KnE Life Sciences

ICASI
3rd International Conference on Advance & Scientific Innovation
ICASI – Life Sciences Chapter
Volume 2022

Knowledge E
enriching | engaging | empowering

Conference Paper

Identification of Gene Diversity of Melanocortin-4 Receptor and its Relationship with the Birth and Body Size of Beef Cattle

MW Albakri[1], LE Anugratama[1], Panjono[2], and T Hartatik[1]*

[1]Department of Animal Breeding and Genetic, Faculty of Animal Science, Universitas Gadjah Mada, Bulaksumur, Yogyakarta, Indonesia 55281
[2]Department of Animal Production, Faculty of Animal Science, Universitas Gadjah Mada, Bulaksumur, Yogyakarta, Indonesia 55281

ORCID
https://orcid.org/0000-0003-2917-3370

Abstract.
Melanocortin-4 Receptor (MC4R) expressed in hypothalamus which is part of the brain that are involved in regulation of appetite, metabolism and bodyweight. This study aimed to identify the MC4R gene diversity in the second cross generation of Belgian Blue, Wagyu, and Brahman crossbred and its relationship with the body weight and size birth. Polymerase Chain Reaction (PCR) and DNA sequencing were used to identify Single Nucleotide Polymorphism (SNP). The result of this study showed that MC4R genes was polymorphic with one SNP at 1133 C>G and included missense mutation. SNP 1133 C>G has two of allels C and G with three genotypes. Test of Hardy Weinberg equilibrium showed that the population of cattle was in equilibrium. The heterozigosity values showed that the population of cattle had a low diversity of genetics. The result of association showed that SNP 1133 C>G in MC4R Genes was not able to indicate the difference between the weight, body length, heart girth and wither's height at birth. Based on this study, it can be concluded that the polymorphism of MC4R genes can only be used to distinguish cattle genotypes but cannot be used as the selection tools for body weight and size birth.

Keywords: Crossbreed beef cattle, Diversity, Heterozigosity, MC4R gene, SNP.

Corresponding Author: T Hartatik

Published: 13 Sepetmber 2022

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the ICASI Conference Committee.

1. Introduction

The national demand of beef in 2017 was reached in 784 thousand tons, whereas the beef production in 2017 is estimated at 532 thousand tons [1]. The alternative solutions from the goverment is Introduction of new breeds cattles which are Belgian Blue and Wagyu to complete the national demand of beef. The Belgian Blue cattle has developed muscle/ meat caused by muscular hypertrophy (mh). The muscular hypertrophy is enlargement of muscle cells as a result of mutations in the myostatin gene, as known as double-muscling [2]. Based on the case, the muscular hypertrophy has function to increas the meat products. The Wagyu cattle has the physical strength

advantage and it has evenly distributed of fat in the meat (marbling) [3]. Based on the case, it functions to improve the quality of the meat. The Brahman Cross cattle has adaptable in tropical climates advantage but it has a small of body size. The Belgian Blue and Wagyu cattle has low adaptability in tropical climates. On the developments, breeds of Belgian Blue, Wagyu, and Brahman Cross were crossed and it will produced cattle with has superior in production then Brahman cross, but it has adaptibility in tropical climate like a Brahman cross.

Crossing has aimed to get advantage traits in heterozygous. The results are not usually expected because the crossing has most diversity. To increase the efficiency, it should be to selected. The genetic marker is the one way to do selection. *Melanocortin 4 receptor* (MC4R) is an expression of a protein released by the hypothalamus gland in humans and has an effect on appetite, metabolism regulation and body weight. The difference of base in the MC4R gene or usually called Single Nucleotide Polymorphysm (SNP) were associated with appetite, and body weight of several spesies. The MC4R gene plays a role in the behavior and growth of rabbits (2). MC4R gene's SNP associated with carcass quality of cattle (3) and broilers (4).

Based on the introduction, we aimed to investigate the diversity of *MC4R* gene in the second cross generation of Belgian Blue, Wagyu, and Brahman cross and its relationship between of the body weight and size birth. The result of this study can be used to the futher studies of the *MC4R* gene in the second cross generation of Belgian Blue, Wagyu, and Brahman cross for genetic marker cattle selection.

