J I T A A
Journal of the Indonesian Tropical Animal Agriculture
Accredited by Ditjen Penguatan Risbang No. 60/E/KPT/2016

J. Indonesian Trop. Anim. Agric.
pISSN 2087-8273 eISSN 2460-6278
http://ejournal.undip.ac.id/index.php/jitaa
44(2):125-134, June 2019
DOI:10.14710/jitaa.44.2.125-134

Polymorphism of the SNP g. 1180 C>T in leptin gene and its association with growth traits and linear body measurement in Kebumen Ongole Grade cattle

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Received November 30, 2018; Accepted April 21, 2019

ABSTRAK

Penelitian ini bertujuan mengetahui polimorfisme gen leptin sapi PO Kebumen dan asosiasinya dengan sifat pertumbuhan. Seratus sampel darah sapi dikoleksi untuk analisis molekuler. Polimorfisme gen leptin dianalisis menggunakan 2 teknik: *Polymerase Chain Reaction - Restriction Fragment Length Polymorphism* (PCR-RFLP) menggunakan enzim restriksi *HpyCH4V* dan sekuensing. Asosiasi antara gen leptin dan sifat pertumbuhan dianalisis menggunakan T-test. Hasil penelitian menunjukkan ada SNP g. 1180 C>T pada gen leptin populasi Sapi PO Kebumen, yang merubah asam amino dari arginin menjadi sistein. Dua alel, C dan T, dengan frekuensi masing-masing 0,885 dan 0,115 dan 3 genotip, CC, CT dan TT, dengan frekuensi masing-masing 0,78; 0,21 dan 0,01 telah dideteksi. Populasi yang diamati berada dalam *Hardy-Weinberg equilibrium*. Terdapat asosiasi yang signifikan antara genotip dan lingkar dada saat sapih. Dapat disimpulkan bahwa gen leptin merupakan kandidat gen yang dapat digunakan sebagai marker seleksi untuk lingkar dada saat sapih pada sapi PO Kebumen.

Kata kunci: identitikasi, polimorfisme, gen Leptin, sapi Peranakan Ongole Kebumen

ABSTRACT

The aim of this study was to identify the polymorphism of leptin gene and its association with growth traits in Kebumen Ongole Grade cattle. One hundred blood samples were collected for molecular analysis. Polymorphism of the leptin gene was analyzed using Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) with *HpyCH4V* restriction enzyme and DNA sequencing. Association analysis of the leptin gene with growth traits was analyzed by T-test. The results showed that SNP g. 1180 C>T was found in the population. The SNP changed amino acid from arginine to cysteine. The SNP was significantly associated with a high chest circumference at weaning age in animal having CC genotype ($P<0.05$). There were two identified alleles, namely C and T, with frequencies were 0.885 and 0.115, respectively. The genotype frequencies of CC, CT and TT were 0.78, 0.21 and 0.01, respectively. Allelic and genotypic distribution in the studied population were in Hardy-Weinberg equilibrium. Animals with CC genotype had a higher circumference at weaning age (WCC) than those with CT genotype. In conclusion, SNP g. 1180 C> T in the leptin gene is potential as genetic marker for growth traits in Kebumen Ongole Grade cattle.

Keywords: identification, polymorphism, leptin gene, Kebumen Ongole Grade cattle

INTRODUCTION

Kebumen Ongole Grade cattle are potential for beef production in Indonesia. They are registered as local cattle by the Indonesian Ministry of Agriculture (No.47/Kpts/SR.120/ I/2015). They are widely distributed throughout Kebumen regency, making it as a source of breeding for the cattle (No. 358/Kpts/PK.040/6/2015). Productivity of Kebumen Ongole Grade cattle could be maintained and increased by selection. Quantitative traits, such as growth traits and carcass characteristics are important economic traits, which can be used as a selection criteria in cattle (Vann *et al.,* 2017; Thompson *et al.,* 2014). Studies on quantitative genetics in Kebumen Ongole Grade cattle was previously carried out by Sumadi *et al.* (2017) and Maharani *et al.* (2017) in analyzing of breeding value and prediction of body weight at puberty using a nonlinear mathematical model, respectively.

