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Identification Polymorphism of FSH β-Sub Unit Gene and FSHR Gene in Madura Cattle Results of Artificial Insemination with Limousin Cattle with PCR-RFLP Technique

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Abstract

The aim of this study was estimate of polymorphisms located in the FSH β sub unite and the receptor Follicle Stimulating Hormone (FSHR). Fourteen blood samples were collected from Madura cattle results of Artificial Insemination with Limousin Cattle called *"Madrasin"* cattle which lived in Bangkalan, Madura Island. The DNA was extracted following standard methods. The purified DNA was subjected to PCR-RFLP techniques to identify polymorphisms of the FSH β sub unite and the receptor Follicle Stimulating Hormone (FSHR) genes in "*Madrasin"* cattle ecotypes. The *Polymerase Chain Reaction – Restriction Fragment Lenght Polymorphism* (PCR-RFLP) amplified fragments of FSH β sub unite (313 bp) were disgested with restriction enzymes PstI resulting in 255 bp, 237 bp and 150 bp fragments, that produced 2 allelic is A and B. The allelic A and B frequencies for FSH β sub unite of *"Madrasin"* cattle is 0,50 and 0,50. While, The *Polymerase Chain Reaction – Restriction Fragment Lenght Polymorphism* (PCR-RFLP) amplified fragments of FSHR (306 bp) were disgested with restriction enzymes *Alu*I resulting become two homozigote genotypes, there are GG in the 243 bp, CC in the 243 bp and 193 bp. So the conclusion of this research was that FSH and FSHR genes not changed.

Key words: *Polymorphisym, MAS, FSH β-sub unite, FSHR, PCR-RFLP*

1. Introduction

Madura cattle are one of the local Indonesian cattle that develops on the island of Madura and surrounding islands. Morphologically, Madura cattle have almost the same characteristics as Bali cattle except their smaller body size and horn. Skin color in male and female Madura cows is more brown than Bali cattle, lower legs to knees (Rouse, J.E, 1972). In addition, Madura cattle are more resistant to hot weather, food-efficient, have good meat quality, and more resistant to parasites (Payne and Hodges, 1997).

Exploitation of Madura cattle through wider crossing with exotic cattle will have a change in phenotypic and genetic traits. Crosses are carried out by farmers to obtain superior performance, especially at the speed of growth in body weight and reproductive power. Madrasin cattle have exterior characteristics that resemble Madura and Limousin cattle or a combination of the characteristics of the two cattles. Growth and Performance of Madrasin cattle is a combination of Madura and Limousin cattle (Volkandari, *et al*. 2013).

Progress in the field of molecular biology provides new opportunities in the effort to detect the occurrence of genetic variations (polymorphism) as a basis for improving genetic quality in livestock. Molecular techniques that are potentially used to detect these variations are Restriction Fragment Length Polymorphism (RFLP). With the existence of effective and accurate technology through the use of diagnoses based on deoxyribonucleic acid (DNA), it will greatly assist the breeding program of cattle. Provision of genetic maps through recombinant DNA methods can help breeding programs through molecular data obtained, which regulates the characteristics of production (Hetzel, D.J.S. 1989).

Gene products in the form of hormones (bioregulator) will affect the process of regulating metabolism and appearance of livestock

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morphology. Growth hormone as one of the gene products has a major effect on growth, lactation and development of mammary glands in cattle (Hoj, *et al*. 1993a, b). In addition to growth hormones, it is also necessary to do research on the reproductive side, to obtain a picture of reproductive hormone polymorphism in cross-bred calves with Limousin cattle. Follicle Stimulating Hormone (FSH) is a glycoprotein hormone produced by the pituitary gland, which functions to regulate reproduction in mammals, both male and female (Grigorova, *et al*. 2007). The FSH gene is one of the important things in regulating the properties of cattle that have high economic value, so the FSH gene can be a candidate gene in the Marked Asissted Selection (MAS) program in cattle.