2. Materials and Methods

The materials used in this study were 24 crossbreeds of Belgian Blue, Wagyu, and Brahman Cross. Belgian Blue Cross-Wagyu Cross (BBBX-BX, n=5), Belgian Blue Cross-Wagyu Cross (BBBX-WX, n=2), Wagyu Cross-Belgian Blue Cross (WX-BBBX), and Wagyu Cross- Brahman Cross (WX-BX, n=14). Three ml blood samples were collected and extracted . DNA isolation was performed using gSYNC^{TM}-DNA Extraction kit. The data recording were used weight, body length, heart girth and wither's height at birth.

The primer that used on this study is forward F: 5'-GTC GGG CGT CTT GTT CAT C-3' and reverse R: 5'-GCT TGT GTT TAG CAT CGC GT-3' base on *MC4R* Genbank (EU366350) with 493 bp DNA product size [4]. *Polymerase Chain Reaction* (PCR) performed in total reaction of 20 µl, containing 10 µl PCR kit (Kappa, Biosystem), 7 µl aquabidest, 1 µl of both forward and reverse primer and 1 µl of genome DNA. PCR analysis were amplified protocol in pre-denaturation 94˚C for 3 minute, denaturation

94°C for 45 second, annealing 64°C for 45 second, extention 72°C for 60 second, followed by 34 cycles and the end of step is Post extension 72°C for 10 minute. Then, temperate were decreased until 94°C. The quality of the PCR product were visualized by electrophoresis using 1% agarose, The PCR product was finally analyzed using UV transilluminator. All of PCR sample was sequencing by LPPT UGM to determine position of SNP and genotyping used BioEdit software and for identification of amino acid changed using Ekspassy software.

Genetic frequency and allele frequency can be calculated by allele frequency prediction. Pearson Chi square to verify Hardyweinberg equilibrum.

1. Hardyweinberg equilibrum

$$X^2 = \sum \frac{(O-E)^2}{E}$$

Where:

χ2 = *chi-kuadrat*;

O = observation value; dan

E = expected value.

1. Observed heterozigosity

$$H_{o=}\sum_{i\neq j} \frac{N_{ij}}{N}$$

Where:

H_o = observed heterozigosity;

N_{ij} = number of heterozygous individuals -i; and

N = number of individuals observed

1. Expected heterozigosity

$$He_{=}1 - \sum_{i=1}^{q} x_i^2$$

Where:

He = expected heterozigosity;

xi = allel frequency -i; and

q = number of allels

1. Anova one Way

$$Yik = \mu + \alpha_i + e_{ik}$$

Where :

Yik = number of observation (size birth and body weight)

μ = average of growth traits

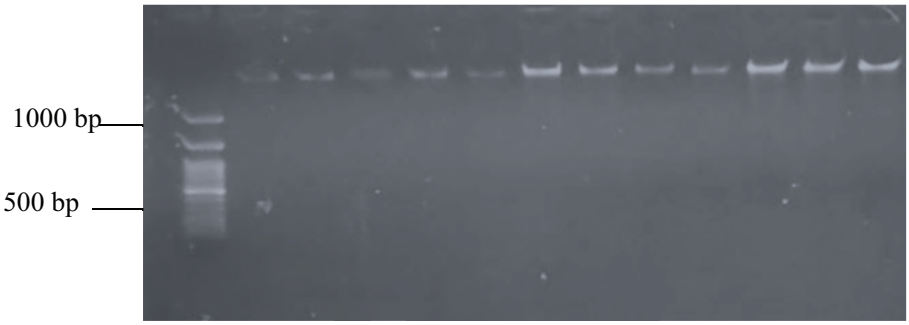
α_i = effect of treatment - i (genotipe)

e_{ik} = random errors

3. Results and Discussion

3.1. DNA Isolation

Twenty-four genomic DNA samples were isolated using kit DNA isolation. The quality of the PCR product were visualized by electrophoresis using 1,5% agarose. The result of electrophoresis can be seen in Figure 1.



Lane M = 1000 bp DNA *ladder* ; lane S 1-12 = DNA Sample

Figure 1: DNA isolation product.

