this study, 5 point mutations were found in exon 9 and intron 9, namely 3 points of transition mutation and 2 points of transversion mutations. 3 point transition mutations that occur are A>G at position 8, T>C at position 213 and G>A at position +252. Meanwhile, 2 points of transversion mutations occurred, namely A>T at the +49 position and A>C at the +232 position. Windelspect (2007) states that mutations occur due to the substitution of pyrimidine base (thymine and cytosine) with another pyrimidine base or between one purin base (adenine and guanine) and another purin base. Mutations or changes in nucleotide bases are widely used as the basis for identifying genetic diversity.

Based on the results of the sequencing, there were 30 haplotypes in exon 9 and intron 9 in the FSHR gene of Pesisir cattle. Base differences between individuals and diversity in the population of Pesisir cattle are shown in Table 2.

Table 2. The result of haplotype in exon 9 and intron 9 of FSHR gene in Pesisir Cattle (aligned with GenBank reference).

Haplotype	Control Area Sequence Number				
	8	213	+49	+232	+252
	A	T	A	A	G
1	AG	.	.	AC	.
2	.	.	.	AC	.
3	AG	.	.	CC	.
4	.	.	.	.	.
5	.	.	.	AC	.
6	.	.	.	CC	GA
7	.	.	.	AC	.
8	AG	.	.	AC	.
9	AG	.	.	CC	.
10	GG	CC	TT	CC	.
11	AG	.	.	AC	.
12	AG	.	.	CC	GA
13	.	.	.	AC	GA
14	.	.	.	CC	AA
15	.	.	.	.	.
16	AG	TC	.	AC	.
17	AG	.	.	CC	GA
18	AG	.	.	CC	.
19	AG	TC	.	CC	GA
20	GG	CC	TT	CC	.
21	.	.	.	AC	GA
22	.	TC	.	AC	.
23	AG	.	.	AC	.

The sequencing result of 70 samples of Coastal cattle DNA using the service of 1st base in Singapore showed that there were differences in base composition (haplotypes) indicating differences in individual livestock. According to Akbar *et al*. (2014), different haplotype types in each individual can be used as a reference in individual identification. The more diverse types of haplotype composites in a population, the higher the level of genetic diversity and vice versa.

Genotype Frequency and Allele Frequency

Based on the number and types of genotypes in the exon 9 and intron 9 FSHR genes in Pesisir cattle, the genotype and allele frequencies are shown in Table 3 below.

Table 3. The result of genotype frequency and allele frequency FSHR gene.

SNP Position	N	Genotype Frequency			Allele Frequency	
8	70	GG	AG	AA	G	A
A – G		0,1	0,46	0,45	0,33	0,67
213	70	TT	TC	CC	T	C
T – C		0,83	0,07	0,1	0,86	0,14
+49	70	AA	AT	TT	AC	T
A – T		0,86	0	0,14	0,86	0,14
+232	70	CC	AC	AA	C	A
A – C		0,36	0,47	0,17	0, 60	0,40
+252	70	GG	AA	AA	G	A
G – A		0,82	0,17	0,01	0,9	0,1

Description: SNP = *Single Nucleotide Polymorphism*
N = Number of samples

Table 3 shows that there are 5 positions of SNP, namely 8 A – G, 213 C – T, +49 A – T, +232 A – C dan +252 G – A with 3 pairs of genotypic frequencies each and having the highest allele frequency of 0.9. The results of this study illustrate that the genotype frequency distribution in this study is polymorphic (various) because the value is less than 0.99 or more than 1%. As stated by Nei and Kumar (2000) that the locus of an allele is said to be polymorphic (various) if the frequency is equal to or less than 0.99 or more than 1%. If the opposite happens then it is monomorphic (uniform). Polymorphic properties are very important to analyze because it is one of the requirements for a gene to be used as a genetic marker (Hartl and Clark, 1997).

Hardy-Weinberg Equilibrium

The results of the data analysis obtained in this study need to be chi-square tested to see whether the data deviate or not from the Hardy-Weinberg law. The results of the chi-square test in this study are shown in Table 4.

Table 4. The result of chi-square test on Hardy-Weinberg equilibrium of FSHR gene in Pesisir cattle.

No	Posisi Mutasi	χ^2 hitung	χ^2 tabel 0,05	χ^2 tabel 0,01	Keterangan
1	18 A → G	187,56	5,991	9,210	**
2	213 T → C	62,46	5,991	9,210	**
3	+49 A → T	72,53	5,991	9,210	**
4	+232 A → C	146,93	5,991	9,210	**
5	+252 G → A	104,59	5,991	9,210	**

Description: $x^2_h > x^2_t(0,05)$ = significantly different

The results of the analysis show that the results obtained from Pesisir cattle at position 8, 213, +52, +232 and +252 have x^2 count greater than x^2 table ($x^2_h > x^2_t$). This means that the genotype frequencies of the research results are very significantly different and are not in Hardy-Weinberg equilibrium.

This can be due to the population of Coastal cattle used has been selected, the population is small and the mating occurs not randomly. In accordance with Hardjosubroto's (1998) statement, that things that can affect the Hardy-Weinberg imbalance are mutations, gene flow, migration, selection, genetic drift, and the absence of random mating. Vasconcellos *et al*. (2003) also suggested that some events such as accumulation of genotypes, divided population, mutation, selection, migration and mating in the same group/population (endogamy) can cause imbalances in the population.

Conclusion

In the exon 9 and intron 9 regions of the FSHR gene, there were 5 polymorphic (various) SNPs. From the results of the Chi-Square test calculation shows that the allele frequency and genotype frequency in the population of Pesisir cattle are not in Hardy-Weinberg equilibrium. (χ^2 h> χ^2 t).

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