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GENETIC MUTATION AND DELETION IN MADURA'S CATTLE AS THE RESULTS OF CROSSBREEDING

Budi Utomo[1] and Erma Safitri*[1,2]
[1]Departement of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga
[2]Stem Cells Research Division of Institute Tropical Disease (ITD), Universitas Airlangga

BU: budi_reprovet@yahoo.com
*Correspondence author: erma-s@fkh.unair.ac.id

Abstract

Crossbreeding between Madura's cattle with superior cattle from other nations will have consequences on germplasm conservation Madura's cattle will be not valid, except in Sapudi Island. Required the use of Mitochondrial Deoxyribonucleic acid (mtDNA) as a genetic markers in intraspecific variability, can be used to track the relatively new events, can be used for a historical reconstruction phyilogenic of several species that are close together. This study uses the D-loop region fragment Madura's cattle to get the data so that it can determine the genetic diversity between individual nucleotide composition of Madura's cattle and identify its phenotypes. Such data can be used to identify the genetic purity of Madura's cattle that exist in the area of Sapudi island, Sumenep, Pamekasan, Sampang, Bangkalan. DNA fragment size is 980 bp in D-loop region of mitochondrial DNA that is located in the area of individual mtDNA 15795-16341. Madura's cattle have been successfully amplified by PCR using primers BIDLF and BIDLR. The results of PCR successfully read the nucleotide sequence is different each individual cow Madura (BP 716; 756 BP; BP 964; 1098 BP; BP 1113). In conclusion, from the sequence analysis of mtDNA D-loop region have found 50 sites of genetic diversity: Pamekasan Madura's cattle mutation, there were 15 transitions, 9 transversions. Sampang's Madura cattle have 13 transitions, 8 transversions. Madura's cattle from Sumenep have 2 transitions, 2 transversions and 1 deletion. Despite this high level of variation in this study

Key words : Madura's cattle , Germplasm, Genetic Characters, mtDNA, Genetic Purity

1. Introductiom

'World Watch List for Domestic Animal Diversity' report (3rd ed.) Reports that there are approximately 6300 race (breed) cattle in the world of about 30 species of animal domestication and most of the breed is now a local species originating from countries growing. The genetic diversity of local breeds owned a large role in the success of breeding programs in developing countries during the period of the 19th to 20th century (1).

Food and Agriculture Organization (FAO) predicts that at least one traditional breeds become extinct every week and more than 30% of livestock in Europe is now estimated threatened with extinction (2). Many traditional breeds have disappeared as farmers focus more on the nation's new cow. Approximately 16% of the nation's traditional cattle have become extinct and less than 15% are rare (3).

As science and technology of cattle breeding, biotechnology, market demand, the mechanization of agriculture and livestock production, will encourage the exploitation of animals through crossbreeding to make new breed (4-5).

Groups of cattle were included in the first category is Bali cattle because Bali cattle are known to be the result of domestication directly from Bull (6-7) and has the physical characteristics that only has a minor change compared to its predecessor. The second group of cattle are Madura cattle because according (8) and (9); they are the result of cross between bull or Bali cattle with zebu cattle that has lasted more than 1,500 years ago, even though it is not well documented (without obvious recording).

Exploitation of Madura cattle through crossbreeding increasingly broad and uncontrolled with exotic cattle which would give unfavorable impact on Madura cattle that have adapted to the local environment. Genetic studies in cattle Madura Madura is interesting because genetic variation is quite large. It is important related to business improvements and maintain the properties of the genetic code so that local livestock did not decrease even extinct genetic quality of Indonesian country.

Molecular tagging using DNA (Deoxyribonucleic Acid) both on the nuclear DNA and mitochondrial DNA (mtDNA) will get results that can reveal the difference with more precision in differentiating intra and interspesies concerning the structure, composition and organization of the genome at the DNA level.

This study uses the D-loop region fragment Madura cattle to get the data so that it can determine the genetic diversity between individual nucleotide composition of Madura cattle and identify sightings fenotifnya. Such data can be used to identify the genetic purity of

Madura cattle that exist in the area of Sapudi island, Sumenep, Pamekasan Pamekasan, Sampang, Bangkalan.

2. Materiala and Methods

2.1. Materials Research

Sample : 5 ml of whole blood from each cattle taken from vein puncture was filled in venoject with 10% EDTA.

Materials : 20 samples of whole blood from cattle, *Wizard Genomic DNA Purification Kit* from Promega (*cell lysis solution, nuclei lysis solution, protein precipitation solution,* RNA*ase*, DNA *rehydration solution*), isopropanol, 70 % ethanol*,* PCR *Core System* I from Promega (MgCl 2, 10 X *buffer reaction Taq* DNA *polymerase*, PCR *nucleotida mix, Taq* DNA *polymerase*), restriction enzymes *Hind* III from Gibco BRL, agarose from Promega, 1X *tris acetic acid* EDTA (TAE), *ethidiumbromida*, aquades, primer D-Loop which consist of primers, primer DL-F:5'TTCTTCAGGGCCATCTCATC-3' and primer DL-R:5'GCATCTTGAGCACCAGCA TA-3', *blue loading dye*, tissue, parafilm, ice krystal, *ultra pure water* from Biotech, 50-2000 bp *marker* from Bio Rad.

Agarose Gel Electrophoresis : *agarose gel* 1.5% → solution of 0.5x TBE (Tris-Borate EDTA) 30 ml, 0.45 grams of agarose powder, EtBr 2.5 µl. Materials needed in agarose gel electrophoresis using a sample of DNA produced by PCR, loading dye (0.01 xylene Cyanol%, 0.01% Bromtimol Blue, 50% glycerol), danmarker 100 bp.

2.2. Research Instruments

Centrifuged (Hettich), a micropipette (size 20 mL, 200 mL, 1000 mL), tips 20µl, 200 µldan 1000 mL, 1.5 mL micro tube (Axygen), 0.6 ml PCR tube, a set of horizontal electrophoresis apparatus and power supply (Consort), microwave, incubators, GeneAmp PCR System 2400 thermocycler (Perkin Elmer), Gel Doc 2000 (Bio Rad), autoclave (Ogawa Saiki Co.), measurring cup, erlenmeyer, tube venoject, vortex mixer (Gemmy Industrial Corp.), gloves, water bath (Haake), freezer with temperature of 4° - 20° C, ice crystals maker (Cornelius), electric scales (Denver instrument). a set of tray scorer, digital scales, a 100 volt power supply, micro pipette, tips, glass beaker, microwave, stirrer, and UV Transilluminator.

2.3. Location and Time Research

Research conducted at the Laboratory of Molecular Biology, Institute of Tropical Disease (ITD) . The research was conducted in January and February 2015.

3. Results

3.1. Bases Nucleotide Composition In Madura's Cattle : Sapudi, Bangkalan, Sampang, Pamekasan, Sumenep.

PCR products were sequenced cattle's DNA from five lengths and gained different nucleotide bases that is 710 bp, 1110 bp, 964 bp, 756 bp, 716 bp. Analysis of nucleotide diversity arrangement made after the arrangement of mtDNA D-loop region Madura's cattle Sapudi, Bangkalan, Sampang, Pamekasan, Sumenep aligned with MEGA application 6 with particular reference to the nucleotide composition of the island of Madura's cattle Sapudi. The number of nucleotides of the five bulls is not the same after comparison. This is because there are some deletions of nucleotides in Madura's cattle Sapudi, Bangkalan, Sampang, Pamekasan, Sumenep. Results of multiple alignment can be analyzed as many as 156 bp (Partial) and derived nucleotide diversity of the site as much as 25 pieces. This proves that the D-loop region is an area with very varied nucleotide bases. According to (10), and (11) note that the D-loop region of mitochondrial DNA has the nucleotide bases variation is high, so it is suitable to distinguish the differences between individuals both within the family and between families. Results of multiple alignment of the three cows can be seen in Table 1.

The results of the nucleotide alignment composition of Madura's cattle in Sapudi, Bangkalan, Pamekasan, Sampang, Sumenep which can be analyzed by MEGA 6 along 156 bp. Of the five cows were studied, each of which has the nucleotide composition Thymine (T), Cytosin (C), adenine (A), Guanine (G) are different. This can be seen in Table 2

From table 2, it can be seen from the table comparison of the percentage of nucleotide T (Thymine) D-loop region highest to lowest in a row owned by Madura's cattle Sumenep 32.9%, Sapudi 32.7%, 32.7% Bangkalan, Sampang and Pamekasan 31.4% 29.5%. Comparison of percentage of nucleotides C (Cytosin) D-loop region highest to lowest consecutive Pemekasan Madura cattle owned by 19.9%; 17.9% Sampang, Sumenep 16.1%, Bangkalan Sapudi 16% and 16%. A percentage of nucleotides (adenine) region D-loop highest to lowest consecutive Sapudi Madura cattle owned by 42.9%; Bangkalan 42.9%, 42.6% Sumenep, Pamekasan and Sampang 41.7% 40.4%. The percentage of nucleotide G (Guanine) D-loop region highest to lowest consecutive Sampang Madura's cattle owned by

10.3%; 9% Pamekasan, Sumenep 8.4%, 8.3% and Bangkalan Sapudi 8.3%. Differences in the composition of the nucleotides A, T, G, and C show the differences in composition of amino acids that it contains (Riddly 1991) (12).

The percentage of A + T nucleotides on all cattle studied were Madura's cattle Sapudi, Bangkalan, Pamekasan, Sampang and Sumenep has a higher amount than the percentage of G + C nucleotides. The percentage of A + T nucleotides from the highest to the lowest, respectively, are Cow Sapudi 75.6%; Bangkalan 75.6%; 75.5% Sumenep, Pamekasan, Sampang 71.8% and 71.2%. The percentage of G + C nucleotides from the highest to the lowest, respectively, are 28.9% Pamekasan Madura's cattle; Sampang 28.2%.%, 24.5% Sumenep, Sapudi 24.3%, 24.3% Bangkalan. In this study, the composition of the nucleotide bases A + T has a higher frequency than the composition of the G + C.