Based on the visualization in the UV transilluminator, that the DNA bands were contained thicker and brighter band and some band were thin and faint. This result showed that the bright and thick of the band indicates the concentration of DNA were higher (6). The DNA fragments produced above 1000 bp which indicate that the DNA product were obtained whole genom. Then, The DNA isolation products analyzed by PCR method.

3.2. DNA Amplification

The amplification DNA sequence of the MC4R gene used PCR technique. The primer that used for PCR amplification is forward 5'-GTC GGG CGT CTT GTT CAT C-3' and reverse 5'-GCT TGT GTT TAG CAT CGC GT-3' with 493 basepair [4]. PCR products were visualized by UV transilluminator and were documented by digital camera (fig. 2)

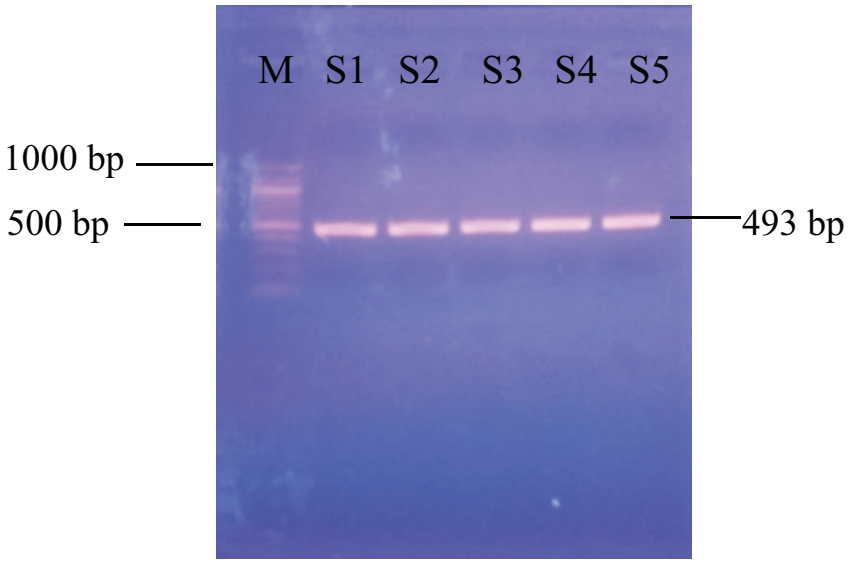


Figure 2: Visualization of PCR product.

3.3. Visualization of PCR product (Fig. 2)

PCR product was visualized and showed in Fig. 2. It has thick, clear, and bright band. PCR product has 493 basepair. It showed that the DNA band were below 500 basepair. That PCR product was indicated similar to expected size 493 basepair. PCR product was sequencing to find the position of SNP and to determine the genotypes of beef cattles.

3.4. SNP Identifiation and Genotyping

SNP identification in targeted product was conducted by sequencing method in the Laboratorium Penelitian dan Pengujian Terpadu Univesitas Gadjah Mada (LPPT UGM). The results of sequencing were performed by electrophoregram, analyzed by Bioedit program to determine the position of SNP in the targeted sequence and to identification based on the peak which indicated the heterozygot genotypes.

Allignment by ClustalW in the accesoris tab on BioEdit. The result of identification and the result of allignment can be seen in fig 3.

The identification of SNP has three genotype CC, CG, and GG. These results are in accordance with previous study [5] and [6]. The SNP were in exon position which are indicated that the SNP can be affect to protein expression. The result of sequencing was registered to genbank database in National Center of Biotechnology (NCBI) with accession number MN692245 and MN692247. According to [7] that the SNP in the

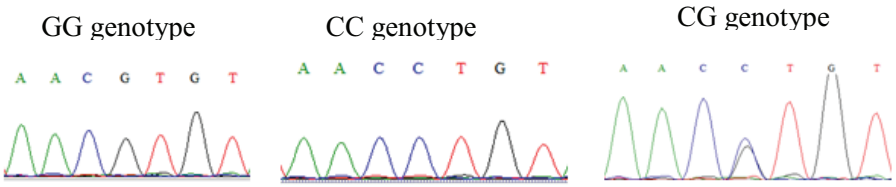


Figure 3: Conformation of SNP 1133 C>G from *MC4R* gene based on electropherogram using BioEdit program.