Nowadays, molecular technology based marker-assisted selection (MAS) has been widely used in cattle genetic improvement (Putri *et al.,* 2015; Seong *et al.,* 2012). PCR-RFLP is commonly used to determine gene polymorphism by identifying the individual genotype (Agarwal *et al.,* 2009). Genetic characterization of Kebumen Ongole Grade cattle has been carried out in the analysis of MC4R and Cyt *b* genes (Maharani *et al.,* 2018; Hartatik *et al.,* 2018). Many of studies reported the usefulness of molecular markers in the genetic analysis of several cattle populations, i.e. Malaysian (Romaino *et al.,* 2014), Jabres and Rambon (Sutarno and Setyawan, 2015; Sutarno *et al.,* 2015), Aceh and Pesisir (Hartatik *et al.,* 2015), Madura (Hartatik *et al.,* 2014) and Bali cattle (Jakaria and Noor, 2015).

Leptin is a protein hormone released from adipose tissue and plays an important role in animal growth such as body weight regulation, fat mass and energy metabolism (Sainz *et al*., 2015; Wasim, 2015; Sharifzadeh and Doosti 2010). Leptin gene in bovine located in BTA 4q32. It consists of three exons and two introns. The first intron is more than 8 kb and the second intron has a length of 1.6 kb (Liefers *et al.,* 2002). The activity of leptin is regulated by leptin receptor (LEPR). LEPR is included in the gp130 family of cytokine receptor. There are six isoforms from LEPR (LEPRa, LEPRb, LEPRc, LEPRd, LEPRe, LEPRf) but LEPRb was the most important isoform because it is the strongest signal which affect in animal growth (Nanjappa *et al.,* 2011). Several studies revealed that mutations in the exon 2 based on SNP 1047C>T/R25C influenced body weight, fatness and muscle fat composition (Woronuk *et al.,* 2012; Orru *et al.,* 2011, Kawaguchi *et al.,* 2017). Identification of the bovine leptin gene shows a correlation between polymorphism of this gene and body weight and fatness (Woronuk *et al.,* 2012; Kononof *et al.,* 2017), and meat quality (Shin and Chung, 2006). SNP mutation in exon 2 of the leptin gene is strongly associated with carcass quality (DeVuyst *et al.,* 2011). A causative mutation of A1127T disturbs the leptin function in the body's physiological processes (Fortes *et al.,* 2009). Identification of the leptin gene polymorphism in 5'flanking (promoter) region has been reported in Brangus (Corva *et al.,* 2009) and Holstein Friesian cattle (Matteis *et al.,* 2012). Recently, one synonymous (SNPs g.1025T>C/S17S) and two non synonymous SNPs (g.1047C>T/R25C and g.1048G>A/R25H) found in Ongole grade cattle (Hilmia *et al.,* 2018). The SNPg.1025T>C/S17S indicated no change amino acids and SNP g.1047C>T/R25C and g.1048G>A/R25H changed amino acids from arginine to cysteine, and arginine to histidine, respectively. Moreover, A SNP g. 1180 C>T was found and converted amino acid from arginine to cysteine. Detection those mutation of the leptin gene in Kebumen Ongole Grade cattle has not been performed. Thus, this study was conducted to identify the polymorphism of leptin gene and its association with growth traits.

MATERIALS AND METHODS

Animals, Data Collection and Genomic DNA Extraction

One hundred blood samples of Kebumen Ongole Grade cattle were used in this study. All animals studied were reared in Klirong district, Kebumen regency, Central Java province. An approximately 3 ml blood sample for each animal was collected from the jugular vein using sterilized venoject and vacutainer EDTA. Genomic DNA was extracted by using gSYNC™ DNA Extraction Kit (Geneaid, New Taipei City, Taiwan). This genomic DNA product was used as a template for PCR amplification. Growth data, such as body weight and body size were obtained from recording, provided by Kebumen Ongole Grade cattle Breeder Association and used for

association analysis with the leptin gene. The maintenance management such as feeding and housing is almost uniform under the authority of the Kebumen Ongole Grade cattle Breeder Association. The samples are unrelated family indicated based on their recording.

