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The Variability of Growth Hormone Gene Associated with Ultrasound Imaging of *Longissimus dorsi* Muscle and Perirenal Fat in Rabbits

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ABSTRACT

Identification of genes in rabbits correlated to economic traits were intended to improve and develop their genetic quality. The objective of this research was to analyze the variability of growth hormone gene (GH) in three rabbit breeds, i.e. Rex, Satin, and Reza (Rex and Satin crosses) then was associated with ultrasound imaging of *Longissimus dorsi* muscle and perirenal fat thickness. Identification of the variability of growth hormone gene was analyzed using PCR RFLP technique from blood samples of 33 mature male rabbits in Indonesian Research Institute for Animal Production (IRIAP). Thickness of *Longissimus dorsi* muscle and perirenal fat were imaged and measured by using ultrasound unit at 2nd to 3rd lumbar vertebrae in the left body side. PCR product of GH gene fragment (231 base pair /bp) was digested with restriction enzyme *Bsh1236I*. PCR-RFLP patterns were allele T resulted in an undigested fragment of 231 bp; allele C resulted in fragment of 169 bp and 62 bp. The result showed that *Bsh1236I* GH gene had three genotypes, i.e. CC, TT, and CT. There were signifficant association of *Longissimus dorsi* muscle thickness between rabbit breed (P<0.05). There was no significant association between GH *Bsh1236I* gene polymorphism and imaging ultrasound of *Longissimus dorsi* muscle and perirenal fat thickness. The association of characteristic genotype of GH|*Bsh1236I* gene with measurement phenotype was not significant, however it had potency as marker assisted selection (MAS).

Key words: growth hormone gene, Longissimus dorsi, perirenal fat, rabbit, ultrasound

ABSTRAK

Identifikasi gen yang berhubungan dengan sifat ekonomis sangat diperlukan untuk perbaikan dan perkembangan kualitas genetik kelinci. Penelitian ini bertujuan untuk menganalisis keragaman gen hormon pertumbuhan (GH) pada tiga bangsa kelinci, yaitu Rex, Satin dan Reza, yang selanjutnya dihubungkan dengan pencitraan ultrasonografi ketebalan otot *Longissimus dorsi* dan lemak perirenal. Identifikasi keragaman gen GH menggunakan teknik PCR-RFLP sampel darah dari 33 ekor kelinci jantan dewasa di Balai Penelitian Ternak (BALITNAK). Ketebalan otot *Longissimus dorsi* dan lemak perirenal dicitrakan dan diukur menggunakan alat ultrasonografi pada posisi lumbar vertebrae ke 2 hingga ke 3 pada sisi kiri tubuh. Produk PCR dari fragmen gen GH (231 pasang basa/pb) dipotong dengan enzim pemotong *Bsh1236I*. Pola PCR-RFLP yang dihasilkan, yaitu alel T dihasilkan dari fragmen yang tidak terpotong dengan posisi 231 pb; alel C dihasilkan pada fragmen 169 pb dan 62 pb. Hasilnya menunjukkan bahwa gen GH|*Bsh1236I* memiliki tiga genotipe, yaitu CC, TT, dan CT. Bangsa kelinci berpengaruh terhadap pencitraan USG ketebalan otot Longissimus dorsi (P<0.05). Hubungan polimorfisme gen GH|*Bsh1236I* tidak berpengaruh nyata terhadap hasil pencitraan USG ketebalan lemak perirenal dan otot *Longissimus dorsi*, namun demikian keragaman gen GH|*Bsh1236I* memiliki potensi untuk dijadikan *marker assisted selection* (MAS).