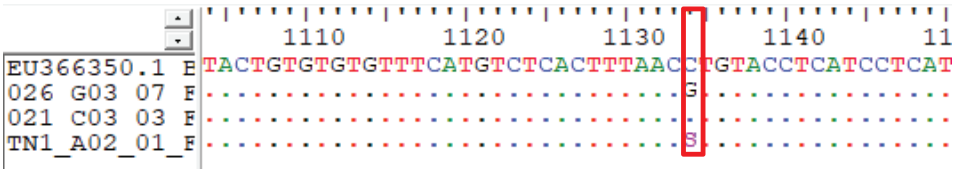


Figure 4: Allignment of SNP 1133 C>G using allignment method.

exon position indicated acid amino expression. It can be affect to protein express in individual fenotipe.

3.5. Analysis of amino acids changed

The Position of SNP 1133 C>G were in exon or CDS indicated that the nucleotides can be changed to protein. The exon in *MC4R* gene started in 278 until 1276 with 999 basepair of nucleotides. The total of amino acids are 333 proteins started from ATG to stop codon TAA. Coding DNA sequence (CDS) is part of DNA sequence organized by exon and encode particular the proteins [8]

The SNP 1133 C>G from the *MC4R* in the first exon was analyzed the amino acids changed by Expassy program. The program can be accessed in https://web.expasy.org/translate/. The fenotypes were not affected by missense mutations. The result of amino acids changed can be seen in fig 5. and Table 1.

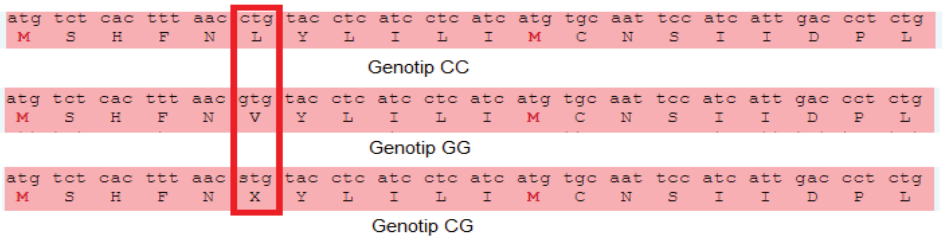


Figure 5: Analysis of amino acids changed in SNP 1133 C>G by *Expassy*.

The SNP 1133 changed amino acids Leucine (L) to Valine (V). It is a point mutation (a missense mutation). This mutation results in fenotipe expression of affected on growth.

Table 1: Amino Acids Mutation Analysis in SNP 1133 C>G.

SNP	Codon	Amino Acid	Mutation
1133 C>G	CTG SGC GTG	*Leucine (L) Leucine/Valine (L/V) Valine (V)*	*Missense*

Note: S (Strong) is G/C

The SNPs being potentially valuable as genetic markers. This result are similar to those reported in study [5].

3.6. Allele and Genotype Frequencies and Hardy Weinberg Test

The result of product PCR has 493 basepair in BBBX-BX (G2), BBBX-WagyuX (G2), WagyuX-BBBX (G2), and *Wagyu*X-BX (G2). The SNP 1133 C>G has two alleles C and G which is CC, CG and GG genotype (Table 2.). Allele and Genotype frequencies can be seen in Table 2.

Table 2: Allele and Genotype Frequencies the SNP 1133 C>G for *MC4R* gene X^2 table *Person's Chi-square test* 0,05;2 = 5,99.

