Identification of growth hormone gene polymorphism and its association with body weight in PO Kebumen cattle

B D P Soewandi¹, Hartati² and A A R Hapsari¹

¹ Indonesia Research Institute of Animal Production (IRIAP), Jl. Veteran III, Ciawi 16002, West Java, Indonesia
² Beef Cattle Research Station, Pahlawan RD no. 2 Grati, Pasuruan, East Java 67184, Indonesia.

Corresponding author: bayu.dewantoro@gmail.com

Abstract. PO-Kebumen cattle was one of local genetic resources that famous with good body performance and high growth rate. The aim of this research was to detect growth hormone (GHR, GH and Pit-1) polymorphism and its association with body weight performance of PO-Kebumen cattle. The sample used in this research was 69 heads PO-Kebumen cattle belongs to the farmers of PO-Kebumen Cattle Farmer Association (ASPOKEB). This research observed the growth of PO Kebumen cattle, birth weight, weaning weight and the weight at one year. The parameters observed were birth weight, weaning weight and the weight at one year. The DNA extraction was carried out using blood samples. After DNA extraction, PCR-RFLP method was used to observe the genotype of three growth gene (GH, GHR and Pit1). The results showed that the GH and GHR genes were polymorphic, meanwhile Pit1 gene was monomorphic. The analysis showed that the three growth genes were not significantly associated to body weight (birth weight, weaning weight and the weight at one year) on PO-Kebumen cattle.

1. Introduction

Peranakan Ongole (PO) cattle were widely developed in Java, and one of the superior PO cattle was PO-Kebumen cattle [1]. Kebumen PO cattle were excellence in body performance which was bigger compared to PO cattle in general. Based on (Badan Standarisasi Nasional, 2015) that at the age of 18–24 months, the minimum size on shoulder height, body length and chest circumference of female cattle of 1st class breeding stock was 119 cm, 120 cm and 138 cm respectively. Based on previous research by [1,3,4] that PO-Kebumen cattle can reach shoulder height size to 133 cm, body length to 124–144 cm and chest circumference to 145–166 cm. Therefore, the superiority of PO-Kebumen cattle needs to be maintained so that there will no genetic quality decreasing due to uncontrolled mating patterns and selection.

The decline of genetic quality in cattle is caused by several factors. One of the factors was the genetic drainage livestock within country [5]. In addition, another factor was the problem of the availability of PO-Kebumen cattle superior breeds [6]. The genetic quality decreasing can also be caused by cases of inbreeding. According to [7] that the channel to get the breeding stock was from the cattle broker, purchased from the breeders in the Kebumen area itself. Therefore, it was necessary to prevent genetic quality decreasing in PO-Kebumen cattle.
Genetic quality decreasing can be prevented by selection. Selection was an act of select male and female livestock to be developed in a breeding area based on their genetic quality [8]. Marker assisted selection can improve genetic quality. The application of Marker assisted selection improves genetic progress in beef cattle [9,10]. Therefore, the genome application to improve the genetic quality of PO-Kebumen cattle can be one of the ways to prevent genetic quality degradation of PO-Kebumen cattle.

The aim of the genome research on cattle was to identify genotypes between cattle. Genome technology, was a marker that can be used as a tool to identify genotypes between livestock [8,11]. One method that frequently used to identify genotypes was PCR-RFLP technique. This technique was increasingly used as a genetic marker because it has several advantages including rapid DNA multiplication using polymerase chain reaction (PCR) and the polymorphism of the fragments was carried out with restriction enzymes, thus able to clearly identify the genotype [12].

Growth hormone genotype detection in beef cattle, because according to [13] states that the association of growth hormone genes with growth traits was important to identify because the candidate gene can be used as a program for selection based on genotype. Growth traits in livestock including body weight, body size, carcass weight and several reproductive traits [13,14]. The growth hormone genes group has several genes that regulate the growth hormone (GH), insulin like growth factor I and II (IGF-I dan IGF-II) and its receptor, and related to protein binding (growth hormone receptor (GHR)) [15], pituitary transcription factor 1 (Pit-1 (serves to activate the expression of GH and prolactine genes (PRL)) [16,17].