Polymerase Chain Reaction (PCR) Ampification

Genbank, primer, gene target and SNP in according to Shin and Chung (2006) and Hernandez *et al.* (2016) are presented in Table 1. PCR was performed in 25 µL total volume containing 2 µL of genomic DNA, 12.5 µL PCR kit (*MyTaq™ HS Red Mix*), 0.5 µL of each primer forward and reverse, and 9.5 µL double-distilled water (DDW). PCR condition was performed in 5 min at 94˚C (pre-denaturation) and 35 cycles of 30 s at 94˚C (denaturation), 30 s at 60˚C (annealing), 30 s at 72˚C (extension), and 5 min at 72˚C for the final extension in a SEDI Thermo Cycler PCR machine. PCR products were visualized onto 1.5% standard agarose gels stained with ethidium bromide.

Sequencing and SNP Identification

Sequencing was carried out to confirm the gene target and SNP position based on reference study and eight Kebumen Ongole Grade cattle PCR product. The PCR products were used for direct sequencing which was performed by 1st BASE DNA Sequencing Service Company (Malaysia). Determination of the nucleotide sequences was performed in one direction using the forward primer. Nucleotide sequences results were analyzed using MEGA 7.0.9 (Tamura *et al.,* 2011). SNP g. 1180 C>T was further used to genotype all animals investigated by using PCR-RFLP.

PCR-RFLP and Genotyping

Restriction enzyme was determined using NEBcutter V2.0 (http://nc2.neb.com/NEBcutter2/). *HpyCH4V* restriction enzyme was used to digest the 466 bp of the leptin gene with a recognition site of 5'-TG│CA-3' (Figure 1). PCR-RFLP was carried out in 20 µL reaction volume containing 15 µL of PCR product, 2 µL 10x buffer, 0.2 µL *HpyCH4V* and 2.8 µL DDW. The reaction mixture was incubated at 37^{o}C for 4 hours in multi-heater. The digestion products were visualized onto 3% agarose gels.

Statistical Analysis

Allelic and genotypic frequencies were calculated by a simple allele counting method according to Warwick *et al.* (1990):

The Frequency of C allele = ∑ C locus/∑ (C locus + T locus)

The Frequency of T allele = ∑ C locus/∑ (C locus + T locus)

The Frequency of CC genotype = (∑ CC/N) x 100%

The Frequency of TT genotype = (∑ TT/N) x 100%

The Frequency of CT genotype = (∑ CT/N) x 100%

Where :

∑ CC = the number of individual of CC genotype

∑ TT = the number of individual of TT genotype

∑ CT = the number of individual of CT genotype

N = total of individual samples

Hardy-Weinberg equilibrium for identified locus was determined in a Pearson's Chi-square test, with the following mathematical model (Kang and Shin, 2004):

$$X^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)(O_i - E_i)^2}{E_i}$$

Where:

X^2 = Chi-square test value

Table 1. Genbank, Primer, Target Location of Leptin Gene, SNP to Location of Leptin Gene

Genbank	Target Location	Primer (5'-3')	SNP	Region	Fragment size (bp)
U50365	877-1342	GATTCCGCCGCACCTCTC CCTGTGCAAGGCTGCACAGCC	1127A>T	Exon 2	466
			978C>T	Intron	
			1180 C>T	Exon 2	

O_i = observed frequency

E_i = expected frequency

n = the number of compared data

Association analysis between leptin gene and growth traits was analyzed with IBM SPSS Statistics v. 25 using the following formula (Kim, 2015) :

$$t = \frac{\bar{x}_1 - \bar{x}_2}{Sp\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}}$$

with

$$Sp = \sqrt{\frac{(n_1 - n_2)S_1^{\,2} + (n_1 - n_2)S_2^{\,2}}{n_1 + n_2 - 2}}$$

where:

$\bar{x}_1$ = Mean of the first sample

$\bar{x}_2$ = Mean of the second sample

n_1 = Sample size (i.e., number of observations) of the first sample

n_2 = Sample size (i.e., number of observations) of the second sample

S_1 = Standard deviation of the first sample

S_2 = Standard deviation of the second sample

Sp = Pooled standard deviation

RESULTS AND DISCUSSION

Growth Traits Profile

Mean and standard deviation for each growth trait, including birth weight (BW), birth body length (BBL), birth chest circumference (BCC), birth shoulder height (BSH), weaning weight (WW), weaning body length (WBL), weaning chest circumference (WCC), weaning shoulder height (WSH), average daily gain (ADG), yearling weight (YW), yearling body length (YBL), yearling chest circumference (YCC) and yearling shoulder height (YSH) are presented in Table 2. The results of this study were in agreement with previously reports in Kebumen Ongole grade cattle (Maharani *et al.,* 2018).

Polymorphism, Genotyping and Allele Distribution of Leptin Gene

Based on reference sequence, only SNP g. 1180 C>T was confirmed, while SNP 1127A>T and 978C>T were not identified in our population (Figure 2). The SNP was identified by the presence of "double peak" at position 1180 based on GenBank accession number U50365 (Figure 3). The SNP resulting two alleles, C and T (Figure 4). Digestion of 466 bp of the leptin gene by *HpyCH4V* restriction enzyme generated three

Table 2. The Growth Profile of Kebumen Ongole Grade Cattle

Variable	n	Mean±SD	Minimum	Maximum
Birth Weight (kg)	99	29.76±3.09	22.00	41.00
Birth Shoulder Height (cm)	99	71.44±5.14	48.00	80.00
Birth Body Length (cm)	99	57.03±5.82	44.00	74.00
Birth Chest Circumference (cm)	98	68.12±4.19	56.00	76.00
Weaning Weight (kg)	67	129.98±40.33	66.00	275.00
Weaning Shoulder Height (cm)	67	103.30±11.82	69.00	125.00
Weaning Body Length (cm)	67	92.55±12.02	70.00	127.00
Weaning Chest Circumference (cm)	67	114.67±12.17	74.00	151.00
Average Daily Gain (kg/d)	48	0.58±0.15	31.00	101.00
Yearling Weight (kg)	8	237.45±59.23	158.00	325.00
Yearling Shoulder Height (cm)	8	115.45±14.73	73.00	125.00
Yearling Body Length (cm)	8	110.91±14.39	84.00	127.00
Yearling Chest Circumference (cm)	8	137.27±12.88	122.00	159.00

n= number of animal; Means in the same row with different superscripts differ significantly (P<0.05); n= number of animal;