Sites such diverse nucleotide bases due to mutations in the nucleotide bases. Mutations include substitutions transition mutations (replacement of a purine base by another purine base is A↔G or substitution of pyrimidine bases by another pyrimidine bases are T↔C) and transversion substitution mutations (replacement of a purine by a pyrimidine bases, namely A↔ C, A↔T, G↔C, G↔T, or replacement of a purine pyrimidine bases by which T↔A, T↔G, C↔A, C↔G). In addition there is also a deletion mutation, namely the reduction of base pairs that caused loss of base pairs that originally there. From these results, Pamekasan on Madura cattle there are 15 transition substitution (Si), 9 transversion substitution (ST). In cattle there are 13 mutations Sampang transitions, 8 transversions mutations. In cattle there are 2 Mutation Tranversi Bangkalan and Sumenep Cow tranversi there are two mutations, one deletion.

3.2. Genetic distance between Madura's Cattle Sapudi, Bangkalan, Pamekasan, Sampang, Sumenep.

Close genetic relationship between Madura's cattle in Sapudi, Bangkalan, Pamekasan, Sampang and Sumenep are seen by measuring the genetic distance. Genetic distance is measured using Pairwise analysis indicated Distance Calculation matrix genetic differences between the three cows that have made sequencing the nucleotide at D-Loop region MtDNA.

The genetic distance from this model is used to look at the level of substitution of transition and tranversi through many different nucleotides per couple (13). The cows that have the lower value of genetic distance, then the animal has a closer kinship. Instead of cattle that have high genetic distance, the relationships are more distant. Genetic history between Sapi Madura Sapudi, Bangkalan, Pamekasan, Sampang, Sumenep (Table 3).

From table 3 showed that the genetic distance Madura's cattle Sapudi (0.0000) with Beef Bangkalan and Sumenep (0:13) has a close kinship level. While in the cow Sampang Madura (0186) and Pemekasan (0173) have genetic distance farther. This is because of differences in nucleotide significantly in Madura's cattle with cattle Sapudi Pemekasan and Sampang.

4. Discussion

Information on genetic diversity can be obtained in several ways, such as by using microsatellite DNA [14], AFLPs, SNPs, Y-chromosomal DNA or analyzing the mitochondrial DNA (mtDNA) [15]. Mitochondrial DNA (mtDNA) has represented the most informative genomic elements for describing the origin of livestock. Until now, the mitochondrial sequences have been widely studied in cattle, pigs, sheep, horses, dogs, donkeys, and goats (16).

Mitochondrial deoxyribonucleic acid (mtDNA) in this study were isolated from cattle's blood cells with the same quality with mitochondrial DNA isolated from tissue / muscle. This is in line with the statement from Tapio and Grigaliunaite (17), expressed the mitochondrial DNA can be isolated from the hair, bones, teeth, body fluids (saliva, semen, blood). According (18), mitochondrial DNA found in cells or tissues that have the activity of metabolites high or in areas that require ATP in large quantities, such as the tail of sperm cells, epithelial cells are actively dividing the skin epidermal tissue and heart muscle cells.

MtDNA segments that can be used for the analysis of genetic diversity of an organism is the mtDNA control region or D-loop region, namely the non-coding part of the mitochondrial hypervariable (19). The high rate of nucleotide polymorphisms or differences in the sequence of the second part of the non-coding hypervariable region is used to distinguish between individuals of a species (20). In addition D-loop region analysis is also used to see the variation among subspecies and between populations (21). MtDNA D-loop regions known to be rapidly developed compared with other parts of mtDNA. This is due to the accumulation of base substitution, insertion and deletion processes that speed is very fast when compared to nuclear of DNA (22).

The results of comparative studies from Madura cattle in Bangkalan, Pemekasan, Sampang and Sumenep in this study have a high nucleotide diversity arrangement. From these data can be analyzed that nuklotida bases A (adenine) has a higher percentage of nucleotide bases C (cytosine). Studies Prusak and Grzybowski (2004) (23) of 20 species of

mammals discovered cases in humans and zebra have sequences with more base A compared to C on the strand mild mitochondrial DNA, sheep and cattle (Zebu) bases A lot more than the base C, and this phenomenon is common in mammals. The results also showed the same phenomenon. According to (24) This phenomenon has to do with the level of amino acid identity associated with a redox reaction center protein content (both Qo and Qi) which is involved in electron transfer (25-26).

Differences in the rate of mutation or variation in the mitochondrial regions in this study may be influenced by many factors (27-28). Studies to determine the barrier effect of the mutation rate of the level of replication, DNA repair efficiency, and exposure to mutations are not done immediately, and is generally associated with biological variables, such as body size, generation time and SMR (Standard Metabolic Rate). According to the theory, the individual taxa with large body size, a long life and value of low metabolic, usually have low mutation rate, but according to the results of research on ribosomal genes and P12, mtDNA mutation is not related to body size and SMR (29-32) argues that the effect of the rate of metabolism is proposed as a factor affecting the level of mitochondrial mutations, because the pollutants contained in the free radicals of oxygen can affect the metabolism rate. Although discovered a correlation between mutation rate and SMR, but only found in the case of an improved model of mitochondrial damage (DNA repair) due to insufficient oxidation. This indicates that mitochondria in higher organisms have been equipped excellent mechanism through excision bases within the system repair oxidative damage (33), as happened in the nuclear genome of the cell , hence the existence of a specific DNA repair efficiency in the taxon has never been proven. (34) reported that the composition of mtDNA was not associated with oxidative damage but more on spontaneity during the replication process. Therefore, the discovery of cases of mutations in this study is likely to occur at the level of DNA replication. This is in line with the opinion of (35) that genetic diversity can arise due to natural selection, environmental influences, mutation and mating. The genetic diversity and a high mutation rate in this study is not interpreted as being detrimental. Maintain the genetic diversity of a population is very important in conservation because of the high genetic diversity will greatly help a population to adapt to the changes that occur in the surrounding environment. Polymorphic high level in the D-loop region of mtDNA does not cause changes in third phenotype cows were tested. This is because the D-loop region is non-coding regions that are not expressed. This area only play a role in the regulation and initiation of replication and transcription of mtDNA (36).

Conclusion in this research : Mitochondrial Deoxyribonucleic acid (mtDNA) region Diplacement Loop (D-Loop) Sapudi Madura cattle, Bangkalan, Pamekasan, Sampang, Sumenep can be amplified well with the size of 980 bp. From the sequence analysis of mtDNA D-loop region found 50 sites of genetic diversity that is Madura cattle from Pamekasan substitution mutation, there were 15 transitions, 9 transversions substitution mutation. Sampang Madura cattle have 13 substitution mutations transitions, 8 transversions substitution mutation. Madura cattle from Sumenep have 2 substitution transitions mutation, 2 transversions substitution mutations and 1 deletion. Despite this high level of variation in this study did not result in a change in phenotype as D-Loop region is non-coding regions that are not expressed.

Acknowledgments

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Table 1. Alignment of Nucleotide sequences of D-Loop Regions between Madura cattle Sapudi, Bangkalan, Sampang, Pamekasan, Sumenep.

	Sequence	
Sapudi	**ATG TAC ATA ACA TTA ATG TAA TAA AGA CAT GAT ATG TAT ATA GTA CAT**	**48**
	TAA ATT ATA TAC CCC ATG CAT ATA AGC AAG	**78**
CB	... ...	48
	... ...	78
CP	.A. ... ... .T. ... ... A..	48
	..C ... .C.	78
CS	.A. ... ... .T. ... ... A.. ... ... G..	48
	..C ... .CT ... .G.	78
CSm	... ...	48
	... ...	78

	Sequence	
Sapudi	**TAC ATG ATC TCT ATA ATA GTA CAT AAT ACA TAC AAT TAT TAA TTG TAC**	**126**
	ATA GTA CAT TAT ATC AAA TCC ATC CTC ACA	**156**
CB	... ...	126
	... ... .AC	156
CP	... T.. .A. ..C ... .C. ... ... .G. ... .TA .TC C.C ... .C. ...	126
	... ...C ... .T .C. ..T GAC	156
CS	... T.. .A. ..C ... .C. ... ... .G. ... .TA .TC C.C ... .C. ...	126
	... ...T ... ..T GAC	156
CSm	... ...-.	126
	... ... .AC	156

Note:

Sapudi : Madura cattle Sapudi
CB : Madura cattle Bangkalan
CP : Madura cattle Pamekasan
CS : Madura cattle Sampang
CSm : Madura cattle Sumenep
Dot (.) Denotes nucleotide same Madura cattle Sapudi.

Table 2. Comparison of Nucleotide composition D-loop Region Partial Madura cattle Sapudi, Bangkalan, Pamekasan, Sampang, Sumenep.

Madura Cattle	**%**					
	T	**C**	**A**	**G**	**T + A**	**C+G**
Sapudi	32.7	16.0	42.9	8.3	75.6	24.3
Bangkalan	32.7	16.0	42.9	8.3	75.6	24.3
Pamekasan	29.5	19.9	41.7	9.0	71.2	28.9
Sampang	31.4	17.9	40.4	10.3	71.8	28.2
Sumenep	32.9	16.1	42.6	8.4	75.5	24.5

Table 3. Genetic Distance Based Method D Pairwise-loop Regional Distance between Madura cattle Sapudi, Bangkalan, Pamekasan, Sampang, Sumenep.

