DNA Sequence Analysis of Follicle-Stimulating Hormone (FSH) Gene and Follicle-Stimulating Hormone Receptor (FSHR) Gene in Madrasin Cattle with Ovarian Hypofunction

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DNA Sequence Analysis of Follicle-Stimulating Hormone (FSH) Gene and Follicle-Stimulating Hormone Receptor (FSHR) Gene in Madrasin Cattle with Ovarian Hypofunction

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Abstract: The research aims to determine the profile of the follicle-stimulating hormone (FSH) gene and follicle-stimulating hormone receptor (FSHR) gene polymorphism in Madrasin cattle with ovarian hypofunction. The method applied was carried out using laboratory techniques according to PCR and DNA sequencing procedures. The samples used were 14 Madrasin cattle with ovarian hypofunction. The results of PCR of the FSH gene were shown by the presence of a band with a size of 313 bp and an FSHR gene with a band size of 306 bp. The analysis of the results for the sequen of the PCR products was carried out by comparing the nucleotide sequence of the sample with the reference nucleotide sequence. The reference nucleotide sequence is analyzed with the BioEdit ver.8 software to align the nucleotides sample and check the nucleotides' control. One sample of Madrasin cattle FSH gene PCR products and one sample of PCR control products were best selected from 14 samples that are best sequenced according to the selection after purification of DNA samples by First Base Malaysia. The samples were sequenced then BLAST to see the nucleotide sequence from the results with the available database on the website www.ncbi.nlm.nih.gov to find the similarity between a nucleotide or protein sequence (query sequence) with a database sequence (subject sequence). The sequence alignment was carried out using the BioEdit ver 8 Program. The BLAST results showed that the nucleotide sequence of FSH sequencing with the NCBI database was 94% homologous with the Bos Indicus cross Bos taurus Follicle Stimulating Hormone Beta-Subunit Gene (XM_027562775.1) and 98% homologous with the Bos Indicus cross Bos taurus Follicle Stimulating Hormone Receptor (FSHR) (XM_027555230.1). The FSH gene experience a transition mutation at base positions 16 and 21, and transversion mutations at base position 19, while the FSHR gene experience a transition mutation at bases 16 nd 169.

Keywords: follicle-stimulating hormone, follicle-stimulating hormone receptor, sequencing, homolog.

卵巢功能低下的马德拉斯牛的促卵泡激素（促卵泡激素）基因和促卵泡激素受体（费舍尔）基因的脱氧核糖核酸序列分析

摘要：该研究旨在确定卵巢功能低下的教师牛的促卵泡激素（促卵泡激素）基因和促卵泡激素受体（费舍尔）基因多态性的概况。所应用的方法是根据聚合酶链反应和脱氧核糖核酸测序程序使用实验室技术进行的。所使用的样品是14头卵巢功能低下的教师牛。促卵泡激素基因的聚合酶链反应结果由大小为313bp的条带和大小为306bp的费舍尔基因的存在显示。通过将样品的核苷酸序列与参考核苷酸序列进行比较，对聚合酶链反应产物的序列结果进行分析。使用生物编辑版本8软件分析参考核苷酸序列，以比对核苷酸样品并检查核苷酸的对照。从马来西亚第一基地纯化脱氧核糖核酸样品后，根据选择的最佳顺序，最好从14个样品中选出教师牛促卵泡激素基因聚合酶链反应产物的一个样品和聚合酶链反应对照产物的一个样品。对样品进行测序，然后通过爆破进行测序，以从结果中找到核苷酸序列，网址为www.ncbi.nlm.nih.gov，可找到核苷酸或蛋白质序列（查询序列）与数据库序列（受试者）之间的相

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似性顺序）。使用生物编辑版本8程序进行序列比对。爆破结果显示，通过全国工商联数据库进行的促卵泡激素测序的核苷酸序列与老板印度杂交金牛座卵泡刺激激素β-亚基基因（XM_027562775.1）具有94％的同源性，与老板印度杂交的金牛座卵泡具有98％的同源性。刺激性激素受体（费舍尔）（XM_027555230.1）。促卵泡激素基因在第16和21位碱基处发生过渡突变，在第19个碱基处发生转化突变，而费舍尔基因在第168和169位处发生过渡突变。