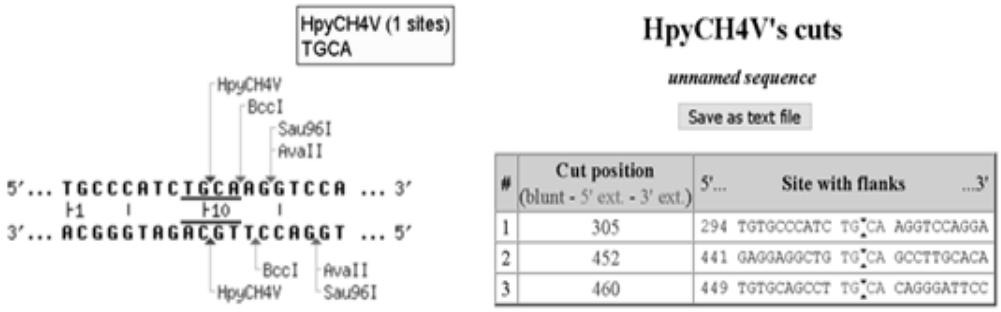

Figure 1. The Site and Cut Position of *HpyCH4V* Restriction Enzyme

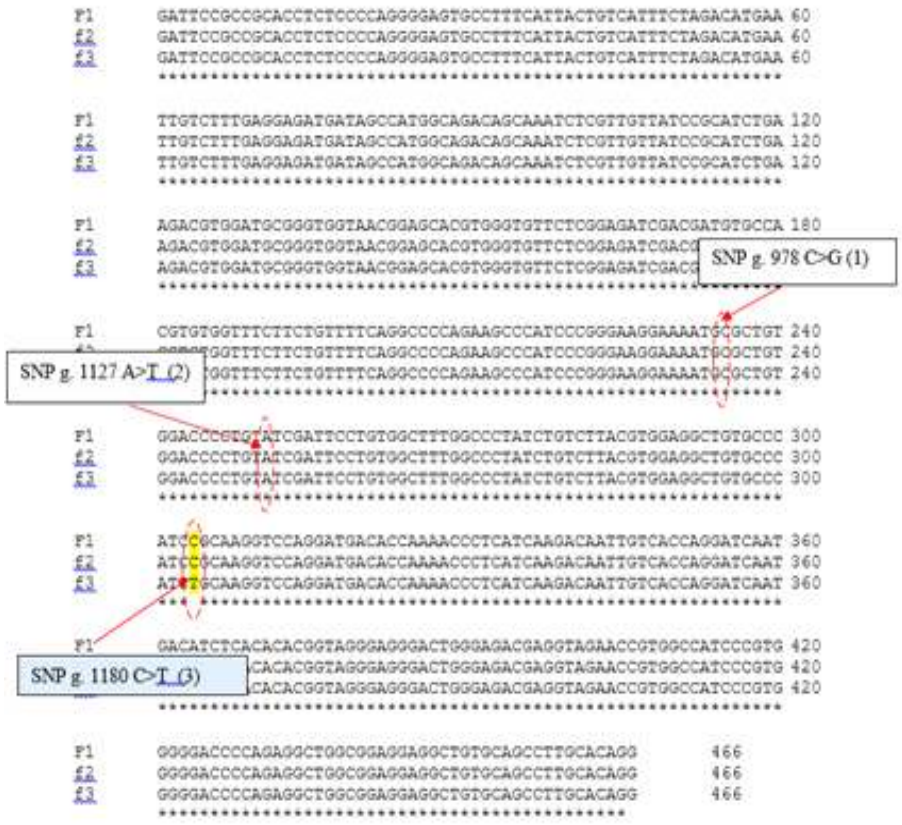

Figure 2. SNPs Identification using Sequences Alignment (466 bp). Note : SNP 1 and 2 were not found in the Kebumen Ongole Grade cattle; SNP 3 was indicated in the samples

genotypes: an undigested PCR product (466 bp) fragment of genotype TT; 6, 8, 147 and 305 bp fragments of genotype CC; and 6, 8, 147, 305 and 466 bp fragments of genotype CT. CC and CT genotypes were found in 78 and 21 animals, respectively, while TT genotype was found only in 1 animal. Therefore, only 2 genotypes (CC and CT) were used for next steps. The fragment size below 100 bp were not visible during the visualization process. Allelic frequencies of C and T were 0.885 and 0.115, respectively, while genotypic frequencies of CC, CT and TT were 0.78, 0.21 and 0.01, respectively (Table 3). Based on the frequency values, the population was dominated by C alleles and CC genotypes. The Chi-square test revealed that SNP g. 1180 C>T in

the studied population was in the Hardy-Weinberg equilibrium.