Several research related to genes that play a role in growth regulation have been conducted. The studies that have been conducted and found an association between growth genes and body weight was the GH gene in Pesisir cattle [13], GHR gene in Zebu cattle [15] and Pit-1 gene Limousin cattle [18]. Therefore, the aim of this study was to detect growth gene polymorphisms (GHR, GH and Pit1) and its and its association with the body weight performance in PO-Kebumen cattle.

2. Materials and methods

2.1. PO-Kebumen cattle performance data

The parameter data of PO-Kebumen cattle were obtained from individual PO-Kebumen cattle belongs to the farmers of PO-Kebumen Cattle Farmer Association (ASPOKEB) including birth weight, weaning weight (205 days) and the weight at one year (365 days).

2.2. Blood samples and DNA extraction

Blood samples were collected from 69 heads of PO-Kebumen Cattle belongs to the farmers of PO-Kebumen Cattle Farmer Association (ASPOKEB). The blood was drawn from the jugular vein using a vacuum tube containing EDTA. The DNA was extracted from 200 µl of blood using an extraction kit (Zymo research) according to the factory protocol.

2.3. PCR-RFLP analysis

The method used to detect DNA variations was PCR-RFLP.

The primer used for the GH gene designed by [19], GHR designed by [20] and Pit-1 designed by [21] which the forward and reverse primers sequences were shown in Table 1. The composition of the PCR reagent consists of PCR kit MyTaq (MyTaq, Bioline), forward dan reverse primers (200 ng µl⁻¹), DNA samples (5–50 ng µl⁻¹) and double destilated water (ddH₂O) to final volume 20 µl. PCR was performed using a Thermocycler (AB System). The PCR program for 3 gene set at temperature of: pre denaturation 95°C for 1 minute; 35 cycle for denaturation 95°C for 15 second, respectively 53.8°C; 65.7°C and 54.6 °C (for GHR, GH and Pit-1 gene) for 15 second, extention at 72°C for 10 second and the final extention 72°C fof 5 minutes.
Table 1. Genebank accession number, length, location and primers sequence of 3 growth genes in PO-Kebumen cattle.

| Gene   | Genebank accession number | Length (pb) | Location   | Primers Sequence                          |
|--------|---------------------------|-------------|------------|--------------------------------------------|
| GHR    | AF140284.1                | 342         | Exon 10    | F: 5’-GCT AAC TTC ATC GTG GAC AAC-3’       |
|        |                           |             |            | R: 5’-CTA TGG CAT GAT TTT GTT CAG-3’       |
|        |                           |             |            | GH5: 5’-CCCAGGGCAAGAATGAGGC-3’            |
|        |                           |             |            | GH6: 5’-TGAGGAACGTGACGGGCCC-3’            |
| GH-L2  | JQ711182.1                | 327         | Exon 3 and 4| F: 5’-CTA TGG CAT GAT TTT GTT CAG-3’       |
|        |                           |             |            | R: 5’-CTA TGG CAT GAT TTT GTT CAG-3’       |
| Pit-1  | Y15995.1                  | 1301        | Exon 5 and 6| F: 5’-CAATGAGAAAGTTGGTGTC-3’              |
|        |                           |             |            | R: 5’-TCTGCACTCGAGATGCT-3’                |

The restriction enzyme used to detect variations in the GH gene was MspI, for GHR gene variation was AluI, and variation of Pit-1 gene was Hinfl. The PCR and RFLP product were visualized using agarose gel 2% and following GelRed™ Nucleic Acid Gel Staining and visualisation were carried out on geldoc (Infinity VX2).

2.4. Data analysis

2.4.1. Allele frequency. The genotype of individual cattle in this study was determined based on the differences in the number and size of the bands shown in the RFLP results. The genotype and allele frequencies were calculated based on [22] formula with statistical model formula:

\[ X_i = \frac{(2N_{ii} + \sum_j N_{ij})}{2N} \]

with the \( x_i \) as the number of individuals on genotype \( X_i \), \( N \) was the total sample size.