关键词：促卵泡激素，促卵泡激素受体，测序，同源物。

1. Introduction

Indonesia is known as one of the countries with different genetic resources [1]. One of the genetic resources with high economic and socio-cultural value is local beef cattle [2]. Furthermore, its diversity plays an important role in developing livestock because it is a genetic base material. It is needed in assembly to form superior clumps to increase productivity [3]. Local beef cattle are Indonesian native beef cattle; that is, they have been reared in the country for a long time, and cattle originating from outside the country have long been bred and reared, and therefore they have certain characteristics [4]. Madura cattle are local Indonesian cattle species developed on the island of Madura and the surrounding islands. Morphologically, it has almost the same characteristics as Bali cattle, apart from their smaller body size and horns. The skin color of male and female Madura cattle is brown from the lower leg to the knee, making it different from that of Bali cattle [5]. Furthermore, Madura cattle are more resistant to hot weather, feed more efficiently, have good quality meat, and are more resistant to parasites [6].

Ovarian hypofunction is the reduction of the function of the ovaries, which includes the decreased production of hormones. Furthermore, it is a common reproductive disorder. It is an event in which the ovary has restricted its function so that follicular development and ovulation do not occur. According to [7], anesthesia due to ovarian hypofunction is often associated with an inability of the follicular cells to respond to hormonal stimulation, changes in the quantity and quality of hormonal secretions, a decreased stimulation associated with hypothalamic-pituitary-ovarian function, which causes decreased gonadotropin secretion, and therefore, there is no ovarian activity after childbirth. The lack of nutrition will affect the function of the anterior pituitary resulting in the low production and secretion of *Follicle Stimulating Hormone* (FSH) and *Luteinizing Hormone* (LH), which prevent the ovaries from growing or experiencing hypofunction [8].

Follicle Stimulating Hormone is a gonadotropin hormone produced by the anterior pituitary gland that plays a role in forming follicles in the ovaries. The synthesis and secretion of FSH are regulated by gonadotropin-releasing hormone (GnRH), which is secreted by special neurons in the hypothalamus. FSH induces follicular development in the ovary until it forms a tertiary follicle (follicle de Graaf). The tertiary follicle then forms estrogen, which suppresses the anterior pituitary, and therefore the FSH secretion stops. In the testes, the target of the FSH is the Sertoli cells reported in the seminiferous tubules and plays a role in spermatogenesis [9]. In addition, FSH works in cells through special receptors, namely FSHR, which is located exclusively on the gonads. The expression of FSHR in humans and several other mammals that have been identified are only reported in the testes and ovaries. In the testes, FSHR expression is reported exclusively in Sertoli cells. Meanwhile, in the ovaries, it is only expressed on granulosa cells [9-11].

Based on the description above, it is necessary to study the FSH profile in the blood and FSHR gene polymorphisms in Madrasin cattle with ovarian hypofunction. Since limited research is carried out, the results are expected to be used as a reference for improving the reproductive quality of Madura cattle, especially Madrasin cattle with ovarian hypofunction.

2. Materials and Methods

The research was carried out in 2019 at the Institute of Tropical Disease, Airlangga University, Surabaya. The material used was genomic DNA obtained from the blood of 14 Madrasin cattle.

2.1. Blood Sample Collection

The blood samples were obtained from Bangkalan Regency. Furthermore, it was approximately 5 ml of Madrasin cattle blood sample, drawn through the jugular vein using a venojet and a vacutainer tube with EDTA (whole blood) and then stored at the temperature of 4° C.