PCR-RFLP products revealed polymorphism of the leptin gene, resulting two alleles, C and T. Similarly, C and T alleles at SNP g.1180 C>T were also identified in Brahman (Hernández *et al.,* 2016) and Simmental bulls (Orrù *et al.,* 2011). The frequency of C allele was higher than T allele. This results with the same SNP g.1180 C>T indicated similar to those observed in Korean cattle, with frequencies of 0.64 for C allele and 0.36 for T allele (Shin and Chung, 2006). The frequencies of C allele was also higher than T allele in Turkey cattle which were 0.52 and 0.28 respectively (Kaygisiz *et al.,* 2011). In this study, the genotypic frequencies of CC, CT and TT were 0.78, 0.21 and 0.01, respectively. CC genotype had a higher frequency than CT and TT genotypes. Previously, CT genotype had the highest frequency in Korean (Shin and Chung, 2006), Sistani and Sarabi cattle (Aslaminejad 2010). The genotype of CC was predominat genotype found in crossbred cattle and CT was the most frequent genotype observed in Friesian Holstein (Choundhary *et al.,* 2005). In contrast, TT genotype was absence in Golpayegani cattle (Nassiry *et al.,* 2007). The differences of allelic and genotypic distribution might be due to difference of cattle breeds, which may produce different genetic expression.

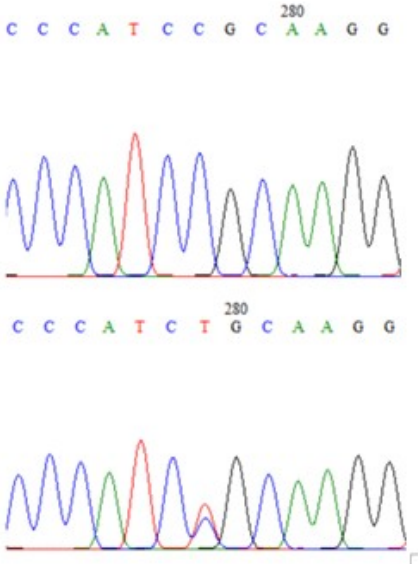

Figure 3. The Electrophoregram which Indicated the Polymorphism of Leptin Gene (SNP g. 1180 C> T)

A Chi-square test showed that the distribution of allele and gentoype in the population was in to Hardy-Weinberg equilibrium. This indicate the population will remain constant from one generation to the next generation in absence of disturbing factors (Moonesinghe *et al.,* 2010). This is similar with reports in Hanwoo (Han *et al.,* 2010), Qinchuan (Zhang *et al.,* 2009), Zebu (Mukesh *et al.,* 2008) and Holstein Turkey cattle (Ozdemer, 2012). HWE in a particular population is determined by the absence of several factors, such as gene recombination, genetic drift, inbreeding, mutation and gene flow from other populations (Banos *et al.,* 2008).

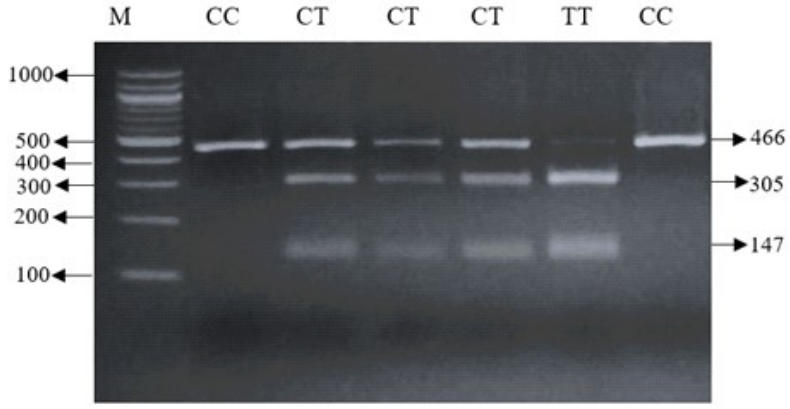

Figure 4. The Results of PCR-RFLP with *HpyCH4V* Restriction Enzyme. Note: M=Marker, CC, CT, TT=Genotype Sample

Effect of Leptin Gene to Growth Ttraits

Genotypes of animals based on the SNP g. 1180 C>T were associated with their growth traits. TT genotype was not used in association analysis due to it contains only 1 individual in the population. The results showed that SNP g. 1180 C>T had a significant effect (P<0.05) on WCC (Table 4). Animals with CT genotype had higher WCC than those with CC genotype. However, there was no significant association between genotype and several growth traits, such as BW, BSH, BBL, BCC, WW, WSH, WBL, WCC, ADG, YW, YSH, YBL and YCC (P>0.05).