No	**Sapi Madura**	**1**	**2**	**3**	**4**
1	**Sapudi**				
2	**Bangkalan**	0.013	-		
3	**Pamekasan**	0.173	0.154	-	
4	**Sampang**	0.186	0.166	0.033	-
5	**Sumenep**	0.013	0.000	0.154	0.166

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Polymorphism of Growth Hormone (GH) Genes in The Artificial Insemination Result of Madura Cattle with Limousin Cement as A Reference for Genetic Selection ~~of Superior Seeds~~

Abstract

Research on genetic polymorphism of growth hormone (GH) and receptor growth hormone (rGH) has not been done in crossbred of Limousin cattle, so it is interesting to be examined. Blood samples were taken from 14 Madura calves that were artificially inseminated with

Limousin cement. DNA amplification done by using Polymerase Chain Reaction (PCR) method, Restriction Fragment Length Polymorphism (RFLP) method to determine the genotype. DNA sequencing was done to determine nucleotide sequences of GH unit genes. The results showed that identification of GH gene polymorphisms and rGH was done by breaking DNA fragments from 432 bp and 298 in Madura and Limousin cattle (Madrasin) ie, L and V alleles have a frequency of 0.67 and 0.33 for the GH gene, respectively. This proves that the crossed-breeding of Madrasin have V alleles that are not owned by the Madura cattle. While in the rGH gene, the A allele is 0.92 and the G allele is 0.08, with the frequency of the A allele larger than the G allele. This research concluded : the GH and rGH undergo changes on polymorphisms in Madrasin cattle and this research information can be used as a basis for superior seed selection.

Key words: Polymorphism, GH Gene, rGH Gene, Madrasin, PCR, RFLP, V alleles

1. Introduction

Madura Cattle is one of Indonesia's germplasm wealth. Several laws enacted in an effort to maintain its purity. One of the regulations concerning the preservation of Madura cattle issued since the Dutch colonial era is staatsblad (sheets of the country) No. 226/1923, No. 57/1934, and No. 115/1937. Post-independence, chapter 13a of Law No. 6/1967, had set

out the main points of animal husbandry and animal health, in an effort to maintain the population, shape, colour of the skin, and improve the quality of Madura cattle production.

Madura cattle is one type of local Indonesian cattle that grow on the island of Madura and surrounding islands. Morphologically, Madura cattle have almost the same characteristics as Balinese cattle except for their smaller body size and horns. The skin colour of male and female Madura cattle is browner than Bali cattle, lower legs to knees (1). In addition, Madura cattle are more resistant to hot weather, efficient to food, have good meat quality, and are more resistant to parasites (2).

Madura cattle become a local breed of beef cattle that is formed as a result of natural isolation and environmental influences, so it has a uniformity characteristic that stands out among other local beef breeds in Indonesia. With the contribution of genetic characteristics of zebu cattle such as tolerance to stress due to climate and resistance to tick attack and strict natural and environmental selection in a long time, so Madura cattle become a cattle breed that has a very high adaptability to the environment. In addition, Madura cattle have a good response to the improvement of feed and resistance to feed with high crude fibre content (3).

The exploitation of Madurese cattle through an increasingly widespread crossing with exotic cattle will have the effect of changing phenotypic and genetic traits. Crosses are done by breeders to obtain superior performance especially at the speed of weight growth and reproductive power.

Therefore, genetic studies of Madura cattle that have been crossed with Limousin breed become interesting to do in order to see the calves produced have a good quality in terms of body growth and reproduction. Furthermore, the results of the genetic selection will be used as a reference for obtaining superior seeds, especially for crossbred calves from Madura cattle that are inseminated with limousin cement.

Advances in the field of molecular biology provide new opportunities in an attempt to detect the occurrence of genetic variation (polymorphism) as a basis for improving genetic quality in farms. Potential molecular techniques used to detect variations include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphism DNA (RAPD), Double Strand Conformation Polymorphism (DSCP), and Marker-Assisted Selection (MAS). With effective and accurate technology through the use of diagnostics based on deoxyribonucleic acid (DNA), it will greatly assist cattle crossing programs. Provision of genetic maps through recombinant DNA methods can assist cattle crossing programs through obtained molecular data, which regulate the properties of production (4).

Gene products in the form of hormones (bioregulators) will affect the regulatory process of metabolism and appearance of livestock morphology. Genetic variation (polymorphism) in gene loci especially that encodes hormone is very important because it determines the genetic character of a population that can assist in improving the genetic quality of the population (5).

Growth hormone as one of the gene products has a major effect on the growth, lactation and development of mammary glands in cattle (6). Polymorphisms in genes that encode and regulate growth hormone are very potential as genetic markers for phenotypic properties with high economic value productivity.

In addition to growth hormone, research needs to be done also on the reproductive side, to obtain a picture of polymorphism of reproductive hormone in calves from cattle with Limousin cattle. Research on genetic polymorphism of growth hormone (GH) gene has not been done in crossbred calves from Limousin cattle, so it is interesting to be investigated deeper so that the results obtained can be used as a reference for obtaining cattle breeds that have a good quality of performance and power reproduction.

In addition to growth hormone, research needs to be done also on the reproductive side, to obtain a picture of polymorphism of reproductive hormone in calves from cattle 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 (7). In females, it functions for the proliferation and development of follicles until ovulation (8). While in men, the combination of FSH and testosterone is a tropical hormone that regulates Sertoli cell function, which is necessary for initiation and maintenance of quality and quantity in spermatogene (9). FSH hormones consist of α and β-sub units. β-sub units play a role in determining the specificity of bonding with receptors (FSHR) (10). It ha been reported that the presence of exon 3 mutations of the FSH β-sub-unit genes in bul were identified to have lower fresh cement concentrations, a lower percentage of acroson integrity on fresh and frozen cement, more low motility in frozen semen (11). Research genetic polymorphism of growth hormone (GH) and β-sub-unit genes has not been done crossbred calves from Limousin cattle, so it is interesting to be investigated deeper so that results obtained can be used as a reference for obtaining cattle breeds that have a good qua of performance and power reproduction.

2. Materials and Methods

2.1. Research Materials

The main material of this research is DNA Genome obtained from the bloo Madura calves from Limousin cement artificial insemination with 14 blood sam Genomic DNA was then extracted using a DNA Extraction QIAamp Mini spin column k degrade the cell walls, proteins, and fats. The DNA samples were then ready for reactions. The primer used to amplify GH gene (Table 1)

2.2. Materials and Equipments

Tools used include DNA Extraction Kit, venoject, vacuuttainer tube, cem refrigerant, small and large eppendorf tube, agarose gel, micropipette, tip, tub electrophoresis, autoclave, scales, and gloves.

The main ingredient is DNA samples taken from the whole blood of Madu from Limousin cement artificial insemination from Bangkalan, Madura Distric samples. Supporting materials include: Primer (GH gene Primer), HaeIII Restricti PstI restriction enzyme, DNA Extraction Materials (K Proteinase, Absolute Et Buffer, A & B Wash buffer), PCR materials (dNTP mix, Taq DNA polymer Electrophoresis Materials (Triss Base, boric acid, agarose, Na2 EDTA, Ethid DNA Marker, DNA Loading dye), tissue and mica plastics.

2.3. Research Methods

2.3.1 Collection of Blood Samples

Blood samples were obtained from Bangkalan District. Blood samp cattles and Limousine cattle crossbred calves did by collecting about 5 m from the cattle through the jugular vein by using venojet and vacutainer t then kept at 4°C.

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The DNA was isolated and purified using a QIAamp Mini s DNA kit following the provided extraction protocol. A total of 200 lysed by adding 200 µl lysis buffer solution and 20 µl K proteinase

was then incubated at 56°C for 60 minutes in the waterbath shaker. After incubation, the solution was then added 200 µl 96 % absolute ethanol and centrifuged 8000 x g for 1 min.

DNA purification was done by spin column method with the addition of 500 µl wash buffer I then continued with centrifugation at 8000 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 min. After the supernatant was removed, the DNA was then dissolved in 200 µl elution buffer and sterilized at 8000 x g for further extraction of DNA to be stored and stored at -20°C.

2.3.3 PCR-RFLP Technique

The PCR reaction composition was conditioned on 25 µl reaction volume comprising 100 ng of DNA, 0.25 mM each primer, 150 µM dNTP, 2.5 mM Mg2 +, 0.5 Taq DNA polymerase and 1x buffer. The condition of the PCR machine begins with the initial denaturation at 94°C x 2 minutes, followed by 35 subsequent cycles with each denaturation at 94°C x 45 seconds, with annealing temperature: 65°C x 30sec (GH), followed by one end extension cycle at temperature 72°C for 5 minutes using GeneAmp PCR System 2400 ThermoCycler (Perkin Elmer), for FSH β-sub unit primer with anealing at 60°C. The PCR product was then electrophoresed on a 1.5% agarose gel with 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na2EDTA) containing 100 ng/ml ethidium bromide. Then visualized on UV transilluminator (gel documentation system). The allele is determined by interpreting the bands that are most migratory to the anode pole as allele 1, allele 2, and so on.

The PCR products obtained from each of the target genes were then analyzed using RFLP by cutting using restriction enzymes having cutting sites in the HaHII gene and the FSH β-PstI gene. A total of 4 µl DNA PCR product added *0.5 µl*, then incubated for 17 hours at 37°C.

2.3.4 Sequencing

The determination of nucleotide sequences of GH (Growth Hormone) rGH (receptor Growth Hormone) unit genes was done by DNA sequencing that is the final step to obtain data of nucleotide sequence from fragment result of PCR-RFLP propagation. The DNA bands that already restricted on an agarose gel as PCR-RFLP products serve as a mould in the sequencing reaction by using forward and reverse primers as in the time of amplification.

2.3.5 Data Analysis

The diversity of individual genotypes can be determined from the DNA bands of the gene found. Each sample was compared to the same size (marker) and calculated the frequency of the allele. The frequency of alleles can be calculated using the Nei and Kumar formulas (12). Sequencing data analysis using UGENE 1.21.0 software.

3. Resuts

3.1 The PCR Result of GH Gene

PCR of 14 Madrasincattle's blood samples was performed to detect the presence of the Growth Hormone (GH) gene, the result of PCR showing 14 gene band GH using GH gene primers. Positive results are shown in Figure 1.