Breed	N	Genotype Frequency			Allele Frequency		X^2(HWE)
		CC	CG	GG	C	G	
BBBX-BX (G2)	5	0,4 (2)	0,4 (2)	0,2 (1)	0,6	0,4	0,1388
BBBX-*Wagyu*X (G2)	2	0 (0)	0,5 (1)	0,5 (1)	0,25	0,75	0,2222
*Wagyu*X-BBBX (G2)	3	0 (0)	1 (3)	0 (0)	0,5	0,5	3
*Wagyu*X-BX (G2)	14	0,21 (3)	0,28 (4)	0,5 (7)	0,35	0,65	1,9980
Second Generation	24	0,21 (5)	0,42 (10)	0,38 (9)	0,42	0,58	0,491

The results showed that the second-generation of crossbred beef cattle had the highest CG genotype (0,42%) and lowest CC genotype (0,21%). These results are in accordance with previous studies [9][10] stated that the *Qinchuan* cattle at *MC4R* had the highest CG genotype frequency (0,54) followed by CC genotype (0,28) and GG genotype (0,18), while genotype frequencies of Korean cattle are 0,42, 0,19, and 0,39 [4]. According to [6] that the presence of high heterozygous genotype is due to mating with outside male cattle. This is in accordance with the fact that Belgian blue and Wagyu males are imported cattle.

Based on the allele frequencies were estimated, it can be known that the population of the *Belgian Blue, Wagyu,* and *Brahman Cross* from second-generation crossbreed are polymorphism. Based on X^2 test which was viewed from each breed, the X^2 value from

Belgian Blue Cross-Brahman Cross (BBBX-BX) (G2) was 0,1388; *Belgian Blue Cross-Wagyu Cross* (BBBX-WX) (G2) was 0,2222; *Wagyu Cross-Belgian Blue Cross* (WX-BBBX) (G2) was 3; *Wagyu Cross-Brahman Cross* (WX-BX) (G2) was 1,9980, whereas viewed from the second generation of crossbred *Belgian Blue, Wagyu*, dan *Brahman Cross* was 0,491. All of the obtained results are smaller than X^2 table result (5,99) indicated that the second generation of crossbred *Belgian Blue, Wagyu*, and *Brahman Cross* are in *Hardy-Weinberg* equilibrium state. The equilibrium state indicates that the allele and genotypes frequencies in cattle population are constant from generation to generation while the disturbing factors are absent.

3.7. Heterozygosity

Heterozygosity estimate or observed heterozygosity average (H_o) and expected heterozygosity (He) aimed to find out the genetic variability information in a population which is used as a cattle selection program so that it can be used for next generation [11].

Inbreeding cattle or known as heterosis. This occurs in a cross characterized by the high performance of the crossbred cattle which exceeds the average performance of the two parental breeds [12]. High heterosis coefficient is related with the results of crossbred between two different breed and distant biodiversity [12].

Accordsing to [13] stated that the Heterozygosity is the most accurate indicator to determine genetic variation from the average percentage of heterozygous loci of each individual or the average percentage of heterozygous in a population. The heterozygosity average is influenced by the size of population and the number of loci. 30 samples (minimum sample size) are required to estimate the heterozygosity average.

Table 3: Heterozigsity Estimate.

Breed	N	Ho	He
BBBX-BX (G2)	5	0,4	0,48
BBBX-*Wagyu*X (G2)	2	0,5	0,375
*Wagyu*X-BBBX (G2)	3	1	0,5
*Wagyu*X-BX (G2)	14	0,285	0,455
Second generation	24	0,416	0,487

Observed Heterozygosity (H_o) in the second generation of crossbred *Belgian Blue, Wagyu*, and *Brahman Cross* is less than 0,5 (50%) or 0,416 which indicates that the variation of *MC4R* gene at the population is low [14] (Javanmard *et al.,* 2005). When

viewed from each cattle order, observed heterozygosity in BBBX-BX (G2) and *WagyuX*-BX (G2) have observed heterozygosity under 0,5 , while the value for BBBX-*WagyuX* was 0,5 and *WagyuX*-BBBX was 1. Measured population has not genetic relationship or genetic linkage if the heterozygosity value was in 1 [13].

The observed heterozygosity (H_o) in the second generation of crossbred *Belgian Blue, Wagyu*, and *Brahman Cross* are 0,416 lower than expected heterozygosity which are 0,4875. It indicates that in the population occurs endogamy degree due to intensive selection process [15].

This resulted was similar to field situation because there was selection of bulls. The observed heterozigosity (Ho) in BBBX-BX (G2) and WagyuX-BX (G2) has under value from expected heterozigosity (He), while in WagyuX-BBBX (G2) and BBBX-WagyuX (G2) has higher observed heterozigosity (Ho) than expected heterozigosity (He).