Kata kunci: gen hormon pertumbuhan, Longissimus dorsi, lemak perirenal, kelinci, ultrasonografi

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INTRODUCTION

Rabbits have several advantages besides as pets, laboratory animals, may also serve to produce meat and leather-fur (Rogel-Gaillard *et al*., 2009). Rabbit is potential livestock that produces meat. The rabbit meat product in Indonesia is not as popular as in other countries. The world's largest rabbit meat production was reported in Chinese state with an estimated annual production 550,000–600,000 tons of carcass each year (Lebas, 2009). Rabbit populations in Indonesia at 2011 reached 915,140 heads and spreaded in 12 provinces (Ditjen PKH, 2011). Along with change the way the community views the rabbit meat, this can increase the need for seed broiler rabbits. However, rabbit meat consumption in Indonesia is relatively still low due to the less supply. Rabbit meat has different characteristics from beef, chicken, pork, or lamb. Rabbit meat is white meat, has a smooth and soft fiber and higher protein with cholesterol and low fat (Rogel-Gaillard *et al*., 2009).

Rex, Satin, and Reza (crosses Rex and Satin) are rabbit developed breed in IRIAP which germplasm rabbit meat in Indonesia. Currently, the existence of selection and crossbreeding conducted on the rabbits with potential for meat and leather-fur had only seen from phenotypic aspects include performance and productivity, while the genetic aspects (gene) has not been done. Identification of genes related to the economic trait were needed for improvement and development of the quality of rabbit genetic.

Target gene used was growth hormone gene (GH) having an important role in growth and development. Effects of the GH gene on the growth are observed in several tissues (Akers, 2006). Polymorphisms in this gene have been used as a genetic marker associated with different performances and productions traits such as body weight, birth weight and weaning weight in goat (Wickramaratne *et al.,* 2010; Supakorn & Pralomkarn, 2013), milk yield and body weight in cattle (Jakaria *et al.,* 2007; Katoh *et al.,* 2008; Misrianti *et al.,* 2012), sheep (Adams & Briegel, 2005), carcass traits in pig (de Faria *et al.,* 2006). The rabbit GH gene has been already sequenced by Wallis & Wallis (1995) and has been investigated as a gene associated with market weight on commercial rabbit (Fontanesi *et al.* 2012).

Analysis of genetic variation that associated with measurement of phenotypic was determined by using ultrasound imaging of *Longissimus dorsi* muscle and perirenal fat thickness. Ultrasonography is one of the most widely used techniques for *in vivo* prediction of carcass in swine, sheep and cattle (Moloney *et al*., 2002; Schroder & Staufenbiel, 2006; McEvoy *et al.,* 2007). Some reports have also shown the suitability of this technique for the evaluation of body composition in rabbits (Pascual *et al*., 2010; Cardinali *et al*., 2008), estimated of Longissimus muscle in sheep (Sahin *et al*., 2008; Esquivelzeta *et al*., 2012), cattle (Wall *et al*., 2004; Yokoo *et al*., 2008), pig (Micklander *et al*., 2005). Taking imaging with ultrasound does not interfere of livestock, low cost and useful method to evaluate subcutaneous and visceral adipose tissue in anatomy and different metabolic conditions (Stouffer, 2004).

Percentage of perirenal fat depot (PFD) is a predictor of percentage fat in whole carcass (Blasco & Ouhayun, 1993). PFD in rabbit very sensitive to variation of feed and can increase to 40 % in rabbit with high diet. Using of USG is practical method to predict carcass traits included *Longissimus dorsi* muscle and perirenal fat (Silva *et al*., 2012).

This research therefore was aimed to analyze the GH|*Bsh1236I* gene polymorphism in Rex, Satin and Reza rabbit breed that associated with ultrasound imaging of *Longissimus dorsi* muscle and perirenal fat thickness to improve genetic potential through molecular selection.

MATERIALS AND METHODS

Sample Sources

Total 33 blood samples of adult male rabbits were collected from 3 breeds, consisting of Rex (11 samples), Satin (11 samples), and Reza (11 samples) at Indonesian Research Institute for Animal Production (IRIAP). Blood samples were already extracted as DNA collections at the Animal Molecular Genetic Laboratory, Faculty of Animal Science, Bogor Agricultural University.

DNA Extraction

About 5 mL of blood samples were collected from each rabbit in non anticoagulant polypropylene tubes. Blood samples were then mixed with 96% ethanol. The process of DNA isolation used phenol-chloroform method (Sambrook *et al.,* 1989) that was modified by Andreas *et al.* (2010). Genomic DNA was stored at -20 ^{o}C until amplification with polymerase chain reaction (PCR).

