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Identification of growth hormone gene variation in exon region at Indonesian Local Cattle based on PCR-SSCP method

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Abstract. *Rahmatullah SN, Jakaria, Noor RR. 2016. Identification of growth hormone gene variation in exon region at Indonesian Local Cattle based on PCR-SSCP method. Biodiversitas 17: 492-497.* The aim of this study was to identify the polymorphisms of the growth hormone gene (GH) of Indonesian local cattle as well as two exotic cattle, as the outside group, using polymerase chain reaction and single strand conformation polymorphism (PCR-SSCP) and using five primers DNA to identify polymorphism of GH gene. Twenty DNA samples of each Indonesian local cattle, consists of Bali, Pesisir, Madura and Katingan, and ten DNA samples of Simmental and Limousine cattle were used. The results showed that the polymorphism of the GH gene was found in three exons which are exon 1, 2 and 5 for Indonesian local cattle except for the Bali and Madura cattle that showed polymorphism was only at exon 2. Bali and Madura cattle also showed monomorphism in exon 3 and 4. On the other hand, the exotic breed showed the polymorphism in all exons, except for exon 2 in Simmental cattle which was found to be monomorphic.

Keywords: Growth hormone gene, Indonesian local cattle, PCR-SSCP, polymorphism

INTRODUCTION

Indonesia has a diverse local cattle population including Bali, Aceh, Pesisir, Madura and Katingan cattle and their genetic information is still limited. Cattle breeds mentioned above have enormous potential as local genetic resources. There are two main factors that affect performance of local cattle, namely; genetic and environmental factors. From the genetic aspects, there are several genes that have a large influence (major gene) on the properties of economic value. One of the most influential genes is the growth hormone gene for they produce growth hormone (Carnicella et al. 2003). Growth hormone gene (GH) plays an important role as a regulator of feed and nutrient metabolism and absorption in the growth processes (Pawar et al. 2007). In addition GH gene also plays a role in the development of mammary gland cells, lactogenesis and mammary cell proliferation (Lagziel et al. 2000). The other function of GH gene is as a candidate gene to associate with sperm quality traits and polymorphisms of GH gene that could be potential markers for testicular growth after puberty and the on set puberty in bulls (Gorbani et al. 2009; Unanian et al. 2002)

The structure of the growth hormone gene consists of 5 exons and 4 introns and is located on chromosome 19 in the bovine (Hediger et al. 1990). Exon is segment of the eukaryotic gene that encodes a portion of the final product of the gene that especially produces amino acid (protein), whereas intron is part of the genes that is not translated at the time of formation of amino acid/protein and, until recent day, part of intron is unknown for its function (Nicholas 2009).

Exploration and information on the diversity of the gene growth hormone (GH) in exons, either in exon 1, 2, 3, 4 or exon 5, on the Indonesian local cattle are still limited. It is important to know this genetic data that is expected to be used in breeding and developmental programs of Indonesia local cattle. This short report determine the diversity of the growth hormone (GH) gene in exons is by using PCR-SSCP (Polymerase Chain Reaction-Single Strand conformation Polymorphism) in Indonesian local cattle.

MATERIALS AND METHODS

Blood samples used were from the Indonesia local cattle namely Bali, Madura, Pesisir and Katingan cattle. Twenty samples were taken from each Indonesian local cattle, while the ten samples were from each of Simmental and Limousin cattle (imported cattle) which are used as comparing cattle. These samples were collected and stored at the Laboratory of Molecular Genetics and Animal Breeding, Faculty of Animal Science, Institut Pertanian Bogor, West Java, Indonesia.

Primer amplification of GH gene in exons 1, 2, 3, 4, and 5 uses base primer Lagziel et al. (1996) and modified primer of Kioka et al. (1989). It can be seeing in Table 1.

Table 1. Position, fragment length and primer sequences used for amplification of GH gene.