2.2. DNA Extraction

According to the extraction protocol, the DNA was isolated and purified using the QIAamp Mini spin column DNA extraction kit. A total of 200 µl of blood samples was lysed by adding 200 µl of lysis of buffer

solution and 20 µl of proteinase K (10 mg/ml). The mixture was then incubated at 56° C for 60 minutes in a water bath shaker. After incubation, the solution was diluted with 125 µl of absolute ethanol 96% and centrifuged 8,000 x g for 1 minute. The DNA purification was carried out by the spin column method with the addition of 500 µl of wash buffer I washing solution, which was then followed by centrifugation at 8,000 x g for 1 minute. After the supernatant was removed, the DNA was then washed again with 500 µl wash buffer II and centrifuged at 14,000 x g for 3 minutes. Furthermore, it was then dissolved in 200 µl of elution buffer and centrifuged at 8,000 x g, and the extracted DNA was collected and stored at -20° C.

2.3. PCR Technique

The composition of the PCR reaction was adjusted to a reaction volume of 25 ul, consisting of 1 ul of each primer, 12.5 ul of master mix (Promega), 8.5 ul of nucleus free water (NFW), 2 ul of DNA samples. The PCR machine condition started with an initial denaturation at 94° C x 2 minutes, followed by the next 35 cycles of 94° C x 45 seconds each, with annealing temperature: 65° C x 30 seconds (GH), followed by one final extension cycle at the temperature of 72° C for 5 minutes using the GeneAmp PCR System 2400 ThermoCycler (Perkin Elmer), for FSH β-sub unit annealing primer 60° C. The PCR product was 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. The PCR results of the FSH gene were shown by the appearance of a band with a size of 313 bp and the FSHR gene with a band size of 306 bp.

2.4. Sequencing

PCR products were purified and sequenced at First Base Malaysia sequencing.

2.5. Data Analysis

The sequencing results were analyzed using the BioEdit ver 8 software.

3. Results

3.1. Results of FSH (Follicle Stimulating Hormone) Gene Sequencing in Madrasin Cattle

The PCR product was sequenced to see the nucleotide composition of Madrasin cattle. Furthermore, the sequenced nucleotides were analyzed with BioEdit ver 8.0 software to perform multiple alignments using the ClustalW software in BioEdit ver 8. Fig. 1 shows the results of the Madrasin Cattle FSH gene electropherogram with good quality because from the electropherogram, there was no noise, the peaks were not overlapping. Adenine (A) nucleotides are shown in green, Guanine (G) is shown in black, Thymine (T) is shown in red, and Cytosine (C) is shown in blue. The results of the blast and multiple alignments with homology levels above 95% indicated that the target gene was FSH. Complete results can be seen in Fig. 1.

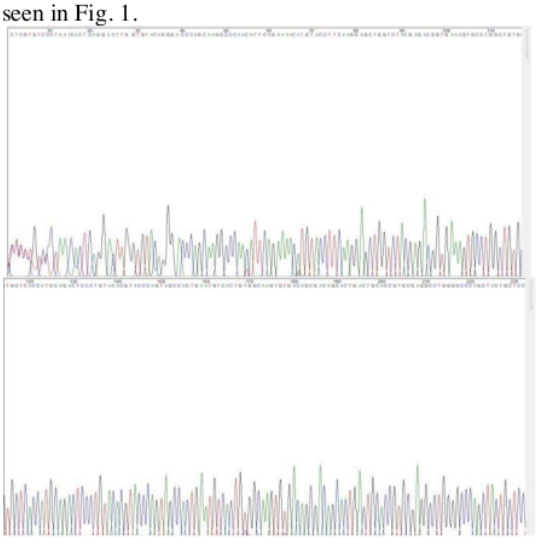

Fig. 1 Follicle-stimulating hormone (FSH) gene electrophoregram results

3.2. Results of FSHR (Follicle Stimulating Hormone Receptor) Gene Sequencing in Madrasin Cattle

The PCR product was sequenced to see the nucleotide composition of Madrasin cattle. The sequenced nucleotides were analyzed using the BioEdit ver. 8.0 software to perform multiple alignments using the ClustalW software in BioEdit ver. 8.