Polymerase Chain Reaction (PCR)

Amplification of PCR was carried out by using specific primer (Fontanesi *et al.,* 2012) for parts of the 5'-flanking region and 5'untranslated region, exon 1 and with method of two step gradient cycle PCR (Lopez & Prezioso, 2001; Xiong, 2004). Primers used were for forward 5'- GTATAGTGGGATGGGGTTGG -3 'and reverse 5'- TTACGCTCCCATTCAGAAGC -3' (Gen Bank access number Z28137). The PCR was performed in a final volume of 15 μL for each reaction containing 1 μL of DNA sample, 9.35 μL destillated water, 0.3 μL primers, 0.05 μL Taq polymerase, buffer 3 μL, 0.3 μL dNTPs, and 1 μL $MgCl_2$. The reaction mixture was subjected to an initial 5 min of denaturation 95 ^{o}C, followed by first 15 cycles of denaturation 95 ^{o}C for 30 s, annealing 30 s at 68 ^{o}C, extension 30 s at 72 ^{o}C, then second of 15 cycles of denaturation at 95 ^{o}C for 30 s, annealing 30 s at 60 ^{o}C, extension 30 s at 72 ^{o}C, and final extension for 5 min at 72 ^{o}C.

PCR - RFLP Analysis

Visualization of amplification was analyzed on Agarose gel 1.5% that containing 2.5 μL EtBr (ethidium bromide), 0.5X TBE buffer (1 M Tris, 0.9 M Boric acid, 0.01 M EDTA pH 8.0) with a 100 bp ladder as a molecu-

lar weight marker for confirmation of the length of PCR product. Digestion by using enzyme and determination of RFLP, 5 µL of PCR products was added to 0.3 µL *Bsh1236I* enzyme, 1 µL destilated water, and 0.7 µL R buffer. The mixture was then incubated at 37 °C for 16 h. The digestion products were separated by horizontal electrophoresis (100 volts, 40 min) in 2% agarose gel in 0.5 X TBE and 2.5 µL ethidium bromide visualized on UV transiluminator.

Ultrasound Measurement

Images were obtained with an ultrasound unit (SonoDop® S5, PT Karindo Alkestron, Indonesia), equipped with a transducer micro-convex with frequency 7.5 MHz. Scanning sites for *Longissimus dorsi* Muscle (LM) and perirenal fat (PF) were located by physical palpation at 2nd to 3rd lumbar vertebrae in the left body side. Ultrasound gel was applied to the scanning sites area. The transducer was always placed in the same position. The images taken were digitalized and ultrasound measurements determined image analysis by using NIH Image J software (ImageJ®, NIH, USA).

Data Analysis

PCR-RFLP data were analyzed by allele calculating and genotype frequencies (Nei & Kumar, 2000). Genotype frequency, determined by the calculation of the ratio of a specific genotype of each population, was calculated by the following formula:

$x_{ii} = n_{ii}/N$

Allele frequency was calculated as ratio of a certain allele to the overall alleles at a certain locus in a population. Allele frequency of GH gene|*Bsh1236I* was calculated by the following formula:

$x_i = (2n_{ii} + \sum n_{ij})/2N$

where x_{ii} is frequency of genotype AiAi, x_i is frequency of allel Ai, nii is number of genotype AiAi, nij is number of genotype AiAj, and N is total samples.

Information content of allele was calculated by PIC values using method described by Botstein *et al*. (1980) and Nagy *et al.* (2012).

$$PIC = 1 - \sum_{i=1}^{n} Pi^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

where Pi and Pj stand for frequency of band i and band j respectively in one population; n is the number of alleles from a certain locus.

Characteristic phenotypic data of each rabbit breed were analyzed using the General Linear Model (GLM) with the model (Steel & Torrie 1995):

$Y_{ij} = \mu + \alpha_i + \beta X_{ij} + \epsilon_{ij}$

where Yij is the observed value,µ is overall mean, αi is effect for breed i, β is coefficient of linier regression; Xij is covarian (body weight), and ϵij is the random error associated with Yij experimental unit.