3.8. Association analysis

The result of beef cattle genotypes were assosiated to body weigt and size birth. Which used the Shoulder height, body length, and chest circumference. The body size assosiation based on SNP 1133 C>G of the *MC4R* gene can be seen in table 4.

TABLE 4: Assosiation genotype analysis the SNP 1133 C>G of MC4R gene with body weight and size.

Breed	Variable	N	Body Size			*p-value*
			CC (cm)	CG (cm)	GG (cm)	
BBBX-BX (G2)	BW BL SH CC	5	n=2 15,67±0,46 50,40±0,85 71,58±3,65 64,21±0,29	n=2 22,73±0,00 58,68±0,00 77,37±0,00 73,35±0,00	n=1 22,73 58,68 77,37 73,35	0.004 0.009 0.249 0.001
WagyuX-BX (G2)	BW BL SH CC	14	n=3 37,61±3,99 57,18±1,90 69,52±2,55^b 77,92±2,26	n=4 39,84±0,00 58,54±0,00 66,58±0,00^a 78,25±0,00	n=7 30,37±7,82 55,58±2,18 69,72±1,80^b 75,62±2,92	0.062 0.066 0.035 0.186
Second Generation	BW BL SH CC	24	n=5 28,84±12,34 54,47±3,97 70,35±2,80 72,43±7,67	n=10 28,49±9,98 57,62±3,85 71,12±5,13 73,94±4,57	n=9 28,92±7,37 57,17±4,19 71,18±3,33 75,39±2,63	0.995 0.355 0.928 0.540

BW: Body Weight; BL: Body Length; SH: Shoulder Height; CC: Chest Circumference.

Genotype assosiation was analysed using the data of weight and size body at birth from twenty-four second generation of *Belgian Blue, Wagyu,* and *Brahman Cross* (5 head of BBBX-BX, 2 head of BBBX-*WagyuX*, 3 head of *WagyuX*-BBBX, 14 head of *WagyuX*-BX) with three types of genotypes are CC, CG and GG. The Analysis correlation

genotypes between weight and size body were not significant. It showed that SNP 1133 C>G of *MC4R* gene has not effect of weight, length, shoulder height and chest circumference at birth. In accordance with previous studies [9]. The un-significant result showed that the SNP 1133 C>G of *MC4R* gene cannot be used as the selection tools or markers in second generation of *Belgian Blue*, *Wagyu,* dan *Brahman Cross.*

If analyzed by the group, the BBBX-*Wagyu*X can not be analyzed because the total of samples were not enough, *Wagyu*X-BBBX can not be analyzed because it has only one of genotype CG. The BBBX-BX (GG) showed that the *MC4R* gene has signifficant effect to body weight, body length and shoulder height, but it can not be tested because the GG genotype has only one sample. The *Wagyu*X-BX has signifficant effect on shoulder height at birth. The tall shoulder expressed by CC and GG genotypes, whereas the short shoulder expressed by CG genotype because the SNP 1133 C>G of *MC4R* gene can be changed the amino acids from leusine to valine. The changed of protein expression were caused by amino acids changed and it related to fenotype expression.

The earlier study about *MC4R* gene of beef cattle showed that the GG genotype of PO cattle has signifficant effect on body length at birth [9] chest circumference and shoulder height [16]. In Madura cattle showed that the GG genotype has effect on higher shoulder height in yearling than CC and CG genotypes [6]. The similar result showed in adult of *Hanwoo* cattle (GG) has higher back fat thickness than others genotypes [4].

4. Conclusions

The *MC4R* gene in the second generation of Belgian Blue, Wagyu and Brahman Cross has one SNP in position 1133 C>G. The SNP C>G were changed amino acids of leucine to valine. The genotypes in SNP 1133 C>G was CC, CG, and GG. The genotype frequency of CG is higher than CC and GG. The allele frequency of G higher than C. The population was in genetic balance. The observed heterozigosity are lower than the expected heterozigosity. *MC4R* gene has no effected on body weight, body length, shoulder length, and chest circumference at birth. We recommended to expended the research for the yearling and adult cattle so the selection by genetic marker can be used.