3.2. The PCR Result of rGH Gene

The result of amplification of 14 Madrasin Cattles in Bangkalan District conducted by PCR method resulted in 14 positive DNA samples using the rGH gene primers. The positive visualization results of electrophoresis can be seen in Figure 2

3.3. The PCR-RFLP Result of GH Gene.

After detecting the presence of the GH gene in Madrasin cattle using the PCR method, the RFLP or GH DNA gene cutting was performed using the AluI retrieval enzyme (5'-AG | CT-3'). Results of RFLP were 14 GH gene samples divided into 4 bands, ie 180 bp, 250 bp, 300 bp, and 400 bp. The result of RFLP of Madrasin cattle GH gene can be seen in Figure 3.

3.4. The PCR-RFLP Result of rGH Gene

The result of amplification test with Polymerase Chain Reaction (PCR) product obtained was then digested with AluI restriction enzyme, obtaining band yield of 167 bp and 81 bp in Madrasin cattle. The electrophoresis results in the process of PCR-RFLP of Madrasin cattle's blood sample can be seen in Figure 4.

4. Discusion

4.1. PCR of GH

The PCR results of the Madrasin cattle's GH gene showed a 432 bp band, *this* corresponds to the bands in the genome library GH itself is a hormone candidate *that plays a* role regulating milk production, carcass and immune system (13). *The GH gene is one of the most important things in managing the properties of high-value livestock so that the GH gene* becomes a gene candidate in the Marked Assissted Selection (MAS) program *in cows.* GH gene plays a role in regulating postpartum growth, tissue, muscle, bone and adipose tissue development, mammary gland growth, lactation, reproduction, and carbohydrate, protein, and body fat metabolism. The GH gene requires a receptor in its expression mechanism to a network mediated by the rGH gene or Growth Hormone Receptor (14).

4.2. PCR of rGH

Based on the results of blood amplification by Polymerase Chain Reaction (PCR) method, continued with electrophoresis readings obtained positive sample results. The rGH genes found in all of Madrasin cattle's blood samples can be detected by PCR with a length of product amplified by the rGH gene segment of 298 bp located in exon 10. The results of this research are similar to those identified by the genetic diversity of RGH genes in Limousin cattle previously performed by Zulkarnaim (15).

The success rate of rGH gene amplification in this research was 100%. The amplification results of the rGH gene segment were visualized on a 1.5% agarose gel presented (Figure 3.2). The temperature and duration of the annealing also determine the degree of amplification specificity and the cause of other factors that play a role in determining the success of amplification is the quality or purity of DNA used as template DNA.

4.3. RFLP of GH

Amplification of Madrasin cattle's GH gene using PCR method is known to have 432 bp length, which will be followed by cutting of Madrasin gene site using AluI restricition enzyme. Based on RFLP result, PCR product along 432 bp produce 2 allele that is L and V, whereas in Madura cattle can only be found 1 allele that is L allele.

Restrictive enzymes can recognize the GH gene at the cutting site, this is because the DNA sequence at the cutting site is not mutated. The triplet codon thus formed is CTG which encodes the Leusin allele (L) (16). L allele itself is shown with fragment length (60 bp, 100 bp, and 300 bp).

The diversity of the Madrasin cattle's GH gene is indicated by the presence of V allele resulting from the presence of a mutation or change of base causing the change of serine amino acid (C) to glycine (G) so that the Madrasin cattle's GH gene cutting phase changes from AGTC to AGGT. As a result of this change is formed codon triplet GTG that encode the valine amino acid (V) (17). V allele itself is shown with fragment length 60 bp, 100 bp, 150 bp and 300 bp.

4.4. Genotype Frequency and GH Gene Allele

According to the Volkandri's research (18), the frequency of genotypes and the L allele of Madura cattle were 1.00 and 1.00 respectively. Whereas in Madrasin cattle got the frequency of genotype and allele respectively participate 0,928 and 0,96. Based on these differences allegedly occurring changes in allele and genotype frequencies between Madura cattle with Madrasin cattle due to cross-breeding with Limousin cattle.

This is in accordance with Rachman's research (19), which found the genotype of Limousin cattle's frozen cement used in artificial *insemination in Larangan sub-district*, Pamekasan District. Successively detected L and V alleles have frequencies of 0.67 and 0.33 and 0.82 and 0.18 respectively. This proves that Limousin cattle have a V allele that is not owned by Madura cattle.

4.5. RFLP of rGH

Based on the results of treatment using PRC-RFLP of the amplified *rGH gene* segment there are two AluI cutting sites known as allele A and allele G, allele A is marked by truncation of 298 bp fragments into two parts along 167 bp and 81 bp. The fragment of the rGH gene that has anAluI enzyme cutting site will indicate that no mutation occurs but if no cutting site is indicated in the absence of a cutting by the AluI enzyme, it can be stated that there is a mutation in the rGH fragment site.

The diversity in the AluIrGH gene segment is thought to be due to the mutation or alteration of the base causing the change of serine amino acids to glycine. The change causes the cutting site not to be recognized by the AluI enzyme, resulting in an 81 bp fragment known as the G allele (13, 20). Results of genotyping on Madrasin cattle's segment of the rGH gene resulted in two fragments that were cut off, ie AAgenotype, which showed fragments along 81 bp and 167 bp and fragments that were cut into one band called AG genotype which showed fragment along 81 bp at (Figure 3.4).

The results of this research differ from the results of previous research conducted by Zulkarnaim [15], that is cutting the fragment of rGH gene in Limousin cattle yield three genotypes namely AA, GG, AG, AA genotype is shown as fragments along 167 bp, 81 bp and 50 bp, and genotype AG is shown as fragments along 167 bp, 131 bp, 81 bp and 50 bp.

4.6. Frequency of Genotype and rGH Gene Allele

The result of the analysis of the rGHaluI gene segment showed that the frequency of the allele A was 86% higher than the frequency of the allele G, the A and G allele frequencies in Madrasin cattle were 0.92 and 0.08, respectively (Table 3), while the AA and AG genotype were 0.85 and 0.14. The results of this research differed greatly from previous results of Limousin cattle which had A and G frequency in Limousin cattle respectively of 0.286 and 0.174 while AA and AG genotype were 0.238 and 0.095. B~~ased on these results it is known~~

In conclusion, in this study, the GH and rGH undergo changes on polymorphisms in Madrasin cattle and this research information can be used as a basis for superior seed selection.

Acknowledgement

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Limousin cement. DNA amplification is done by using Polymerase Chain Reaction (PCR) method, Restriction Fragment Length Polymorphism (RFLP) method to determine the genotype. DNA sequencing was done to determine nucleotide sequences of GH unit genes. The results showed that identification of GH and rGH gene polymorphisms was done by breaking DNA fragments from 432 and 298 bp in Madura and Limousin cattle (Madrasin) ie, L and V alleles have a frequency of 0.67 and 0.33 for the GH gene, respectively. This proves that the crossed-breeding of Madrasin have V allele that is not owned by the Madura cattle. While in the rGH gene, the A allele is 0.92 and the G allele is 0.08, with the frequency of the A allele larger than the G allele. This research concluded: the GH and rGH undergo changes on polymorphisms in Madrasin cattle and this research information can be used as a basis for superior seed selection.

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The exploitation of Madurese cattle through an increasingly widespread crossing with exotic cattle will have the effect of changing phenotypic and genetic traits. Crosses are done by breeders to obtain superior performance especially at the speed of weight growth and reproductive power.

Therefore, genetic studies of Madura cattle that have been crossed with Limousin breed become interesting to do in order to see the calves produced have a good quality in terms of body growth and reproduction. Furthermore, the results of the genetic selection will be used as a reference for obtaining superior seeds, especially for crossbred calves from Madura cattle that are inseminated with limousin cement.

Advances in the field of molecular biology provide new opportunities in an attempt to detect the occurrence of genetic variation (polymorphism) as a basis for improving genetic quality in farms. Potential molecular techniques used to detect variations include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphism DNA (RAPD), Double Strand Conformation Polymorphism (DSCP), and Marker-Assisted Selection (MAS). With effective and accurate technology through the use of diagnostics based on deoxyribonucleic acid (DNA), it will greatly assist cattle crossing programs. Provision of genetic maps through recombinant DNA methods can assist cattle crossing programs through obtained molecular data, which regulate the properties of production (4).

Gene products in the form of hormones (bioregulators) will affect the regulatory process of metabolism and appearance of livestock morphology. Genetic variation (polymorphism) in gene loci especially that encodes hormone is very important because it determines the genetic character of a population that can assist in improving the genetic quality of the population (5).

Growth hormone is one of the gene products which has a major effect on the growth, lactation, and development of mammary glands in cattle (6). Polymorphisms in genes that encode and regulate growth hormone are very potential as genetic markers for phenotypic properties with high economic value productivity.

In addition, the research needs to be done to show the importance the growth hormone on the reproductive side, and to obtain a picture of polymorphism of reproductive hormone in calves from cattle with Limousin cattle. Research on genetic polymorphism of growth hormone (GH) gene has not been done in crossbred calves from Limousin cattle, so it is interesting to be investigated deeper so that the results obtained can be used as a reference for obtaining cattle breeds that have a good quality of performance and power reproduction.

Follicle Stimulating Hormone (FSH) is a glycoprotein hormone produced by the pituitary gland, which functions to regulate reproduction in mammals, both male and female

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2. Materials and Methods

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2.2. Materials and Equipments

The main ingredient is DNA samples taken from the whole blood of Madura's calves from Limousin cement artificial insemination from Bangkalan, Madura District with ten samples. Supporting materials include: Primer (GH gene Primer), HaeIII Restriction enzyme, PstI restriction enzyme, DNA Extraction Materials (K Proteinase, Absolute Ethanol, Lysis Buffer, A & B Wash buffer), PCR materials (dNTP mix, Taq DNA polymerase enzyme), Electrophoresis Materials (Triss Base, boric acid, agarose, Na2 EDTA, Ethidium bromide, DNA Marker, DNA Loading dye), tissue and mica plastics.