Variability data of GH gene|*Bsh1236I* to measurement of perirenal fat thickness and *Longissimus dorsi* muscle were analyzed using the GLM with the model (Steel & Torrie, 1995):

$Y_{ij} = \mu + \alpha_i + \beta X_{ij} + \epsilon_{ij}$

where Yij is the observed value,µ is overall mean, αi is effect for genotype i, β is coefficient of linier regression; Xij is covarian (rabbit breeds), and ϵij is the random error associated with Yij experimental unit.

RESULTS AND DISCUSSION

GH Gene Amplification

The result of GH gene amplification showed that an amplicon with the length of 231 bp, which was located in part of the 5′-flanking region and 5′untranslated region, exon 1. The amplification fragment of the GH gene was performed by Fontanesi *et al.* (2012) with an annealing temperature of 58 °C, but in this research has different temperature to get amplicon. The optimal annealing temperature in this research was 68 °C on 15 cycle and 60 °C on 15 cycle. The amplification of the GH gene fragment was carried on GeneAmp® PCR System 9700 (Applied Biosystem). The success rate of the GH gene amplification in this study was 100%. Gene segment amplification products were visualized on 1.5% Agarose gel as shown in Figure 1. Position of annealing primers of the GH gene sequences was shown in Figure 2.

GH|*Bsh1236I* Gene Polymorphism

Genetic polymorphism of the GH gene was done by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) method using *Bsh1236I* restriction enzyme. This enzyme recognized and cut at nucleotides of CG|CG sites. RFLP process resulted in two fragments with the base lengths of 231 and 169 bp.

Results from the PCR-RFLP analysis shows there were three genotypes identified, namely CC, TT, and CT genotypes that were derived from two alleles, namely C and T alleles (Figure 3). Genotyping the GH|*Bsh1236I* gene, showed for the resulted one fragment of 231 identified for the TT genotype; one fragment of 169 bp for the CC genotype; and two fragments of 231 and 169 bp for the CT genotype. Identification of the GH gene polymorphism in rabbit has been done by PCR-RFLP using *Bsh1236I* restriction enzyme by Fontanesi *et al.* (2012) who reported the presence of two types of alleles, namely C and T alleles with three kinds of genotypes, namely CC, TT, and CT genotypes.

Rex rabbit breed had frequency of genotype CC highest than Satin and Reza rabbit breeds. Satin rabbit breed had the highest frequency of genotype CT. Rex and Satin breeds were rabbit with good fur and Reza (Rex and Satin crosses) mostly influenced by Satin rabbit in this case that frequency of genotype TT same with CT (Table 1). Reza rabbit breed created by crossing between Rex and Satin rabbit with Mendel law did not work in the F2 based on Prasetyo (2007).

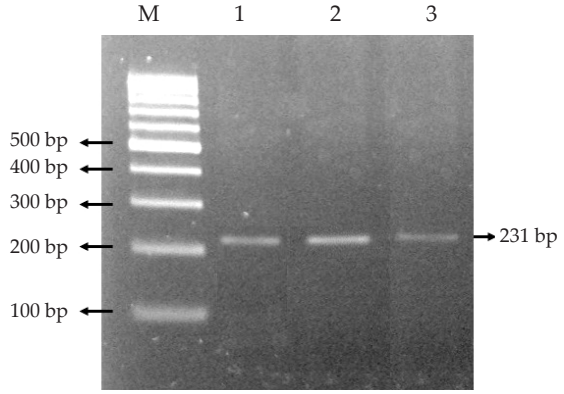

Figure 1. Visualization of GH gene amplification results in 1.5% agarose gel. Note: M= marker, No. 1-3= number of sample.