2.4.2. H-W equilibrium. The Hardy-Weinberg (H-W) equilibrium was calculated by the formula according to [23] as follow

\[ X^2 = \sum \frac{(o-e)^2}{e} \]

with \( X^2 \) was the chi-square test, \( o \) was the number of observed genotype, and \( e \) was the number of expected genotype.

2.4.3. Heterozygosity. Heterozygosity data in PO-Kebumen cattle population and polymorphism information content (PIC) value for GHR, GH dan Pit-1 gene. The heterozygosity was calculated using the formula based on [24] and the PIC value calculated using the formula based on [25]. Both of the value was analyzed using POPGENE ver 1.32 software.

2.4.4. Statistical analysis. Analysis of variance for genotype data from 3 genes with parameters of birth weight, weaning weight and weight at 1 year were carried out using general linear model (GLM) assisted by IBM SPSS ver 20.0 software.

3. Results and discussion

Table 2 shows data on birth weight, weaning weight and weight at one year for PO-Kebumen cattle. The data on birth weight of this study have a higher value when compared to the results of the research from [26,27] both male and female in PO cattle. Meanwhile, the weaning weight of PO-Kebumen cattle in this study obtained a higher average when compared to research conducted by [28]. When compared to other local cattle in Indonesia, the 1 year weight of PO-Kebumen cattle in this study was higher compared to Bali cattle in the research result from [29].
The results of this study indicate that by sex, PO-Kebumen cattle was not significantly different on its birth weight, weaning weight and weight at 1 year (P>.05) (Table 2). However, there was a tendency that male PO-Kebumen cattles have a higher weight from birth to weaning, but at the age of 1 year the weight of the males cattles was lower than the female cattles. According to Sri Rachma et al. (2011) that sex of cattle affects in growth. Therefore, the difference in average weight was influenced by sex.

| Character       | Sex    | n   | Average | Standard deviation |
|-----------------|--------|-----|---------|--------------------|
| Birth weight    | Male   | 28  | 29.71   | 3.31               |
|                 | Female | 41  | 28.44   | 2.16               |
| Weaning weight  | Male   | 28  | 130.00  | 51.43              |
|                 | Female | 41  | 129.95  | 42.25              |
| Weight at 1 year| Male   | 28  | 178.39  | 59.06              |
|                 | Female | 41  | 183.32  | 55.31              |

3.1. Polymorphism identification and amplification of 3 growth genes

![Figure 1](image1.png)

**Figure 1.** Genotype GHR, GH and Pit-1 gene visualisation on PO-Kebumen cattle. A = GHR gene visualisation using AluI enzyme; B = GH gene visualisation using MspI enzyme, C = Pit-1 gene visualisation using HinfI enzyme.

The fragments of GHR, GH and Pit-1 gene were successfully amplified in this study. The GHR gene amplification was derived from part of exon 10 [20], while the GH gene was amplified from some exon 3 and exon 4 [19], and according to [21] Pit-1 gene amplification derived partly from exons 5 and 6. Identification of three growth genes (GHR, GH and Pit-1) in PO-Kebumen cattle carried out by PCR-RFLP using restriction enzymes (*Thermo Fisher Scientific*), where the temperature and
incubation time following the usage instructions. Identification of GHR genes using AluI enzymes with restriction site was AG\|CT, MspI enzymes for GH gene with restriction site was C\|CGG, and Pit-1 gene using HinfI enzymes with restriction site was G\|ATTC. The results of the polymorphism of 3 growth genes based on PCR-RFLP were shown in Figure 1.

On these three growth genes, PCR-RFLP method using 3 restriction enzyme resulted 7 genotype for each growth gene. On GHR gene using AluI enzyme resulted 3 genotype (AA, AG and GG), meanwhile on GH gene using MspI enzyme resulted 2 genotype (-/- and -/+), and for Pit-1 gene using HinfI enzyme resulted 2 genotype (BB and AB). The GHR gene polymorphisms in this study were caused by mutations at 257 bp position with A>G substitution on exon 10, meanwhile on GH gene the polymorphism was caused by mutations at 837 bp with C>T substitution on intron 3 [27]. Polymorphism on Pit-1 gene was caused by mutations at g.1256 G>A substitution on exon 6 [31].