5. Acknowledgement

This research was supported by Research Grant Rekognisi Tugas Akhir (RTA) 2020 contract no. 2488/UN1.P.III/DIT-LIT/PT/2020 and the sample was supported by PT. Widodo Makmur Perkasa, Klaten, Indonesia. The author thanks to Riyan Nugroho Aji, Laurentius

Lintang Adi, Iwan Setiawan, Aditya Pamungkas for helping to collect the samples and also to Retno Setyawati for helping in laboratory analysis

References

[1] Statistik BP. 2017. *Kajian Konsumsi Bahan Pokok Tahun 2017.*

[2] Putra WPB. 2017. Teknik Persilangan Pada Sapi Belgian Blue (Bos Taurus) Untuk Menghasilkan Bibit Unggul Di Indonesia. *BioTrends* **8 :** 1–4.

[3] Ratna MP. 2018. Penerimaan wagyu sebagai shoku bunka jepang di Indonesia. *Humanika* **24:** 110–7.

[4] Kong HS, J Seong, DS Suh, KD Park and HK Lee. 2012. Identification and analysis of MC4R polymorphisms and their association with economic traits of Korean cattle (Hanwoo). *Mol. Biol. Rep.* **39:**3597–601.

[5] Maharani D, A Fathoni, Sumadi, T Hartatik and M Khusnudin. 2018. Identification of MC4R gene and its association with body weight and body size in Kebumen Ongole Grade cattle. *J. Indones. Trop. Anim. Agric.* **43:**87–93.

[6] Prihandini PW. 2018. *Analisis potensi genetik sapi Madura di Kabupaten Pamekasan dan Loka Penelitian Sapi Potong Jawa Timur.* Universitas Gadjah Mada.

[7] Latifah. 2019. *Deteksi Single Nucleotide Polymorphism dan Hubungan Polymorphism Gen Melanocortin 4 Receptor Terhadap Sifat Pertumbuhan dan Feed Intake pada Kambing Bligon*. Universitas Gadjah Mada.

[8] Kurniawati S and NS Hartati. 2018. Optimasi suhu annealing primer degenerate untuk mengamplifikasi fragmen gen arginine decarboxylase (adc) genom ubi kayu lokal maluku tenggara. *J. ILMU DASAR* **19:**135–142.

[9] Fathoni A. 2016. *Estimasi parameter genetik sifat pertumbuhan dan identifikasi gen MC4R sapi peranakan ongole di Kabupaten Kebumen Jawa Tengah*. Universitas Gadjah Mada.

[10] Liu W B, SR Chen, J X Zheng, L J Qu, G Y Xu and N Yang. 2010. Developmental phenotypic-genotypic associations of tyrosinase and melanocortin 1 receptor genes with changing profiles in chicken plumage pigmentation. *Poult. Sci.* **89**: 1110–1114.

[11] Marson E P, JBS Ferraz, FV Meirelles, JCC Balieiro, JP Eler, LGG Figueiredo and GB Mourão. 2005. Genetic characterization of European-Zebu composite bovine using RFLP markers. *Genet. Mol. Res.* **4:**496–505.

[12] Harjosubroto W. 1994. *Aplikasi Pemuliaan Ternak Di Lapangan* . Gasino, Yogyakarta.

[13] Nei M. 1987. *Molecular evolutionary genetics.* Columbia University Press, New York.

[14] Javanmard A, MHB Asadzadeh, and Tavakolian J 2005 The allele and genotype frequencies of bovine pituitary-specific transcription factor and leptin genes in Iranian cattle and buffalo populations using PCR-RFLP *Iran. J. Biotechnol.* **3**:104–8

[15] Machado M A, Schuster I, Martinez M L and Campos A L 2003 Genetic Diversity of Four Cattle Breeds Using Microsatellite Markers. *Rev. Bras. Zootec.* **32**:93–98.

[16] Nasuha. 2020. *Estimasi Output Sapi Potong Dan Identifikasi Gen MC4R Sapi Peranakan Ongole Di Kabupaten Tuban Provinsi Jawa Timur.* Universitas Gadjah Mada, Yogyakarta.