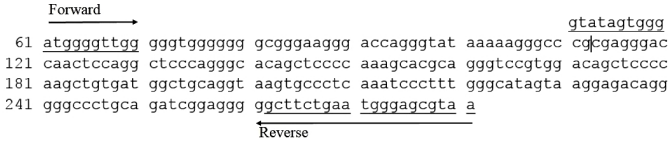

Figure 2. Primer position, PCR product (GenBank access number Z28137) and *Bsh1236I* restriction enzyme
Note: the bottom line shows the primer

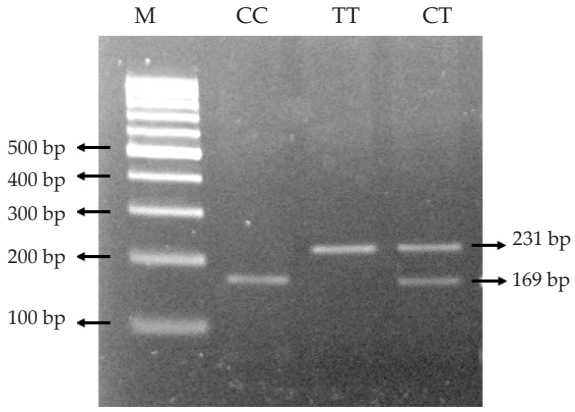

Figure 3. Result of GH gene fragment using PCR-RFLP method with *Bsh1236I* restriction enzyme on 2% agarose gel. Note: M= marker; CC, TT, CT= genotype.

Based on the allele frequency measurement, C allele had higher frequency in most breeds including research breeds (Table 2). But, in this case it did not establish an interesting pattern because Checkered Giant breed, the rabbit with high weight (Rogel-Geillard *et al.* (2009) had T allele frequency higher than C allele. Nei & Kumar (2000) stated that an allele was polymorphic if frequency of that allele was equal or less than 0.99. Analysis of PIC value was shown in Table 3 indicated that all of the rabbit breeds included of PIC in high category based Botstein *et al.* (1980). It mean that fragment of GH|*Bsh1236I* gene had high degree of genetic information in Rex, Satin, and Reza rabbit.

Table 1. Genotype frequencies of the GH gene

No	Breed	n	Genotype frequencies		
			CC	TT	CT
1	Rex	11	0.818	0.091	0.091
2	Satin	11	0.273	0.273	0.455
3	Reza	11	0.273	0.364	0.364

Note: n= number of samples.

Table 2. Allele frequencies of the GH gene

Breed	n	Allele frequencies	
		C	T
Californian	4	0.625	0.375
Champagne d'Argent	13	1.000	0.000
Checkered Giant	16	0.406	0.594
Chincilla	8	0.937	0.063
Giant Grey	4	1.000	0.000
Giant White	3	1.000	0.000
Loop	3	1.000	0.000
New Zealand White	12	0.625	0.375
Rhinelander	3	1.000	0.000
Vienna Blue	4	0.875	0.125
Rex*)	11	0.864	0.136
Satin*)	11	0.500	0.500
Reza*)	11	0.455	0.545

Source: Fontanesi *et al*., 2012; *) result of research; n= number of samples

Table 3. Estimating of polymorphic informative content (PIC) value on Rex, Satin, and Reza rabbit breeds

Breed	n	PIC
Rex	11	0.207
Satin	11	0.375
Reza	11	0.373

Note: n= number of samples.

Phenotypic of Rabbit Breeds

Phenotypic studies included measurement of body weight and ultrasound imaging (USG) to measure the *Longissimus dorsi* muscle and perirenal fat thickness in every breed of rabbits. Ultrasound imaging of *Longissimus dorsi* muscle and perirenal fat in rabbits are shown in Figure 4. Perirenal fat visible white (hyperechoic) curved shape on the wall of the stomach, while the muscle was part black gray (hypoechoic) measured from under the skin. This measurement was performed transversely.