Fig. 2 shows the Madrasin Cattle FSHR gene electropherogram results with good quality because the electropherogram shows no noise, and the peaks are clear and do not overlap. Adenine (A) nucleotides are shown in green, Guanine (G) is shown in black, Thymine (T) is shown in red, and Cytosine (C) is shown in blue. The results of the blast and multiple alignments with homology levels above 95% indicated that the target gene was FSHR.

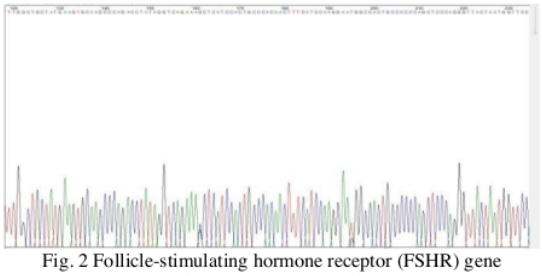

Fig. 2 Follicle-stimulating hormone receptor (FSHR) gene electrophoregram results

3.3. Blast Results & Multiple Alignment for FSH

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
Bos indicus      ---------- --AACTACAC GGGACTTGGT GTACAGGGAC CCAGCAAGGC
FSH Madrasin     CTCNTGTCTC CTAACCACTC AGGACTTGGT GTACAGGGAC CCAGCAAGGC

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
Bos indicus      CCAACATTCA GAAAACATGT ACCTTCAAGG AGCTGGTCTA CGAGACGGTG
FSH Madrasin     CCAACATTCA GAAAACATGT ACCTTCAAGG AGCTGGTCTA CGAGACGGTG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
Bos indicus      AAAGTGCCTG GCTGTGCTCA CCATGCAGAC TCCCTGTACA CGTACCCAGT
FSH Madrasin     AAAGTGCCTG GCTGTGCTCA CCATGCAGAC TCCCTGTACA CGTACCCAGT

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
Bos indicus      AGCCACTGAA TGTCACTGCG GCAAGTGTGA CAGCGACAGC ACTGACTGCA
FSH Madrasin     AGCCACTGAA TGTCACTGTG GCAAGTGTGA CAGCGACAGC ACTGACTGCA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
Bos indicus      CCGTGCGAGG CCTGGGGCCC AGCTACTGCT CCTTCAGGGA AATCAAAGAA
FSH Madrasin     CCGTGCGAGG CCTGGGGCCC AGCTACTGCT CCTTCAGGGA AATCAAAGAA

                 ....|....| ....|....| ....|....| ..
                       260        270        280
Bos indicus      TAAAGAGCAG CGGATGCTTT GAGCTGCCTA C-
FSH Madrasin     TAAAGAGCAG CGGATGCTTT GA-CTGCCTA CA
```

Fig. 3 Multiple alignment results of Madrasin cattle FSH gene and Bos Indicusx Bos taurus cattle FSH gene

3.4. Blast Results and Multiple Alignments for FSHR

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
FSHR Madrasin    TCTTCATTCT GCTGAGCAAG TTTGGCTGCT ATGAAGTGCA AGCCCAGACC
Bos indicus      --TTCATTCT GCTGAGCAAG TTTGGCTGCT ATGAAGTGCA AGCCCAGACC

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
FSHR Madrasin    TATAGGTCAG AAAGCTCATC CACTGCCCAC AACTTTCATC CAAGGAATGG
Bos indicus      TATAGGTCAG AAAGCTCATC CACTGCCCAC AACTTTCATC CAAGGAATGG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
FSHR Madrasin    CCACTGCCCC CCAGCTCCCA GGGTTACTAA TGGTTCCAAT TACACCCTTA
Bos indicus      CCACTGCCCC CCAGCTCCCA GGGTTACTAA TGGTTCCAAT TACACCCTTA

                 ....|....| ....|....| ....|...
                       160        170
FSHR Madrasin    TCCCCCTAAG ACATTTGACC AAGAACTA
Bos indicus      TCCCCCTAAG ACATTTAGCC AAGAACTA
```