Exon	Fragment length (bp)	Primer sequence [a]
1	315	F: 5′-TGG TGG CAG TGG AGA CGG GA-3′
		R: 5′-GGA CAC GCG AAT GGA GGG GA-3′
2	283	F: 5′-GCC CTG CTC TGC CTG CCC TG-3′
		R: 5′-CCC CAC ACA CCC CCG TTT CT-3′
3	158	F: 5′-GTG TGT TCT CCC CCC AGG AG-3′
		R: 5′-CTC GGT CCT AGG TGG CCA CT-3′
4	198	F: 5′-GGA AGG GAC CCA ACA ATG CCA-3′
		R: 5′-CTG CCA GCA GGA CTT GGA GC-3′
5	392	F: 5′-GCT GCT CCT GAG GGC CCT TC-3′
		R: 5′-CCA CCC CAC CCC CCA GAA TA-3′

The extraction of DNA from blood samples followed the standard method of phenol chloroform (Sambrook et al. 1989) that have been modified. There were five sets of primers used for amplification of GH gene fragment in the exons (Table 1). Mix reagent used 1 ml samples of DNA, 25 pmol of primers (forward-reverse), 200 μM dNTP mixture, 1 mM $MgCl_2$, and 0.5 units of *Taq* polymerase and buffer with a total volume of 12 ml. PCR conditions for amplification were as follows: denaturation at a temperature of 95°C for 5 minutes, annealing at a temperature of 60-66°C for 45 seconds and extension at 72°C for 1 minute, and extension end at 72°C for 5 minutes. DNA amplification was cycled 35 times. PCR products were then electrophorezed on 6% of polyacrylamide gel.

SSCP analysis (Single Strand Conformation Polymorphism)

Application of the Single Strand Conformation Polymorphism (SSCP) is a simple and reliable yet sensitive technique because in this technique, the relation of the electrophoretic mobility of a single strand DNA is important for detection of mutation in genomic DNA. SSCP is a powerful method in screening single strand and much more sensitive to the replication of DNA (Zhu et al. 2006). Identification (genotyping) of PCR products with SSCP technique (single strand conformation polymorphism) was performed with 12 mL of PCR product mixed with 10 mL loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol), then denatured at 95^0C for 5 minutes. Samples were cooled immediately in iced water for 3 minutes, then electrophorezed on the SSCP gel polyacrylamide 10-12%. Electrophoresis was carried out using Protean II xi cells (Bio-Rad) at 200-300 V with temperature of 5^0C for 8-14 h at 0.5 x TBE buffer solutions. After gel has undergone electrophoresis and genotyping, the results based on the SSCP banding pattern were appeared on polyacrylamide gel that has undergone silver staining method (Byun et al. 2009) with some modification.

Data analysis

Frequencies of allele and genotype

Frequencies of allele and genotype are calculated using the method of Nei and Kumar (2000) i.e.:

Heterozygosity value

Genetic diversity (genetic variability) is done by estimating the frequency of observed heterozygosity (Ho) Weir (1996).

Frequencies of expected heterozygosity

$$H_e = 1 - \sum_{1=1}^{n} p_{1i}^2$$

Note:
H_e = frequencies of expected heterozygosity
P_{1i} = frequencies of allele number individuals to-iin locus 1
n = number of sample in locus-1

Expected heterozygosity variance

$$V_{s1}(H_e) = \frac{2}{2_n(2n-1)}\left\{2(2_n-2)\left(\sum x_i^3 - \left(\sum x_i^2\right)^2\right) + \sum x_i^2 - \left(\sum x_i^2\right)^2\right\}$$

Note:
V_{sl} (H_e) = expected heterozygosity variant
x_i = frequency of gene to-i
Standard error = $\sqrt{V_{s1}\ (H_e)}$ (Weir 1996).

RESULTS AND DISCUSSION

Amplification of GH gene

The results of study on genetic markers could be applied in breeding since the use of GH gene is as molecular assisted selection to increase daily gain production and improve milk production and composition (Bastos et al. 2001). GH gene amplification results in fragment length shown in each exon are as follows: exon 1: 315 bp, exon 2: 283 bp, exon 3: 158 bp, exon 4: 198 bp, and exon 5: 392 bp. Results of GH gene PCR amplification product in each exon is showed in Figure 1. Based on the results of amplification performed on each fragment in the GH gene, annealing temperature found in exon 1 is at temperature of 62°C, exon 2 is at temperature of 66°C, exon 3 is at temperature of 61°C, exon 4 and exon 5 are at temperature of 60^0 C, whereas the time needed by five exon was 45 seconds for each. The results are different from those performed by Yao et al. (1996), Lagziel et al. (1996), Malveiro et al. (2001), Muhaghegh et al. (2006) that the temperature and time of annealing in exon 1, 2, 3, 4and 5 are, respectively, 63°C for 50 seconds, 68°C for 50 seconds, 60°C for 30 seconds, 70^0 C for 30 seconds, and 63°C for 50 seconds. Amplification of gene fragments had succeed and was determined by: the condition of annealing to DNA target, PCR machine conditions and reagents used, in addition, the type/breeds of cattle delivered different amplification gene fragment process (Viljoen et al. 2005). Amplification of GH gene fragment product was subjected to identify variations in number of bands of DNA sample (Muhaghegh et al. 2006).