Phenotypic measurement of *Longissimus dorsi* muscle in every rabbit breeds showed signifficant association ($P<0.05$). Measurement analysis of muscle thickness from

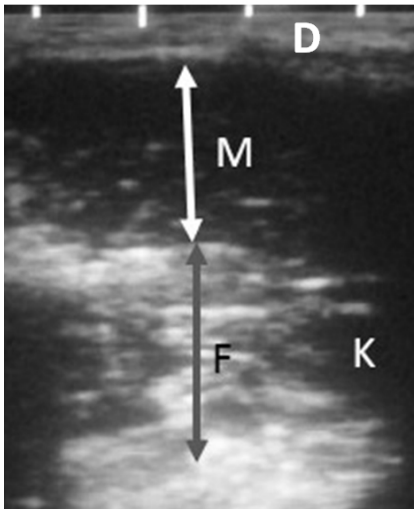

Figure 4. Ultrasound imaging of rabbit perirenal fat and *Longissimus dorsi* muscle. (D= dermis, M= muscle, F= fat, K= kidney).

Rex and Satin rabbit breeds had same effect and both were significantly higher than Reza rabbit breed. This phenomenon showed that Reza rabbit breed in this research had non-additive effect which led to possibility of negative heterosis (Khalil & Youssef, 2010; Fayeye, 2013) and estimated the occurance of transgressive variation (Notter 1999; Wuletaw *et al*., 2006), means phenotype of offspring was lower than their parents.

Perirenal fat thickness from measurement of USG was not affected by rabbit breeds (Table 4). It is related to the sex of a rabbit which measured a male rabbit. Male rabbits have lower fatty liver of female rabbits (Brahmantiyo *et al.*, 2010). Body weight of the experimental rabbit belong to the type of medium weight rabbits. Rex, Satin, and Reza breeds are rabbit with fur skins and meat production as well as having slow growth so that fatty liver is not formed in large quantities (Brahmantiyo *et al.*, 2011). In contrast to the results of Yonkova *et al.* (2011), fat thickness in this case including the results of ultrasound imaging perirenal fat increased with the increase in body weight with statistically significantly different results.

Table 4. The average value of *Longissimus dorsi* muscle and perirenal fat thickness of ultrasound measurement imaging with different rabbit breed

Breed	n	LM (cm)	PF (cm)
Rex	11	1.88 ± 0.35[a]	1.76 ± 0.43[a]
Satin	11	1.86 ± 0.19[a]	1.88 ± 0.38[a]
Reza	11	1.59 ± 0.13[b]	1.73 ± 0.18[a]

Note: n= number of samples; means in the same columns with different superscript differ significantly (P<0.05).

Association of GH Gene Polymorphism with Ultrasound Imaging of Perirenal Fat and *Longissimus dorsi* Muscle

Polymorphism of GH|*Bsh1236I* gene with ultrasound imaging of perirenal fat and Longissimus dorsi muscle thickness at Rex, Satin and Reza rabbit showed that rabbit with CT genotype had thickness of *Longissimus dorsi* muscle higher than CC and TT genotype, although had no significantly different (P>0.05) (Table 5).

It seems that at rabbit with homozygous genotype, rabbit with CC genotype had Longissimus dorsi thickness muscle higher than rabbit with TT genotype. It was related to the thickness of fat that was inversely with the thickness of muscle, and could be assosiated with sexual maturity rate. Animal with late-maturing had a greater proportion of muscle with less fat (Irshad *et al*., 2012).

Table 5. Measurement of ultrasound imaging with different genotype for gene fragment of GH|*Bsh1236I*

Charasteristic	Genotype		
	CC (n=15)	TT (n=8)	CT (n=10)
Perirenal Fat (cm) [ns]	1.71 ± 0.40	1.91 ± 0.26	1.81 ± 0.32
Longissimus dorsi muscle (cm) [ns]	1.78 ± 0.36	1.68 ± 0.21	1.86 ± 0.20

Note: n= number of samples; ns= non signifficant.

CONCLUSION

PCR-RFLP analysis of the GH|*Bsh1236I* gene segments are high polymorphism in Rex, Satin, and Reza rabbit breeds. There are significant association between rabbit breed with *Longissimus dorsi* muscle thickness. The association of characteristic genotype of GH|*Bsh1236I* gene with measurement phenotype is not significant, however it has a potency as marker assisted selection (MAS).

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