The FSH hormone consists of α and β-sub units. β-sub units play a role in determining the specificity of bonds with receptors (FSHR) (Fan and Hendrickson, 2005). FSH receptors are needed by FSH in the ovary to start and maintain follicular development by binding to specific receptors (FSH Receptor) on the surface of granulosa cells. The FSHR gene has an important role in ovarian stimulation and its physiological knowledge can be used to predict differences in FSHR function and ovarian response to FSH.

Therefore, genetic studies of madura cows that have been crossed with the Limousins are interesting to do to see the calves produced have good quality in terms of their body and reproductive growth, especially to be used as a reference as superior seeds.

2. Material and Methods

Research Materials

The main ingredients are DNA samples taken from whole blood of Madura cattle from artificial insemination with Limousin cement from Bangkalan, Madura, Indonesia with a total of 14 samples. Supporting materials include: Primary FSH β-subunits, primary receptor FSH, HaeIII Restriction Enzymes, Restriction Enzymes, DNA Extraction Materials (Proteinase K, Absolute Ethanol, Buffer Lysis, Wash Buffers A & B), PCR Materials (dNTP mix, Taq DNA polymerase enzymes) , Electrophoresis (Triss Base, boric acid, agarose, Na2 EDTA, Ethidium bromide, DNA Markers, DNA Loading dye), tissue and plastic mica.

Research Equipments

The tools used include: DNA Extraction Kit, venoject, vakuttainer tube, centrifuge, cooling device, small large eppendorf tube, agarose gel, micropipette, tip, tube rack, electrophoresis, autoclave, scales, gloves.

Collection of Blood Samples

Blood samples were obtained from Bangkalan, Madura, Indonesia. Blood sampling was carried out by collecting about 5 ml of blood samples from cattle through the jugular vein using venojet and vacuttainer tubes with EDTA and then stored at 4^oC.

Tabel 1. Primers were used to amplify the FSH β-sub-unit and FSHR Gen

Target	Name	Primer	An
FSH β-sub unit	F	5`CTTCCAGACTACTGTAACTCATC‘3	63
	R	5`GTAGGCAGTCAAAGCATCCG‘3	
FSH Receptor	F	5`CTGCCTCCCTCAAGGTGCCCCTC‘3	60
	R	5`AGTTCTTGGTCAAATGTCTTAGGGGG‘3	

Ket : An = annealing temperature, (Dai, *et al*. 2009).

DNA Extraction

DNA was isolated and purified using the QIAamp Mini spin column DNA Extraction Kit by following the extraction protocol provided. A total of 200 µl of blood samples were lysed by adding 200 µl of lysis buffer solution and 20 µl of proteinase K (10 mg / ml), the mixture was then incubated at 56oC for 60 minutes in a waterbath shaker. After incubation the solution is then added 200 µl of absolute ethanol 96% and centrifuged 8,000 x g for 1 minute.

DNA purification is done by spin column method with the addition of 500 µl of wash buffer I washing solution which is then followed by centrifugation at 8,000 x g for 1 minute. After the supernatant is removed, DNA is then washed again with 500 µl of wash buffer II and centrifuged at 14,000 x g for 3 minutes. After the supernatant is removed, the DNA is then dissolved in 200 µl of elution buffer and centrifuged at 8,000 x g for the extracted DNA to be stored and stored at -20 ° C.

PCR-RFLP Technique

The composition of the PCR reaction was conditioned on a reaction volume of 25 ul consisting of 100 ng of DNA, 0.25 mM of each primer, 150 uM dNTP, 2.5 mM $Mg2^+$, 0.5 Taq DNA polymerase and 1x buffer. The PCR engine

conditions began with initial denaturation at 94°C x 2 minutes, followed by 35 subsequent cycles of 94°C x 45 seconds denaturation, with annealing temperature: 63°C x 45 seconds (FSH) and 60°C x 45 seconds (FSHR), followed by one final extension cycle at 72°C for 5 minutes using GeneAmp PCR System 2400 ThermoCycler (Perkin Elmer). PCR products were then electrophoresed on 1.5% agarose gel with 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na2EDTA) containing 100 ng / ml ethidium bromide. Then visualized in the UV transiluminator (gel documentation system). Alleles are determined by interpreting bands in the most remote form of migration to the anode pole as 1 allele, 2 alleles, etc.