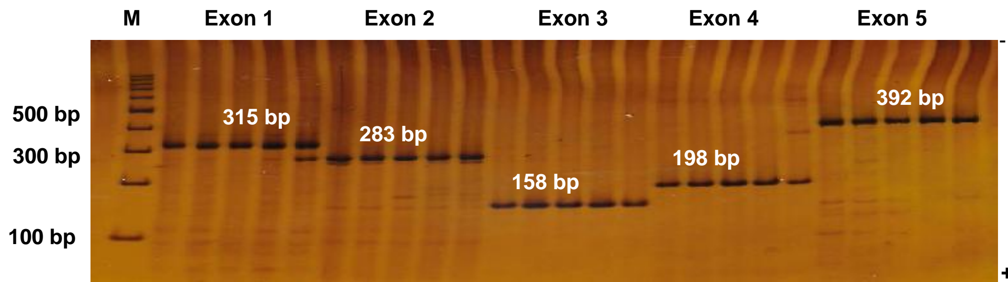

Figure 1. Results of PCR amplification products fragments of GH gene on 6% polyacrylamide gel. M=Marker 100 bp

Figure 2. Pattern genotype of GH gene which was found in Indonesian local cattle

Frequencies of genotype and allele GH gene

Genotype and frequencies of genotype in GH gene obtained from the results of this study indicate that there is variation in range of genotypes and frequencies of genotype on the Indonesian local cattle (Bali, Pesisir, Madura and Katingan cattle) and imports cattle (Simmental and Limousin cattle) (Figure 2 and Table 2). The number of genotypes found in the GH gene exon 1, 2, 3, 4 and 5 are, respectively, three genotypes (AA, AB, AC), four genotypes (FG, DE, GG, DD), two genotypes (HH, HI), two genotype (JJ, JK), and five genotype (LL, LM, LN, NN, MM).

Several alleles are found in each exon and it is shown in (Table 3). The number of alleles found in Indonesian local cattle for each exon varies from 1 - 4 alleles, whereas the Simmental and Limousin cattle have 1-3 alleles. Several studies have shown that the number of alleles found in FH cattle is 2-3 alleles (Lagziel et al. 1996; Yao et al. 1996), while Malveiro et al. (2001) reported 2-6 alleles at the goat Algarvia in Portugal. The results of this study had shown that alleles variation in the Indonesian local cattle are more than that of imported one, and this is like to be caused by several factors from every domestication center, including the use of Indonesian local cattle and the selection system (Talib 2002).

The Indonesian local cattle had high frequencies with value of 1.000 i.e. in Bali cattle, in exon 1, 3, 4 and 5 of alleles A, D, H, J and L. In imported cattle breeds, there are variations in the value frequencies of allele, especially on allele B, C and D. The highest frequencies for allele B is 0.800 in Limousin cattle in exon 3. In Simmental cattle, allele C in exon 5 has the highest frequencies of allele with 0.300, and the highest frequencies value of allele D is 1.000 in Simmental cattle in exon 2. There is something special about Indonesian local cattle, namely the frequencies of allele A at a Indonesian local cattle (Bali, Pesisir, Madura and Katingan cattle) was the highest compared to the imported cattle from the *Bos taurus* (Simmental and Limousin cattle). Like the Bali cattle, other local cattle like Pesisir, Madura and Katingan cattle are polymorphic in each exon in GH gene. Nei and Kumar (2000) states that an allele in a population said to be polymorphic if it has two or more alleles with frequencies of more than 1%. One cause of the GH gene polymorphisms in several breeds such as the breeds cattle of *Bos taurus* and *Bos indicus* is the occurrence of mutations of amino acid leucine-valine (Leu/Val) (Yardibi et al. 2009; Kovacs et al. 2006) and histidine/arginine (Beauchemin et al. 2006). In bovine breeds, in GH polymorphism, some associations between different GH gene genotypes with production traits, growth traits, milk production have been made and the effects of different genotypes were estimated (Bastos et al. 2001).