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3.1 The PCR Result of GH Gene

PCR of 14 Madrasin cattle's blood samples was performed to detect the presence of the Growth Hormone (GH) gene, the result of PCR showing 14 gene band GH using GH gene primers. Positive results are shown in Figure 1.

3.2. The PCR Result of rGH Gene

The result of amplification of 14 Madrasin cattle in Bangkalan District conducted by PCR method resulted in 14 positive DNA samples using the rGH gene primers. The positive visualization results of electrophoresis can be seen in Figure 2

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3.3. The PCR-RFLP Result of GH Gene.

After detecting the presence of the GH gene in Madrasin cattle using the PCR method, the RFLP or GH DNA gene cutting was performed using the AluI retrieval enzyme (5'-AG | CT-3'). Results of RFLP were 14 GH gene samples divided into 4 bands, ie (60 bp, 100 bp, 150 bp, and 300 bp). The result of RFLP of Madrasin cattle GH gene can be seen in Figure 3.

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3.4. The PCR-RFLP Result of rGH Gene

The result of amplification test with PCR product obtained was then digested with AluI restriction enzyme, obtaining band yield of 167 bp and 81 bp in Madrasin cattle. The electrophoresis results in the process of PCR-RFLP of Madrasin cattle's blood sample can be seen in Figure 4.

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4. Discussion

4.1. PCR of GH

The PCR results of the Madrasin cattle's GH gene showed a 432 bp band, this corresponds to the bands in the genome library GH itself is a hormone candidate that plays a role regulating milk production, carcass and immune system (13). The GH gene is one of the most important things in managing the properties of high-value livestock so that the GH gene becomes a gene candidate in the Marked Assisted Selection (MAS) program in cows. GH gene plays a role in regulating postpartum growth, tissue, muscle, bone, adipose tissue development, mammary gland growth, lactation, reproduction, carbohydrate, protein, and body fat metabolism. The GH gene requires a receptor in its expression mechanism to a network mediated by the rGH gene or growth hormone receptor (14).

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4.2. PCR of rGH

Based on the results of blood amplification by PCR method, continued with electrophoresis readings obtained positive sample results. The rGH genes found in all of Madrasin cattle's blood samples can be detected by PCR with a length of product amplified by the rGH gene segment of 298 bp located in exon 10. The results of this research are similar to those identified by the genetic diversity of rGH genes in Limousin cattle previously performed by Zulkarnaim (15).

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The success rate of rGH gene amplification in this research was 100%. The amplification results of the rGH gene segment were visualized on a 1.5% agarose gel presented (Figure 3.2). The temperature and duration of the annealing also determine the degree of amplification specificity and the cause of other factors that play a role in determining the success of amplification is the quality or purity of DNA used as template DNA.

4.3. RFLP of GH

Amplification of Madrasin cattle's GH gene using PCR method is known to have 432 bp length, which will be followed by cutting of Madrasin gene site using AluI restricition enzyme. Based on RFLP result, PCR product along 432 bp produce 2 allele that is L and V, whereas in Madura cattle can only be found 1 allele that is L allele.

Restrictive enzymes can recognize the GH gene at the cutting site, this is because the DNA sequence at the cutting site is not mutated. The triplet codon thus formed is CTG which encodes the Leusin allele (L) (16). L allele itself is shown with fragment length (60 bp, 100 bp, and 300 bp).

The diversity of the Madrasin cattle's GH gene is indicated by the presence of V allele resulting from the presence of a mutation or change of base causing the change of

serine amino acid (C) to glycine (G) so that the Madrasin cattle's GH gene cutting phase changes from AGTC to AGGT. As a result of this change is formed codon triplet GTG that encode the valine amino acid (V) (17). V allele itself is shown with fragment length 60 bp, 100 bp, 150 bp and 300 bp.

4.4. Genotype Frequency and GH Gene Allele

According to the Volkandri's research (18), the frequency of genotypes and the L allele of Madura cattle were 1.00 and 1.00 respectively. Whereas in Madrasin cattle got the frequency of genotype and allele respectively participate 0,928 and 0,96. Based on these differences allegedly occurring changes in allele and genotype frequencies between Madura cattle with Madrasin cattle due to cross-breeding with Limousin cattle.

This is in accordance with Rachman's research (19), which found the genotype of Limousin cattle's frozen cement used in artificial insemination in Larangan sub-district, Pamekasan District. Successively detected L and V alleles have frequencies of 0.67 and 0.33 and 0.82 and 0.18 respectively. This proves that Limousin cattle have a V allele that is not owned by Madura cattle.

4.5. RFLP of rGH

Based on the results of treatment using PRC-RFLP of the amplified rGH gene segment there are two AluI cutting sites known as allele A and allele G, allele A is marked by truncation of 298 bp fragments into two parts along 167 bp and 81 bp. The fragment of the rGH gene that has an AluI enzyme cutting site will indicate that no mutation occurs but if no cutting site is indicated in the absence of a cutting by the AluI enzyme, it can be stated that there is a mutation in the rGH fragment site.

The diversity in the AluI rGH gene segment is thought to be due to the mutation or alteration of the base causing the change of serine amino acids to glycine. The change causes the cutting site not to be recognized by the Alu1 enzyme, resulting in an 81 bp fragment known as the G allele (13, 20). Results of genotyping on Madrasin cattle's segment of the rGH gene resulted in two fragments that were cut off, ie AAgenotype, which showed fragments along 81 bp and 167 bp and fragments that were cut into one band called AG genotype which showed fragment along 81 bp at (Figure 3.4).

The results of this research differ from the results of previous research conducted by Zulkarnaim [15], that is cutting the fragment of rGH gene in Limousin cattle yield three genotypes namely AA, GG, AG, AA genotype is shown as fragments along 167 bp, 81 bp and 50 bp, and genotype AG is shown as fragments along 167 bp, 131 bp, 81 bp and 50 bp.

4.6. Frequency of Genotype and rGH Gene Allele

The result of the analysis of the rGH aluI gene segment showed that the frequency of the allele A was 86% higher than the frequency of the allele G, the A and G allele frequencies in Madrasin cattle were 0.92 and 0.08, respectively (Table 3), while the AA and AG genotype were 0.85 and 0.14. The results of this research differed greatly from previous results of Limousin cattle which had A and G frequency in Limousin cattle respectively of 0.286 and 0.174, while AA and AG genotype were 0.238 and 0.095. Based on these results it is known that Madrasin cattle have different Alel and Genotype with Limousin cattle.

In conclusion, in this study, the GH and rGH undergo changes on polymorphisms in Madrasin cattle and this research information can be used as a basis for superior seed selection.

Acknowledgement

The study was supported by funding from the Directorate General of Higher Education (DIKTI) 2016. The National Education Ministry. Republic of Indonesia.

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Table 1. The Primer Used to Amplify GH Gene

Name	Base Sequens Primer	Location	PCR Product (bp)
F	5’-AGAATCAGGCCCAGCAGAAATC-3’	Exon 3	
R	5’- GTCGTCACTGCGCATGTTTG-3’	and 4	329 bp

Table 2. Genotype and allele frequencies of Madrasin cattle. LL, LV, and VV = homozygot genotype , L and V = Allele

Breed	N	Genotype Frequency			Allele Frequency	
		LL	LV	VV	L	V
Madrasin	14	0,928	0,017	0,000	0,96	0,075
Madura	10	1,00	0,00	0,00	1,00	0,00

Table 3. Genotype and allele frequencies of Madrasin cattle. AA, AG and GG = homozygot genotype, A and G = Allele

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Breed	N	Genotype Frequency			Allele Frequency	
		AA	AG	GG	A	G
Madrasin	14	0,857	0,142	0,000	0,925	0,075
Limousin	21	0,238	0,095	0,667	0,286	0,714

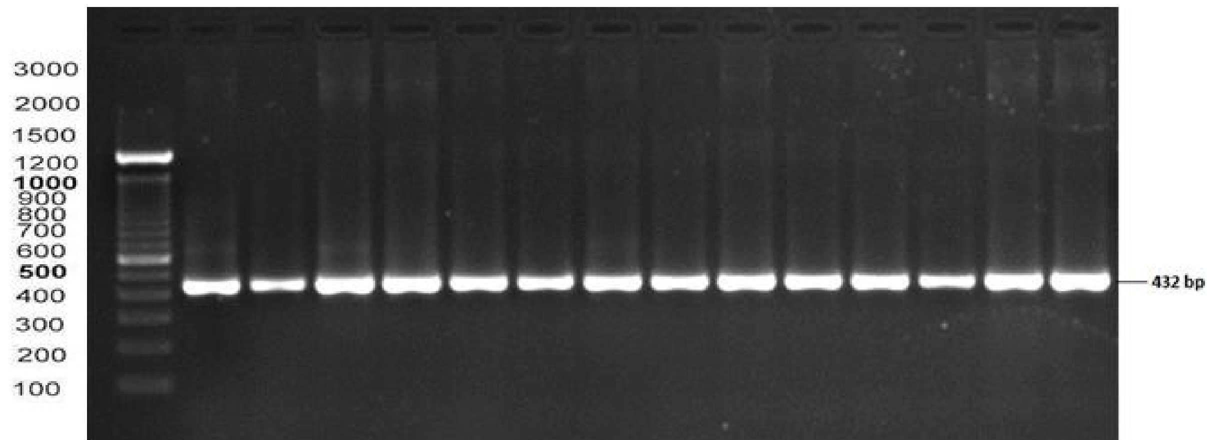

Figure 1. The PCR result of GH genes of Madrasin cattle. Lane M: Marker, Lanes 1-14 is the electrophoresis result of GH genes of Madrasin cattle with 432 bp