Based on [20] that on GHR gene A allele more common to be found in Bos indicus cattles than in subtropical cattles. Therefore, the A allele was more often to be founded compared to the G allele. On Pit-1 gene it was known that the frequency of the A allele tended to be higher in the Bos taurus and cross groups than in the Bos indicus group [31]. Based on the origin the PO-Kebumen cattle were from Bos indicus, in Pit-1 gene many allele B was found in PO-Kebumen cattle.

3.2. Hardy-Weinberg equilibrium (chi-square analysis)
The allele frequency value was over 0.99 then the gene was said to be monomorphic and vice versa, if the frequency value was less than 0.99, the gene was said to be polymorphic [32]. Based on the calculation of the genotype and allele frequencies of the three genes, the GHR and GH genes were polymorphic, while the Pit-1 gene was monomorphic in the PO-Kebumen cattle population (Table 4). Therefore, the Hardy-Weinberg equilibrium test was calculated for the PO-Kebumen cattle population and it was found that the X² values in the three growth genes were 1.95 (gen GHR), 0.52 (GH) and 0.02 (Pit-1) respectively. The X² value of the three growth genes were smaller when compared to X² table in the population of PO-Kebumen cattle.

| Gene | Genotype | Allele | He | Ho | PIC |
|------|----------|--------|----|----|-----|
| GHR  | AA       | AG     | GG | A  | 0.49 | 0.49 | 0.37 |
|      | 0.30     | 0.55   | 0.15 | 0.58 | 0.42 |
| GH   | -/-      | -/+    | +/+ | -  | 0.15 | 0.15 | 0.14 |
|      | 0.15     | 0.16   | 0.92 | 0.08 |
| Pit-1| BB       | AB     | AA | B  | 0.03 | 0.03 | 0.03 |
|      | 0.97     | 0.03   | 0.99 | 0.01 |

He = expected heterozygosity; Ho = observed heterozygosity; PIC = Polymorphism Information Content.

| Gene | Total amount | Genotype | X² |
|------|--------------|----------|----|
| GHR  | Observed (O) | 21 38 10 | 1.95 |
|      | Expected (E) | 23.19 33.62 12.19 |
| GH   | Observed (O) | + + | 0.52 |
|      | Expected (E) | 0.01 |
| Pit-1| Observed (O) | BB AB AA | 0.02 |
|      | Expected (E) | 0.01 |

X² 0.05:2 = 5.99
Based on the results of calculations on the three growth genes (GHR, GH and Pit-1), it was found that the PO-Kebumen cattle population was in equilibrium. Based on research in PO-Kebumen cattle to detect the MC4R gene that conducted by [8] found that the PO-Kebumen cattle population was in equilibrium condition as well. The results of this study indicate that the population of PO-Kebumen cattle has not been subjected for selection process because the allele frequency and genotype of the three growth genes remain constant as long as there are no disturbing factors. The population balance will have an effect because of the evolution within a population due to migration, mutation and selection [33,34].

### 3.3. Genotype frequency and alleles of three growth genes

Genotype and allele frequencies of 3 growth genes in this study (GHR, GH and Pit-1) the PO-Kebumen cattle were calculated and shown in Table 1. Based on the results of the calculation on genotype and allele frequencies in the PO-Kebumen cattle population, which is shown in Table 1 it was find out that the highest genotype frequency of GHR gene was AG, meanwhile on GH gene the highest genotype frequency was -/-, and the highest genotype frequency of Pit-1 gene was BB.

Calculation on the value of the observed heterozygosity (Ho) and the expected heterozygosity (He) of the three growth genes in the PO-Kebumen cattle population were shown in Table 3. The difference between the Ho and He values indicates that the genotypes were imbalance in cattle population [35]. Based on that statement, so in this study these three growth genes were in balance. In addition, it appears that there was no selection process in this PO-Kebumen cattle population, because the Ho and He values were not different. Lower Ho than He can be an indication for the level of endogamy, that can be resulted from an intensive selection process [36]. In general, the He value was an indicator of population diversity and can be used to assist selection programs for future generations [37,38].