The PCR products obtained from each target gene were then analyzed using RFLP through cutting using restriction enzymes which had cutting sites on the FSH β-subunit gene | *PstI* and *AluI* for the FSHR gene. Total of 4 µl of PCR product DNA was added 0.5 µl, then incubated for 17 hours at 37°C.

Sequencing

Determination of nucleotide sequences of the FSH-sub-unit and FSHR genes was carried out by DNA sequencing, which was the final step to obtain the nucleotide sequence data from fragments resulting from the PCR -RFLP multiplication. DNA bands that have been extracted on agarose gel as PCR-RFLP product are used as molds in sequencing reactions using forward and reverse primers such as during amplification.

Data Analysis

The diversity of genotypes of each individual can be determined from the DNA bands of genes found. Each sample is compared based on the same marker and the allele frequency is calculated. The frequency of alleles can be calculated using the formula Nei and Kumar (2000). Analysis of sequencing data using UGENE 1.21.0 software.

3. Results and Discussion

The PCR Result of FSH Gene

From 14 Madrasin cattle blood samples PCR was carried out to detect the presence of FSH gene, the results of PCR showed that there were 14 bands of FSH gene using the FSH gene primer. Positive results are shown in Figure 1. as follows.

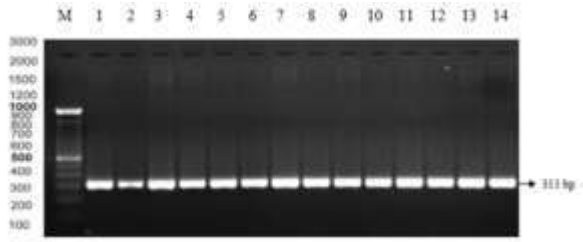

Figure 1. Results of PCR electrophoresis with the primary Madrasin cattle FSH gene. Lane M: Marker, Lane 1-14 is the result of electrophoresis of Madrasin cattle FSH gene with a length of 313bp.

The amplification of the FSH beta sub-unit gene segment was successful for 313 bp using a pair of primers according to the results of (Dai, *et al*. 2009). The condition of PCR used in thermocycler machines, uses an initial denaturation temperature of 94°C for 5 minutes, denaturation of 94°C, primary or annealing attachment of 63°C for 45 seconds, attachment of new DNA at 72°C for 1 minute, and a temperature of 72°C final elongation for 5 minutes. PCR results from Madrasin cattle FSH gene showed a band of 313 bp, this is in accordance with the band in the genome library (Genbank Access Number J00008; Balogh, *et al*. 2008).

Folicel Stimulating Hormone (FSH) is a hormone derived from basophil cells in anterior pituitary which is very instrumental in the process of reproduction. FSH itself is a hormone candidate that plays a role in regulating reproduction, and growing follicles (Ge, *et al*. 2003). FSH gene becomes one of the important things in regulating fertility or fertility in livestock with high economic value, so that the FSH gene becomes a candidate gene in the Marked Asissted Selection (MAS) program in cattle.

The PCR-RFLP Results of FSH Gene

The results of the RFLP were 14 FSH gene samples divided into 3 bands, namely 255 bp, 237 bp, and 150 bp. The RFLP results of Madrasin cattle FSH gene can be seen in Figure 2. as follows.