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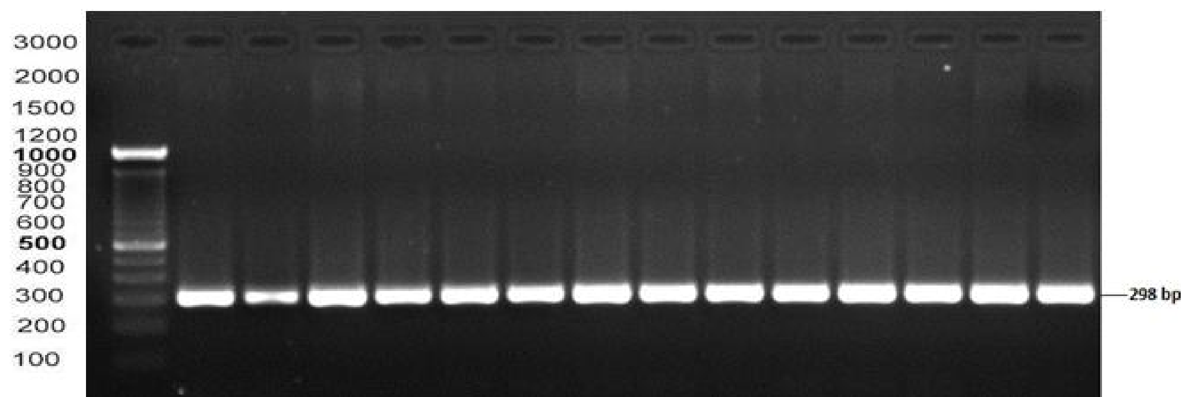

Figure 2. The electrophoresis result of PCR of Madrasin cattle's blood. M= DNA Marker. 1-14 = DNA Samples, lanes 1-14: PCR products (298 bp)

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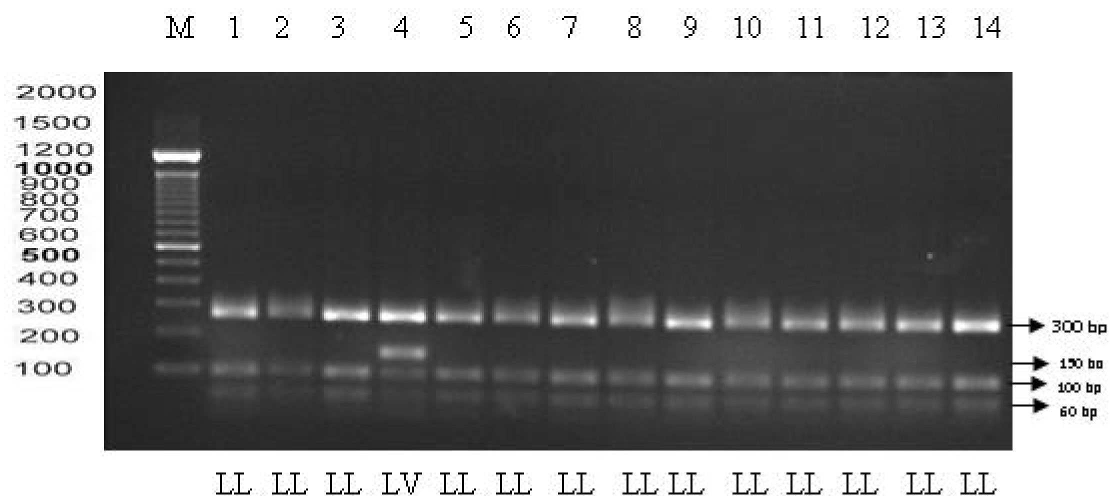

Figure 3. The electrophoresis result of PCR-RFLP using AluI restriction enzyme of Madrasin cattle's GH gene. Lane M: Marker, Lanes 1-3 and 5-14 LL genotype (60 bp, 100 bp, and 300 bp), Lane 4 LV genotype (60 bp, 100 bp, 150 bp, and 300 bp).

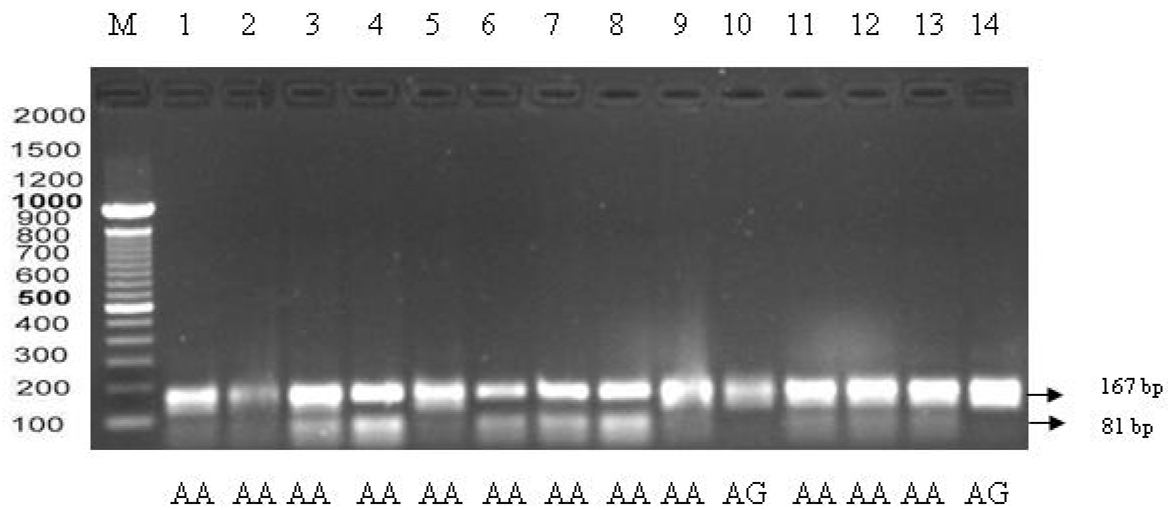

Figure 4. The electrophoresis result of PCR-RFLP using *Alu*I restriction enzyme of Madrasin cattle's rGH gene. M =DNA Marker, AA and AG = Homozygot Genotype. 1-14 = DNA Samples, lanes 1-9 and 11-13 AA genotype (167 bp and 81 bp), lane 10 and 14 AG genotype (298 bp, 167 bp, 81 bp).

Acceptance letter

From: Taha Muneer (muneert16@yahoo.com)

To: rma_fispro@yahoo.com

Cc: erma-s@fkh.unair.ac.id

Date: Tuesday, January 2, 2018 at 08:35 PM GMT+7

Hallo Dr,

In the attachment you can see a copy f acceptance letter, thank you for selection our journal to publish your articles

with my best wishes

Prof. dr. Muneer S> al-Badrany

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From: Taha Muneer (muneert16@yahoo.com)

To: rma_fispro@yahoo.com

Date: Wednesday, May 16, 2018 at 02:10 AM GMT+7

Dear Dr,

In the attachment the approved of your article.Please take a final look at the final form of research and if you wish to modify the writing in Red within 48 hours of receiving this message.
With my best wishes

Prof.Dr. Muneer S. Al-Badrany
Secretary of IJVS

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Ref No156(17-79) 27-11-2017

Date: December 30, 2017

Dear Dr. Budi Utomo

Erma Safitri

I am pleased to inform you that your manuscript entitled:

"Polymorphism of Growth Hormone (GH) Gene in the Artificial Insemination Result of Madura Cattle with Limousin Cement as a Reference for GeneticSelection " **has been accepted for publication in Iraqi Journal of Veterinary Sciences.**

Thank you considering our journal for publishing your research work.

Kind regards

Fouad K. Mohammad

Fouad K. Mohammad, PhD

Editor-in-Chief

Iraqi Journal of Veterinary Sciences

CC/ Editorial Secretary

Polymorphism of growth hormone gene in the artificial insemination result of Madura cattle with Limousin semen as a reference for genetic selection

B. Utomo and E. Safitri*

Department of Reproduction Veteriner, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia
*Correspondence Author, ES: rma_fispro@yahoo.com / erma-s@fkh.unair.ac.id

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Abstract

Research on genetic polymorphism of growth hormone (GH) and receptor growth hormone (rGH) has not been done in crossbred of Limousin cattle, so it is interesting to be examined. Blood samples were taken from 14 Madura calves were artificially inseminated with Limousin cement. DNA amplification is done by using Polymerase Chain Reaction (PCR) method, Restriction Fragment Length Polymorphism (RFLP) method to determine the genotype. DNA sequencing was done to determine nucleotide sequences of GH unit genes. The results showed that identification of GH and rGH gene polymorphisms was done by breaking DNA fragments from 432 and 298 bp in Madura and Limousin cattle (Madrasin) ie, L and V alleles have a frequency of 0.67 and 0.33 for the GH gene, respectively. This proves that the crossed-breeding of Madrasin have V allele that is not owned by the Madura cattle. While in the rGH gene, the A allele is 0.92 and the G allele is 0.08, with the frequency of the A allele larger than the G allele. This research concluded: that GH and rGH undergo changes on polymorphisms in Madrasin cattle can be used as a basis for selection.

Keywords: Polymorphism, GH Gene, rGH Gene, Madrasin, PCR, RFLP, V alleles
Available online at http://www.vetmedmosul.org/ijvs

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Introduction

Madura cattle is one of Indonesia's germplasm wealth. Several laws enacted in an effort to maintain its purity and

set out the main points of animal husbandry and animal health, in an effort to maintain the population, shape, colour of the skin, and improve the quality of Madura cattle production.

Madura cattle is one type of local Indonesian cattle that grow on the island of Madura and surrounding islands. Morphologically, Madura cattle have almost the same characteristics as Balinese cattle except for their smaller body size and horns. The skin colour of male and female Madura cattle is browner than Bali cattle, lower legs to knees (1). In addition, Madura cattle are more resistant to hot weather, efficient to food, have good meat quality, and more resistant to parasites (2).

Madura cattle become a local breed of beef cattle that is formed as a result of natural isolation and environmental influences, so it has a uniformity characteristic that stands out among other local beef breeds in Indonesia. With the contribution of genetic characteristics of zebu cattle such as tolerance to stress due to climate, resistance to tick attack, strict natural, and environmental selection a long time, Madura cattle become a cattle breed that has a very high adaptability to the environment. In addition, Madura cattle have a good response to the improvement of feed and resistance to feed with high crude fiber content (3).