Allele and genotype variations of GH gene in cattle that was shown on monomorphic Bali cattle is caused by domestic cattle in tropical regions of Indonesia and Bali cattle is an indicator that the cattle is different from the breed cattle of *Bos taurus* and *Bos indicus*. Bali cattle's adaptability is obtained from natural selection and the effect of the natural proliferating (Talib 2002).

About Pesisir cattle, the results of this study found that there was polymorphic nature of the bovine GH gene of entire exon of Pesisir cattle. This was evident that the frequencies of allele and genotype of GH gene in the four exons of Pesisir cattle having value less than 0.99 except in exon 2 which was monomorphic. GH gene polymorphism was found in samples of Madura cattle was consistent with the results of research of Purwoko et al. (2003), which stated that there were polymorphisms in the GH gene locus 2 which was positioned at 329 bp.

About Katingan cattle, the results of the study found that there were variations in frequencies alleles namely polymorphic in exon 1, 2, 4 and 5, except exon 3 which was monomorphic. Katingan cattle were one of special genetic diversity cattle due to their genetic specific location and they had associated with the other Indonesian local cattle, for example, Bali cattle, Madura cattle. Katingan cattle are predicted as the crossbreeding results of Bali cattle and native cattle in Katingan region in Central Kalimantan Province, Indonesia.

Within the Indonesian local cattle (Bali, Pesisir, Madura and Katingan), the banding and electrophoretic mobility of the population is analyzed and identified in exon (1, 2, 3, 4 and 5), SSCP patterns were observed and it showed that difference in genotype frequency may be influenced by some factors such as genetic drift and location (Muhaghegh et al. 2006).

Degree of heterozygosity

The degree of heterozygosity is the average percentage of heterozygous lociper individual or the average percentage of heterozygous individuals in the population. Estimation of heterozygosity values is important to know so to get an overview of genetic variability and to determine the level of an allele polymorphism (Nei and Kumar 2000). High heterozygosity showed high genetic diversity within a population.

The average value of the highest heterozygosity was found in Pesisir cattle for 0.390, and the lowest was found in cattle of Madura at 0.0800. The highest heterozygosity values found on the import cattle, Limousin cattle, was at 0.720 and the lowest was in Simmental cattle with 0.620. Based on the results obtained (Table 5), it is shown that Bali cattle had the lowest average of heterozygosity when compared to Pesisir, Madura, Katingan, Limousin and Simmental cattle in each exons 1, 3, 4 and 5. Heterozygosity values obtained from the Indonesian local cattle (Bali, Pesisir, Madura and Katingan cattle) and from the imported cattle (Simmental and Limousin cattle) are polymorphic based on estimations of heterozygosity values. Estimating the value of heterozygosity has important significance namely to know a description of genetic variability (Marson et al. 2005), to know the level of allele polymorphism and the future prospect of population (Falconer and Mackay 1996). The value of heterozygosity could be an indication of the existence of an intensive selection process (Machado et al. 2003). Tambasco et al. (2003) states that if the value of the observed heterozygosity (Ho) is much lower than the expected heterozygosity (He), then it indicates breeding within the group is as a result of an intensive selection process.

Table 2. Number of alleles and genotypes of GH gene in five exons of all cattle breeds

Fragment	Breed cattle											
	Bali cattle		Pesisir cattle		Madura cattle		Katingan cattle		Simmental cattle		Limousin cattle	
	Allele	Genotype	Allele	Genotype	Allele	Genotype	Allele	Genotype	Allele	Genotype	Allele	Genotype
Exon 1	1	1	3	3	2	2	3	3	3	2	2	1
Exon 2	2	2	1	1	1	1	2	3	1	1	2	2
Exon 3	1	1	2	2	1	1	1	1	2	2	2	2
Exon 4	1	1	2	2	1	1	2	2	2	2	2	1
Exon 5	1	1	2	2	2	2	2	3	3	4	3	4