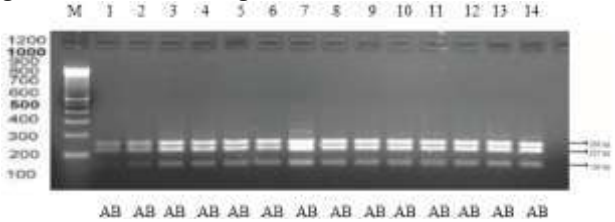

Figure 2. Electrophoresis results of PCR-RFLP FSH gene Madrasin cattle using *PstI* enzyme restriction. Lane M: Marker, Lane 1-14 (255 bp, 237 bp, 150bp).

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Amplification of Madrasin cattle FSH gene using PCR method is known to have a length of 313 bp, which will be continued by cutting the site of Madrasin cattle FSH gene using *AluI* retention enzyme. Based on the RFLP results, 255bp, 237bp, and 150bp PCR products produced 2 alleles, namely A and B, whereas in Madura cattle, only 1 allele, allele B. The restriction enzyme can recognize the FSH gene at the site of cutting, this is because the DNA sequence at the cutting site does not undergo mutation. The A and B alleles themselves are indicated by the length of the fragment (150 bp, 237 bp, and 255 bp).

Frekuensi Genotipe and Alel of FSH Gene

Table 2. Genotype frequency and alleles FSH gene in Madrasin cattle (genotype and allele frequencies of Madrasin cattle). Description AA, AB and BB = heterozygous genotypes, A and B = alleles

Bangsa (*breed*)	N	Frekuensi genotip (*genotype frequency*)			Frekuensi alel (*allele frequency)*	
		AA	AB	BB	A	B
Madrasin	14	0,00	1,00	0,00	0,50	0,50
Madura	10	0,00	0,00	1,00	1,00	0,00

Analysis results showed that allele A frequency was the same as B allele frequency, the frequency of A and B alleles in Madrasin cattles were 0.50 and 0.50 respectively (table 2) while the frequency of genotypes AB and BB were 1, 00 and 0.00. Based on these differences, it was suspected that there was no change in allele frequency and genotype between Madura cattles and Madrasin cattles due to crossbreeding with Limousin cattle.

The PCR Results of FSHR Gene

The results of amplification of 14 Madrasin Cattle blood in Bangkalan, Madura, Indonesia were carried out by PCR method, resulting in 14 positive DNA samples using the FSHR gene primer. The positive results of visualization of electrophoresis can be seen in Figure 3.

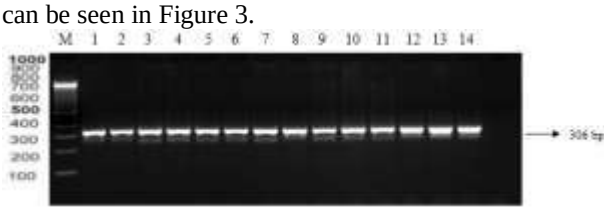

Figure 3. PCR electrophoresis results with the primary Madrasin cattle FSHR gene. Lane M: Marker, Lane 1-14 is the result of electrophoresis of Madrasin cattle FSHR gene with a length of 306bp.

The PCR results of FSH receptor gene in Madrasin cattle with *Alu1* enzyme is 306 bp. The condition of the thermocyler machine on the FSH receptor gene amplification is 94^{o}C initial denaturation for 5 minutes, 94^{o}C denaturation for 45 seconds, annealing temperature of 60^{o}C for 45 seconds, a new DNA elongation temperature of 72^{o}C for 5 minutes.

Based on the results of blood amplication using the Polymerase Chain Reaction (PCR) method, it was continued by electrophoresis reading with positive samples. The FSHR gene found in all Madrasin cattle blood samples can be detected by PCR with the product length of the FSHR gene amplification is 306 bp which is located on exon 10 (Ishak, 2013). The temperature and duration of the annealing also determine the level of specificity of the amplification results and other factors that play a role in determining the success of amplification are the quality or level of DNA purity used as DNA templates.