The polymorphism information content (PIC) value divided into three categories, namely low (≤0.25), medium (0.26–0.49) and high (≥0.5) [25]. In addition, PIC values in the medium to high category can be used as a genetic marker and were quite informative as a marker gene for linkage analysis in the population [39]. In this study, the PIC value which has been shown in Table 3 indicates that the GHR gene has a PIC value in the medium category and has the potency to be used as a gene marker.

### 3.4. Association of three growth genes (GHR, GH and Pit-1) to the body weight of the PO-Kebumen cattle

Table 5 shows the calculation of birth weight, weight of cattle and weight at 1 year in PO-Kebumen cattle in each genotype of the three growth genes (GHR, GH and Pit-1). At birth weight, the GG genotype has the highest weight for the GHR gene, meanwhile on GH gene genotype -/- has the highest weight, and the BB genotype has the highest weight for Pit-1 gene. However, at birth weight there was no significant association between the genotypes of the three growth genes (P>.05). Birth weight that was not associated with the GHR and GH genes shows the same results as the study of research was conducted by [27] on PO-Grati cattle.

Weaning weight and weight at 1 year for PO-Kebumen cattle in this study also showed results that were not significantly different among the genotypes of the three growth genes. Although, Table 5 shows that there was a tendency for one genotype to be higher than the other two genotypes in the same gene. At the weaning weight and weight at 1 year, it was known that the AG genotype in the GHR gene has the highest weight, the GH gene for the -/- genotype has the highest weight and genotype AB had the highest weaning weight for the Pit-1 gene, meanwhile, the highest weight in a year was the BB genotype. Weaning weight and weight in 1 year cattle were no significant differences between the genotypes found in the GH gene [40]. The Pit-1 gene was also not significantly associated even though this gene was found in polymorphic conditions in Angus cattle [41].
Table 5. Average birth weight, weaning weight and weight at 1 year for GH, GHR and Pit-1 genes in PO-Kebumen cattle.

| Characteristics | Gene | Genotype | n  | Average  | Standard deviation |
|-----------------|------|----------|----|----------|--------------------|
| Birth weight    | GHR  | AA       | 21 | 28.24    | 1.76               |
|                 |      | AG       | 38 | 29.08    | 2.72               |
|                 |      | GG       | 10 | 30.00    | 4.11               |
|                 | GH   | +/-      | 58 | 29.09    | 2.89               |
|                 |      | +/-      | 11 | 28.27    | 1.62               |
|                 | Pit-1| BB       | 67 | 28.97    | 2.77               |
|                 |      | AB       | 2  | 28.50    | 0.71               |
| Weaning weight  | GHR  | AA       | 21 | 131.05   | 39.81              |
|                 |      | AG       | 38 | 132.76   | 51.48              |
|                 |      | GG       | 10 | 117.10   | 35.07              |
|                 | GH   | +/-      | 58 | 131.69   | 6.32               |
|                 |      | +/-      | 11 | 123.52   | 14.14              |
|                 | Pit-1| BB       | 67 | 129.81   | 46.49              |
|                 |      | AB       | 2  | 135.50   | 10.61              |
| Weight at 1 year| GHR  | AA       | 21 | 174.76   | 46.80              |
|                 |      | AG       | 38 | 184.53   | 62.75              |
|                 |      | GG       | 10 | 182.90   | 56.49              |
|                 | GH   | +/-      | 58 | 184.47   | 59.76              |
|                 |      | +/-      | 11 | 164.72   | 31.28              |
|                 | Pit-1| BB       | 67 | 181.54   | 56.49              |
|                 |      | AB       | 2  | 174.00   | 22.63              |

4. Conclusion
The conclusion of this study indicated that the two growth genes (GHR and GH) were polymorphic, while the Pit-1 gene was monomorphic. These three growth genes (GHR, GH and Pit-1), however, were not associated with body weight traits (birth weight, weaning weight and weight at 1 year) on PO-Kebumen cattle. Although, the GHR gene was polymorphic and has the potency to be marker that can be used for selection, it was not associated to birth weight, weaning weight and weight at 1 year in PO-Kebumen cattle.

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