The exploitation of Madurese cattle through an increasingly widespread crossing with exotic cattle will have the effect of changing phenotypic and genetic traits. Crosses are done by breeders to obtain superior performance especially for weight growth and reproductive power.

Therefore, genetic studies of Madura cattle that have been crossed with Limousin breed become interesting to do in order to see the calves produced have a good quality in terms of body growth and reproduction. Furthermore, the results of the genetic selection will be used as a reference for obtaining superior calves from Madura cattle that are inseminated with limousin cement.

Advances in the field of molecular biology provide new opportunities in an attempt to detect the occurrence of genetic variation (polymorphism) as a basis for improving genetic quality in farms. Potential molecular techniques used to detect variations include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphism DNA (RAPD), Double Strand Conformation Polymorphism (DSCP), and Marker-Assisted Selection (MAS). With effective and accurate technology through the use of diagnostics based on deoxyribonucleic acid (DNA), it will greatly assist cattle crossing programs. Provision of genetic maps through recombinant DNA methods can assist cattle crossing programs through obtained molecular data, which regulate the properties of production (4).

Gene products in the form of hormones will affect the regulatory process of metabolism and appearance of (bioregulators) livestock morphology. Genetic variation (polymorphism) in gene loci especially that encodes hormone is very important because it determines the genetic character of a population that can assist in improving the genetic quality (5).

Growth hormone is one of the gene products which has a major effect on the growth, lactation and development of mammary glands in cattle (6). Polymorphisms in genes that encode and regulate growth hormone are very potential as genetic markers for phenotypic properties with high economic value productivity.

In addition, the research needs to be done to show the importance the growth hormone on the reproductive side, and to obtain a picture of polymorphism of reproductive hormone in calves from cattle with Limousin cattle. Research on genetic polymorphism of growth hormone (GH) gene has not been done in crossbred calves from Limousin cattle, so it is interesting to be investigated deeper so that the results obtained can be used as a reference for obtaining cattle breeds that have a good quality of performance and power reproduction.

Follicle Stimulating Hormone (FSH) is a glycoprotein hormone produced by the pituitary gland, which functions to regulate reproduction in mammals, both male and female (7). In females, it functions for the proliferation and development of follicles until ovulation (8). While in males, the combination of FSH and testosterone is a tropical hormone that regulates Sertoli cell function, which is necessary for initiation and maintenance of spermatozoa quality and quantity (9). FSH hormones consist of α and β-sub units. β-sub units play a role in determining the specificity of bonding with receptors (FSHR) (10). It has been reported that the presence of exon 3 mutations of the FSH β-sub-unit genes in bulls were identified to have lower fresh cement concentrations, a lower percentage of acrosome integrity on fresh and frozen cement, more low motility in frozen semen (11). Research on genetic polymorphism of growth hormone (GH) and β-sub-unit genes has not been done in crossbred calves from Limousin cattle, so it is interesting to obtained can be used as a reference for obtaining a cattle breeds that have a good performance and power reproduction.

Materials and methods

Research materials

The main material of this research is DNA Genome which obtained from the blood of Madura's calves from Limousin cement artificial insemination with 14 blood samples. Genomic DNA was then extracted using a DNA Extraction QIAamp Mini spin column Kit (Thermo Fisher Scientific Inc. Invitrogen) to degrade the cell walls, proteins, and fats. The DNA samples were then ready for PCR reactions. The primer used to amplify GH gene (Table 1).

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Materials and equipments

The main ingredient is DNA samples taken from the whole blood of Madura calves from Limousin cement artificial insemination from Bangkalan, Madura District with ten samples. Supporting materials include: Primer (GH gene Primer), HaeIII Restriction enzyme, PstI restriction enzyme, DNA Extraction Materials (K Proteinase, Absolute Ethanol, Lysis Buffer, A & B Wash buffer), PCR materials (dNTP mix, Taq DNA polymerase enzyme), Electrophoresis Materials (Triss Base, boric acid, agarose, Na2 EDTA, Ethidium bromide, DNA Marker, DNA Loading dye), tissue and mica plastics.

Collection of blood samples

Madura cattles and Limousine cattle crossbred calves the sample 5 ml of blood collected from jugular vein by using venojet and vacutainer tube with EDTA and then kept at 4°C.

Table 1: The Primer Used to Amplify GH Gene

Name	Base Sequens Primer	Location	PCR Product (bp)
F	5'-AGAATCAGGCCCAGCAGAAATC-3'	Exon 3 and 4	
R	5'- GTCGTCACTGCGCATGTTTG-3'		329 bp

DNA extraction

The DNA was isolated and purified using a QIAamp Mini spin column extraction DNA kit following the provided extraction protocol. A total of 200 µl blood samples were lysed by adding 200 µl lysis buffer solution and 20 µl K proteinase (10 mg/ml), the mixture was then incubated at 56°C for 60 minutes in the waterbath shaker. After incubation, the solution was then added 200 µl absolute ethanol (96%) and centrifuged 8000 x g for 1 min.

DNA purification was done by spin column method with the addition of 500 µl wash buffer I then continued with centrifugation at 8000 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 min. After the supernatant was removed, the DNA was then dissolved in 200 µl elution buffer and sterilized at 8000 x g for further extraction of DNA to be stored and stored at -20°C.

PCR-RFLP technique

The PCR reaction composition was conditioned on 25 µl reaction volume comprising 100 ng of DNA, 0.25 mM each primer, 150 µM dNTP, 2.5 mM Mg2 +, 0.5 Taq DNA polymerase and 1x buffer. The condition of the PCR machine begins with the initial denaturation at 94°C x 2 minutes, followed by 35 subsequent cycles with each denaturation at 94°C x 45 seconds, with annealing temperature: 65°C x 30sec (GH), followed by one end extension cycle at temperature 72°C for 5 minutes using GeneAmp PCR System 2400 Thermocycler (Perkin Elmer, Madison,Wisconsin, United States), for FSH β-sub unit primer with anealing at 60°C. The PCR product was then electrophoresed on a 1.5% agarose gel with 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na2EDTA) containing 100 ng/ml ethidium bromide. Then visualized on UV transilluminator (gel documentation system). The allele is determined by interpreting the bands that are most migratory to the anode pole as allele 1, allele 2, and so on.

The PCR products obtained from each of the target genes were then analyzed using RFLP by cutting using restriction enzymes having cutting sites in the HaHII gene and the FSH β-PstI gene. A total of 4 µl DNA PCR product added 0.5 µl, then incubated for 17 hours at 37°C.

Sequencing

The determination of nucleotide sequences of GH (Growth Hormone) and rGH (receptor Growth Hormone) unit genes was done by DNA sequencing that is the final step to obtain data of nucleotide sequence from fragment result of PCR-RFLP propagation. The DNA bands that already restricted on an agarose gel as PCR-RFLP products serve as a mould in the sequencing reaction by using forward and reverse primers as in the time of amplification.

Data analysis

The diversity of individual genotypes can be determined from the DNA bands of the gene found. Each sample was compared to the same size (marker) and calculated the frequency of the allele. The frequency of alleles can be calculated using the Nei and Kumar formulas (12). Sequencing data analysis using UGENE 1.21.0 software.

Table 2: Genotype and allele frequencies of Madrasin cattle. LL, LV, and VV = homozygot genotype, L and V = Allele

Breed	N	Genotype Frequency			Allele Frequency	
		LL	LV	VV	L	V
Madrasin	14	0,928	0,017	0,00	0,96	0,075

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Madura	10	1,00	0,00	0,00	1,00	0,00

Table 3: Genotype and allele frequencies of Madrasin cattle. AA, AG and GG = homozygot genotype, A and G = Allele

Breed	N	Genotype Frequency			Allele Frequency	
		AA	AG	GG	A	G
Madrasin	14	0,857	0,142	0,000	0,925	0,075
Limousin	21	0,238	0,095	0,667	0,286	0,714

Resuts

The PCR result of GH gene

PCR of 14 Madrasincattle's blood samples was performed to detect the presence of the Growth Hormone (GH) gene, the result of PCR showing 14 gene band GH using GH gene primers. Positive results are shown in Figure 1.

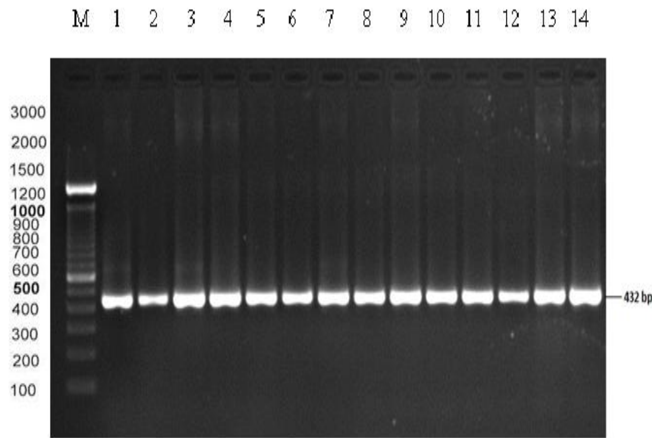

Figure 1: The PCR result of GH genes of Madrasin cattle. Lane M: Marker, Lanes 1-14 is the electrophoresis result of GH genes of Madrasin cattle with 432 bp.

The PCR result of rGH gene

The result of amplification of 14 Madrasin cattle in Bangkalan District conducted by PCR method resulted in 14 positive DNA samples using the rGH gene primers. The positive visualization results of electrophoresis can be seen in Figure 2.