Fig. 4 Multiple alignment results of Madrasin cattle FSHR gene and Bos Indicusx Bos taurus cattle FSHR gene

Table 1 Position and type of nucleotide mutation of FSH gene and FSHR gene for Madrasin cattle

No	Type of Genes	Base Position	Mutation	Type of Mutation
1		16	T>C	Transition
2	FSH	19	A>T	Tranver ion
3		21	G>A	Transition
4		169	C>T	Transition
5	FSHR	167	G>A	Transition
6		168	A>G	Transition

4. Discussion

This research analyzes the Sequencing Follicle Stimulating Hormone (FSH) and Follicle Stimulating Hormone Receptor (FSHR) gene polymorphisms in Madrasin cattle with ovarian hypofunction in a total of 14 blood samples. Sequencing is the final step in determining the nucleotides amplified by PCR. Furthermore, it is performed according to the Sanger method using the Automatic DNA Sequencer based on the dye terminator labeling method. The DNA sequencing stages carried out include: (1) DNA preparation, (2) amplification through PCR using universal primers, (3) DNA purification, (4) electrophoresis, and (5) reading of the sequenced electropherogram.

The data obtained are in the form of an electropherogram in an ABI file, each nucleoid being displayed with a different color. Adenine (A) nucleotides are shown in green, Guanine (G) is shown in black, Thymine (T) is shown in red, and Cytosine (C) is shown in blue. The analysis of the results for the sequence of the PCR products was carried out by comparing the nucleotide sequence of the sample with the reference nucleotide sequence. The nucleotide sequence of the sample results from the PCR genes FSH, FSHR, and the reference nucleotide sequence is entered into a program that automatically sequences the nucleotides of the sample according to the order and position of the standard nucleotides and then checks certain nucleotides other than nucleotides.

A total of 2 samples of PCR control products were selected, the best from 14 samples to be sequenced according to the selection after purification of the DNA samples by First Base Malaysia. The samples were sequenced then BLAST to see the nucleotide sequence from the sequencing results with the available database on the website www.ncbi.nlm.nih.gov to find the similarity of a nucleotide or protein sequence (query sequence) with a database sequence (subject sequence). The sequence alignment was carried out using the BioEdit ver. 8 Program.

The BLAST results show the sequences of nucleotides with NCBI database that are 94% homologous with Bos Indicus cross Bos taurus Follicle Stimulating Hormone (FSH) Beta-Subunit Gene (XM_027562775.1) and 98% homologous with Bos Indicus cross Bos taurus Follicle Stimulating Hormone Receptor (FSHR) (XM_027555230.1).

The transition mutations occur because the purine bases (A) have been replaced by other purine bases (G), or the pyrimidine bases (T) have been replaced by other pyrimidine bases (C). Transversion mutations occur because the pyrimidine base (T) is replaced by a purine base (A), or a pyrimidine base (C) is replaced by a purine base (G) [12]. Based on Table 1, it can be seen that the FSH gene at base positions 16 and 21 has a

transition mutation (T> C and G> A) and that the base position 19 has a transversion mutation (G> A), while the FSHR gene at base positions 167 and 168 has a transition mutation. (G> A and A> G).

The mutations or variations in the FSH and FSHR genes can be influenced by many factors, which include natural selection, environmental factors, mutations, and mating [13]. The presence of mutations in the FSH and FSHR genes is likely due to the cross-breeding of Madura cattle with Limousin breeds, and therefore the possibility of slipping occurs when DNA recombination takes place.

5. Conclusion

The BLAST results showed that the nucleotide sequence of FSH with the NCBI database was 94% homologous with the Bos Indicus cross Bos taurus Follicle Stimulating Hormone Beta-Subunit Gene (XM_027562775.1) and 98% homologous with the Bos Indicus cross Bos taurus Follicle Stimulating Hormone Receptor (FSHR) (XM_027555230.1). The FSH gene experience a transition mutation at base positions 16 and 21, and transversion mutations at base position 19, while the FSHR gene experience a transition mutation at bases 168 and 169.

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