The FSHR gene has an important role in ovarian stimulation and its physiological knowledge can be used to predict differences in FSHR function and ovarian response to FSH. Simoni, *et al*. 1999 reported that changes in DNA sequences could influence FSHR gene activation and they also showed that genotypes play an important role in ovarian physiology. The follicle that stimulates the hormone begins and maintains follicular development by binding to its specific receptors on the surface of granulosa cells in the ovary (Simoni, *et al.* 1999; Dierich, *et al.*1998). This binding allows activation of the follicular stimulation hormone coding gene (Simoni, *et al.* 1999). The importance of identifying DNA polymorphisms lies in their relationship to productive and reproductive genotypes (Allan, *et al*. 2007; Tambasco, *et al.* 2000).

The PCR-RFLP of FSHR Gene

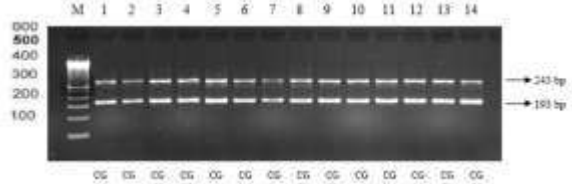

Figure 4. Electrophoresis results of PCR-RFLP FSHR gene Madrasin cattle using *AluI* enzyme restriction. Lane M: Marker, Lane 1-14 (243 bp and 193 bp).

Based on the results of the treatment using the PRC-RFLP the amplified FSHR gene segment contained *AluI* enzyme are two cutting sites known as two genotypes, namely GG visualized on 1 band

(243bp) and visualized CC on 2 bands (243bp and 193bp) marked with a 306 bp fragment cut into two parts along 243 bp and 193 bp. The fragment of the FSHR gene which has an *AluI* enzyme cutting site will indicate that there is no mutation but if there is no cutting site which is indicated by the absence of cutting by the AluI enzyme it can be stated that there is a mutation in the FSHR fragment site (Nawal, *et al*. 2016).

Diversity in the *AluI* FSHR gene segment is thought to be due to mutations or changes in bases causing changes in serine amino acids to glicine. These changes cause the cutting site not to be recognized by the Alu1 enzyme, resulting in a 193 bp fragment known as the G allele (Ge, *et al*. 2003). Genotyping results in Madrasin cattle FSHR gene segment produced two types of truncated fragments namely CG genotype which was shown as 243 bp and 193 bp fragments and fragments cut into one band were called CG genotypes.

The characterization of the allelic variability of the FSHR gene in different cattle breeds allows taking advantage of heterozygosis and selecting individuals that are carriers of specific alleles in loci of reproductive importance. The *FSHR* gene has an important role in ovarian stimulation and the knowledge of its physiology can be used to predict differences in the function of the FSHR and the ovarian response to FSH (Nawal, *et al*. 2016).

Frekuensi Genotipe and Alel of FSHR Gene

Table 3. Genotype and allele frequencies of the FSHR gene in Madrasin cattle (genotype and allele frequencies of Madrasin cattle). Description of CC, CG and GG = heterozygous genotypes, C and G = alleles

Bangsa (*breed*)	N	Frekuensi genotip (*genotype frequency*)			Frekuensi alel (*allele frequency)*	
		CC	CG	GG	C	G
Madrasin	14	0,00	1,00	0,00	0,50	0,50
Madura	10	0,00	0,00	1,00	1,00	0,00

The results of *AluI* FSHR gene analysis showed that the C allele frequency was the same as the G allele frequency, the frequency of C and G alleles in Madrasin cattles were 0.50 and 0.50 respectively (table 3) while the CG and GG genotypes were 1, 00 and 0.00. Based on these differences, it was suspected that there was no change in allele frequency and genotype between Madura cattles and Madrasin cattles due to crossbreeding with Limousin cattle.

4. Conclusion

Therefore, it can be concluded that the isolates in this research that that FSH and FSHR genes not changed.

Competing Interests

The authors declare that they have no competing interests.

Ethical approval

The research does not need ethical approval. However, samples were collected as per standart collection methods without any harm and stress to the animals.

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