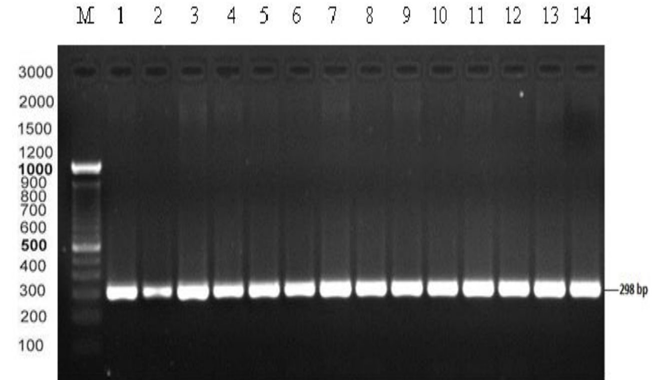

Figure 2: The electrophoresis result of PCR of Madrasin cattle's blood. M= DNA Marker. 1-14 = DNA Samples, lanes 1-14: PCR products (298 bp).

The PCR-RFLP result of GH gene.

After detecting the presence of the GH gene in Madrasin cattle using the PCR method, the RFLP or GH DNA gene cutting was performed using the AluI retrieval enzyme (5'-AG | CT-3'). Results of RFLP were 14 GH gene samples divided into 4 bands, ie (60 bp, 100 bp, 150 bp, and 300 bp). The result of RFLP of Madrasin cattle GH gene can be seen in Figure 3.

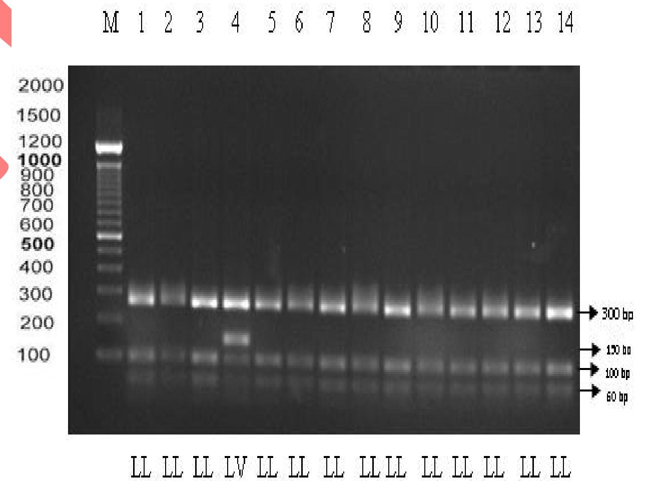

Figure 3: The electrophoresis result of PCR-RFLP using Alul restriction enzyme of Madrasin cattle's GH gene. Lane M: Marker, Lanes 1-3 and 5-14 LL genotype (60 bp, 100 bp, and 300 bp), Lane 4 LV genotype (60 bp, 100 bp, 150 bp, and 300 bp).

The PCR-RFLP result of rGH gene

The result of amplification test with PCR product obtained was then digested with AluI restriction enzyme,

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obtaining band yield of 167 bp and 81 bp in Madrasin cattle. The electrophoresis results in the process of PCR-RFLP of Madrasin cattle's blood sample can be seen in Figure 4.

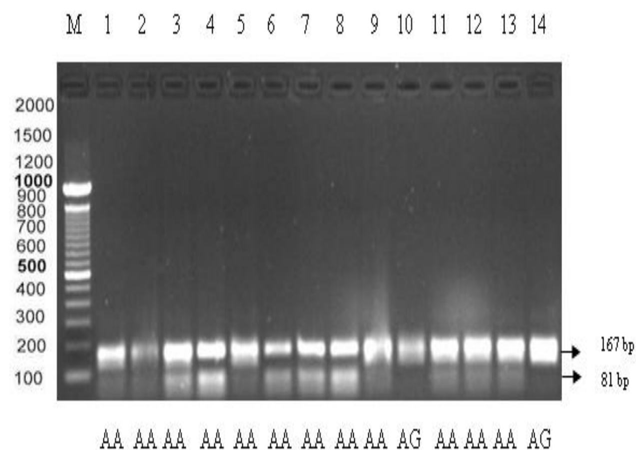

Figure 4: The electrophoresis result of PCR-RFLP using *Alu*I restriction enzyme of Madrasin cattle's rGH gene. M =DNA Marker, AA and AG = Homozygot Genotype. 1-14 = DNA Samples, lanes 1-9 and 11-13 AA genotype (167 bp and 81 bp), lane 10 and 14 AG genotype (298 bp, 167 bp, 81 bp).

Discusion

PCR of GH

The PCR results of the Madrasin cattle's GH gene showed a 432 bp band, this corresponds to the bands in the genome library GH itself is a hormone candidate that plays a role regulating milk production, carcass and immune system (13). The GH gene is one of the most important things in managing the properties of high-value livestock so that the GH gene who Marked Assisted Selection (MAS) program in cows. GH gene plays a role in regulating postpartum growth, tissue, muscle, bone, adipose tissue development, mammary gland growth, lactation, reproduction, metabolism of carbohydrate, protein, and body fat. The GH gene requires a receptor in its expression mechanism to a network mediated by the rGH gene or growth hormone receptor (14).

PCR of rGH

Based on the results of blood amplification by PCR method, continued with electrophoresis readings obtained positive sample results. The rGH genes found in all of Madrasin cattle's blood samples can be detected by PCR with a length of product amplified by the rGH gene segment of 298 bp located in exon 10. The results of this research are similar to those identified by the genetic diversity of rGH genes in Limousin cattle previously performed by Zulkarnaim (15).

The success rate of rGH gene amplification in this research was 100%. The amplification results of the rGH gene segment were visualized on a 1.5% agarose gel presented (Figure 3.2). The temperature and duration of the annealing also determine the degree of amplification specificity and the cause of other factors that play a role in determining the success of amplification is the quality or purity of DNA used as template DNA.

RFLP of GH

Amplification of Madrasin cattle's GH gene using PCR method is known to have 432 bp length, which will be followed by cutting of Madrasin gene site using AluI restricition enzyme. Based on RFLP result, PCR product along 432 bp produce 2 allele that is L and V, whereas in Madura cattle can only be found 1 allele that is L allele.

Restrictive enzymes can recognize the GH gene at the cutting site, this is because the DNA sequence at the cutting site is not mutated. The triplet codon thus formed is CTG which encodes the Leusin allele (L) (16). L allele itself is shown with fragment length (60 bp, 100 bp, and 300 bp).

The diversity of the Madrasin cattle's GH gene is indicated by the presence of V allele resulting from the presence of a mutation or change of base causing the change of serine amino acid (C) to glycine (G) so that the Madrasin cattle's GH gene cutting phase changes from AGTC to AGGT. As a result of this change is formed codon triplet GTG that encode the valine amino acid (V) (17). V allele itself is shown with fragment length 60 bp, 100 bp, 150 bp and 300 bp.

Genotype frequency and GH gene allele

According to the Volkandri's research (18), the frequency of genotypes and the L allele of Madura cattle were 1.00 and 1.00 respectively. Whereas in Madrasin cattle got the frequency of genotype and allele respectively participate 0,928 and 0,96. Based on these differences allegedly occurring changes in allele and genotype frequencies between Madura cattle with Madrasin cattle due to cross-breeding with Limousin cattle.

This is in accordance with Rachman's research (19), which found the genotype of Limousin cattle's frozen cement used in artificial insemination in Larangan sub-district, Pamekasan District. Successively detected L and V alleles have frequencies of 0.67 and 0.33 and 0.82 and 0.18 respectively. This proves that Limousin cattle have a V allele that is not owned by Madura cattle.

RFLP of rGH

Based on the results of treatment using PRC-RFLP of the amplified rGH gene segment there are two AluI cutting

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sites known as allele A and allele G, allele A is marked by truncation of 298 bp fragments into two parts along 167 bp and 81 bp. The fragment of the rGH gene that has an AluI enzyme cutting site will indicate that no mutation occurs but if no cutting site is indicated in the absence of a cutting by the AluI enzyme, it can be stated that there is a mutation in the rGH fragment site.

The diversity in the AluI rGH gene segment is thought to be due to the mutation or alteration of the base causing the change of serine amino acids to glycine. The change causes the cutting site not to be recognized by the Alu1 enzyme, resulting in an 81 bp fragment known as the G allele (13,20). Results of genotyping on Madrasin cattle's segment of the rGH gene resulted in two fragments that were cut off, ie AAgenotype, which showed fragments along 81 bp and 167 bp and fragments that were cut into one band called AG genotype which showed fragment along 81 bp at (Figure 3.4).

The results of this research differ from the results of previous research conducted by Zulkarnaim (15), that is cutting the fragment of rGH gene in Limousin cattle yield three genotypes namely AA, GG, AG, AA genotype is shown as fragments along 167 bp, 81 bp and 50 bp, and genotype AG is shown as fragments along 167 bp, 131 bp, 81 bp and 50 bp.

Frequency of genotype and rGH gene allele

The result of the analysis of the rGH aluI gene segment showed that the frequency of the allele A was 86% higher than the frequency of the allele G, the A and G allele frequencies in Madrasin cattle were 0.92 and 0.08, respectively (Table 3), while the AA and AG genotype were 0.85 and 0.14. The results of this research differed greatly from previous results of Limousin cattle which had A and G frequency in Limousin cattle respectively of 0.286 and 0.174, while AA and AG genotype were 0.238 and 0.095. That Madrasin cattle have different Alel and Genotype with Limousin cattle.

In conclusion, the GH and rGH undergo changes on polymorphisms in Madrasin cattle and this research information can be used in selection.

Acknowledgement

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Acceptance Letter

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Date: Monday, January 8, 2018 at 05:03 AM GMT+7

Hallo
In the attachment you can see a copy of acceptanc letter. Thank you for select our Journal to publish the article
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Ref No 156(17-79) 27-11-2017

Date: December 30, 2017

Dear Dr, Budi Utomo

Erma Safitri

I am pleased to inform you that your manuscript entitled:

" Polymorphism of Growth Hormone (GH) Gene in the Artificial Insemination Result of Madura Cattle with Limousin Semen as a Reference for Genetic Selection " **has been accepted for publication in Iraqi Journal of Veterinary Sciences.**

Thank you considering our journal for publishing your research work.

Kind regards

Iraqi Journal of Veterinary Sciences
ACCEPTED

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