Table 3. Frequency of GH gene genotype in five exon of all breeds

Population	Fragmen																				
	Exon 1 Genotype					*Exon* 2 Genotype						*Exon*3 Genotype			*Exon* 4 Genotype			*Exon* 5 Genotype			
	AA	AB	BB	AC	CC	DD	DE	EE	FF	FG	GG	HH	HI	II	JJ	JK	KK	LL	LM	MM	LN	NN
Bali	1.000	0.000	0.000	0.000	0.000	0.450	0.550	0.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000
Pesisir	0.300	0.600	0.000	0.100	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.950	0.050	0.000	0.400	0.600	0.000	0.400	0.600	0.000	0.000	0.000
Madura	0.850	0.000	0.000	0.150	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.750	0.250	0.000	0.000	0.000
Katingan	0.200	0.650	0.000	0.150	0.000	0.000	0.000	0.000	0.200	0.250	0.550	1.000	0.000	0,000	0.700	0.300	0.000	0.450	0.350	0.200	0.000	0.000
Simmental	0.000	0.800	0.000	0.200	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.300	0.700	0,000	0.100	0.900	0.000	0.300	0.300	0.000	0.200	0.200
Limousin	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.300	0.700	0.200	0.800	0,000	0.000	1.000	0.000	0.400	0.200	0.000	0.200	0.200

Table 4. Allele frequencies in five exon GH genes in all breeds

Population	Fragment													
	Exon 1 Allel			*Exon* 2 Allele				*Exon* 3 Allele		*Exon* 4 Allele		*Exon* 5 Allele		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Bali cattle	1.000	0.000	0.000	0.725	0.275	0.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000	0.000
Pesisir cattle	0.650	0.300	0.050	1.000	0.000	0.000	0.000	0.950	0.050	0.700	0.300	0.700	0.300	0.000
Madura cattle	0.930	0.000	0.070	0.000	0.000	0.000	1.000	1.000	0.000	1.000	0.000	0.875	0.125	0.000
Katingan cattle	0.600	0.325	0.075	0.000	0.000	0.325	0.675	1.000	0.000	0.850	0.150	0.625	0.375	0.000
Simmental cattle	0.500	0.400	0.100	0.000	0.000	0.000	1.000	0.300	0.700	0.550	0.450	0.550	0.150	0.300
Limousin cattle	0.500	0.500	0.000	0.000	0.000	0.150	0.850	0.200	0.800	0.500	0.500	0.650	0.100	0.250

Table 5. Observed heterozygosity (Ho) values and expected heterozygosity (He) values of gene fragment in five exon GH gene in all breeds

Population	n	GH *exon* 1		GH *exon* 2		GH *exon* 3		GH *exon* 4		GH *exon* 5	
		H_{obs}	H_{exp} ± SE	H_{obs}	H_{exp}± SE	H_{obs}	H_{exp}±SE	H_{obs}	H_{exp}± SE	H_{obs}	H_{exp}±SE
Bali cattle	20	0.000	0.000 ±0.000	0.550	0.399±0.028	0.000	0.000±0.000	0.000	0.000±0.000	0.000	0.420±0.000
Pesisir cattle	20	0.700	0.485±0.029	0.000	0.000±0.000	0.050	0.095±0.012	0.600	0.420±0.028	0.600	0.420±0.028
Madura cattle	20	0.150	0.139±0.018	0.000	0.000±0.000	0.000	0.000±0.000	0.000	0.000±0.000	0.250	0.219±0.023
Katingan cattle	20	0.800	0.529±0.028	0.450	0.349±0.028	0.000	0.000±0.000	0.300	0.255±0.025	0.350	0.469±0.026
Simmental cattle	10	1.000	0.580±0.053	0.000	0.000±0.000	0.700	0.420±0.055	0.900	0.495±0.050	0.500	0.585±0.053
Limousin cattle	10	1.000	0.500±0.050	0.300	0.255±0.050	0.800	0.320±0.055	1.000	0.500±0.050	0.500	0.505±0.059

GH gene is polymorphic in five exons (exon 1, 2, 3, 4 and 5) of Indonesian local cattle. The study found allele A, D, H, J and L in Indonesian local cattle which have higher allele frequency value than that in imported cattle. Simmental and Limousin cattle are polymorphic for all the exon (exon 1, 2, 3, 4, 5) except in Simmental cattle that are purely monomorphic in exon 2. The degree of heterozygosity of Indonesian local cattle is the lowest compared to the imported cattle (Simmental and Limousin cattle), so it can be concluded that imported cattle have high polymorphism.

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