The SNP g. 1180 C>T changed amino acid from arginine to cysteine (Javanmard *et al.,* 2010). This mutation may effect on the different expression of leptin gene on WCC in Kebumen Ongole Grade cattle. Circulation of leptin in fat tissue in the human body and animals correlates with body weight which is body weight had possitive correlations with WCC (Friedman, 2011; Sahu *et al.,* 2017). Moreover, leptin which is bounded by neural receptors in the hypothalamus will increase the concentration of Proopiomelanocortin (POMC) and Cocaine and Amphetamine Regulated Transcript (CART). Increased POMC and CART concentrations stimulate the formation of α-melanocortin stimulating hormone (α-MSH). The α-MSH activated the melanocortin receptor (MC4R) signal and stimulated the hypothalamus to secrete thyrotropin releasing hormone (TRH) and

Table 3. Frequency of Alleles and Genotypes based on Leptin Gene with SNP g. 1180 g C>T

Item	Allele Frequency		Genotype Frequency		
	C	T	CC	CT	TT
N	177	23	78	21	1
Value	0.885	0.115	0.78	0.21	0.01

N = Number of sample

Table 4. The Mean and Standard Error of the Growth Traits and Level of Significant for the Leptin Gene using SNP 1180 g. C>T

Variable	n	Genotype	
		CC	CT
Birth Weight (kg)	99	29.53±2.94	30.62±3.54
Birth Shoulder Height (cm)	99	71.15±5.44	72.52±3.76
Birth Body Length (cm)	99	57.54±5.57	55.14±6.47
Birth Chest Circumference (cm)	98	67.99±4.37	68.62±3.51
Weaning Weight (kg)	67	127.05±37.80	146.7±51.69
Weaning Shoulder Height (cm)	67	103.46±11.66	102.8±12.80
Weaning Body Length (cm)	67	92.58±13.71	92.4±8.59
Weaning Chest Circumference (cm)	67	113.42±11.77^{a}	121.8±12.54^{b}
Average Daily Gain (kg/d)	48	0.57±0.12	0.65±0.22
Yearling Weight (kg)	8	297.0±24.43	225.8±61.90
Yearling Shoulder Height (cm)	8	123.67±1.15	118.6±4.67
Yearling Body Length (cm)	8	124.0±2.64	110.8±13.66
Yearling Chest Circumference (cm)	8	146.67±13.65	134.2±12.1

corticotropin releasing hormone (CRH) (Denver *et al.,* 2011). Both of these hormones may increase energy consumption and caused the differences of WCC in Kebumen Ongole Grade cattle.

These results were in agreement with reports from previously study in which the SNP of Leptin gene affected on backfat thickness and marbling score in Korean cattle with CC genotype was more superior than TT genotypes (Shin and Chung, 2006). Schenkel *et al.* (2005) reported that this SNP was significantly associated with carcass quality and composition in cattle. Furthermore, leptin gene had been reported to have an effect on body weight and body size in Chinese cattle (Yang *et al.,* 2007), growth traits in Nellore cattle (Silva *et al.,* 2013).

CONCLUSION

The SNP g. 1180 C>T of the leptin gene is significantly associated with high weaning chest circumference in Kebumen Ongole Grade cattle. The results of this study indicated that the leptin gene may affect on the economic traits in cattle. The SNP g. 1180 C>T could be used as a marker-assiLsted selection in a large population of Kebumen Ongole Grade cattle

ACKNOWLEDGMENTS

This research was supported by Hibah Peningkatan Kapasitas Dosen Muda 2018 from Universitas Gadjah Mada with the contract number: 3126/UN1/DITLIT/DIT-LIT/LT/2018. Authors thank to the Association of Kebumen PO Breeders (ASPOKEB) for their cooperation in data collection (blood samples and recording data).

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