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Melanocortin-4 Receptor (MC4R) gene polymorphism and its effect on growth traits in Madura cattle

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ABSTRAK

Gen Melanocortin-4 Receptor (MC4R) berfungsi sebagai regulator nafsu makan dan kontrol keseimbangan energi. Tujuan dari penelitian ini adalah untuk mengidentifikasi single nucleotide polymorphisms (SNPs) pada gen MC4R dan asosiasinya terhadap sifat pertumbuhan. Materi yang digunakan pada penelitian ini adalah 198 ekor sapi Madura yang dipelihara di Kabupaten Pamekasan. Sepasang primer, berupa PF:5'-GTCGGGCGTCTTGTTCATC-3' dan PR:5'-GCTTGTGTTTAGCATCGCGT-3 digunakan untuk amplifikasi target gen sepanjang 493 bp. Metode *sequencing* digunakaan untuk mendeteksi SNP pada gen MC4R dan metode PCR-RFLP digunakan untuk identifikasi genotip. Hasil penelitian didapatkan dua SNP yaitu SNP g.1133C>G dan g.1108C>T. Namun demikian hanya SNP g.1133 C>G yang dianalisis dan SNP tersebut mempunyai pengaruh yang sangat signifikan (P<0,05) terhadap tinggi gumba pada umur setahun. Sapi Madura dengan genotip GG (110,35 ± 6,40 cm) tinggi gumbanya dideteksi paling tinggi, diikuti dengan genotipe CG (105,96 ± 6,23 cm) dan genotip CC (102,00 ± 8,00 cm) paling rendah. SNP g.1133 C>G gen MC4R terdeteksi merubah asam amino dari valin menjadi leusin. Berdasarkan hasil penelitian ini, dapat disimpulan bahwa SNP g.1133C>G pada gen MC4R dapat digunakan sebagai alat seleksi untuk sapi Madura di Kabupaten Pamekasan berdasarkan sifat tinggi gumba.

Kata kunci: gen MC4R, marker SNP, sifat pertumbuhan, dan sapi Madura.

ABSTRACT

Melanocortin-4 receptor (MC4R) gene has an important role in the regulation of feed intake and energy balance control. The objective of this study was to identify the single nucleotide polymorphisms (SNPs) of MC4R gene and their association with growth traits in Madura cattle. A total of 198 calves were used in this study. Forward primer: 5'-GTCGGGCGTCTTGTTCATC-3'and reverse primer: 5'-GCTTGTGTTTAGCATCGCGT-3' were used to amplify approximately 493 bp of MC4R gene. The results showed that two SNPs, g.1133C>G and g.1108C>T were identified by direct sequencing. The PCR-RFLP method was performed to genotype all individuals studied based on SNP g.1133C>G, and its SNP was significantly associated with shoulder height (SH) at yearling age (P<0.05). Animals with GG genotype had a higher SH (110.35±6.40cm) than those with CC (102.00±8.00 cm) and CG genotype (105.96±6.23 cm). The SNP g.1133 C>G changed amino acid from valine to leucine. In conclusion, the SNP g.1133C>G of the MC4R gene may be used as a marker-assisted selection for SH trait in Madura

cattle.

Keywords: melanocortin 4 receptor gene, single nucleotide polymorphisms, growth trait, and Madura cattle

INTRODUCTION

Madura cattle is native cattle originated from the Madura Island. It is commonly raised for beef and draught animal, as well as racing and beauty contest. Historically, Madura cattle is a crossbred between *Bos Javanicus* and *Bos Indicus* (Nijman *et al*., 2003; Kusdiantoro *et al*., 2009). It has reddish brown hair color with some white color on the back and rump (non-specific). In addition, it has small and upward horns in both cows and bulls.

Current strategies for beef production in Indonesia, the focus is the appropriate utilization of local cattle breeds. Madura cattle as one of the local cattle breed in Indonesia should be preserved its existence and increased their production to meet the need for beef production. Madura cattle have been reported to have good marbling and rapid growth rate compared to Aceh and Pesisir cattles (Bamualim *et al*., 2006; Kutsiyah, 2012).

It is well known that variation in growth traits is determined by genetic and environmental factors. Growth is an important economic trait for cattle genetic improvement. Along with conventional breeding methods, the use of DNA marker based-selection in animal genetic improvement has received much attention to date. It is therefore necessary to determine and assess candidate genes that contribute to the growth traits of cattle.

Melanocortin-4 receptor (MC4R), belonging to the super family of seven transmembrane G protein-coupled receptors, is involved in appetite regulation and energy balance (Zhang *et al*., 2009; Razquin *et al*., 2011). The MC4R has an important role in controlling the effect of leptin on the hypothalamic control of food intake and body weight (Lisyova et al., 2014). Bovine MC4R gene has been isolated and mapped to BTA 24q27 by radiation hybrid mapping. Several SNPs in the exonic region of bovine MC4R gene have been detected by various methods and are associated with growth traits, fatness, carcass weight and meat quality in cattle (Kim *et al*., 2000; Haegeman *et al*., 2001; Bruun *et al*., 2006; Zhang *et al*. 2009; Liu *et al*. 2010; Mc Lean and Schmutz, 2011; Maharani *et al*. 2018). However, there have been no reports on SNPs in the MC4R gene and their effect on economic traits in Madura cattle. Therefore, the objective of this study was to identify the single nucleotide polymorphisms (SNPs) of the MC4R gene and their association with growth traits in Madura cattle.

MATERIALS AND METHODS

Animals and Samples

A total of 198 calves of Madura cattle were used in this study. The cattle were reared under the same condition in a tradisional management by local farmers. Data on growth traits including body weight (BW), chest circumference (CC), body length (BL) and shoulder height (SH) were measured for association analysis. Growth traits were measured based on National Standardization Agency of Indonesia (BSN, 2015). The whole blood samples were collected for genomic DNA isolation.

DNA Extraction and PCR Amplification

Genomic DNA samples were extracted from whole blood samples using GeNetBio DNA Exstraction Kit (GeNetBio, Daejeon, Korea). Detail information, including primer set, annealing temperature and restriction enzyme is presented in Table 1.

Polymerase chain reaction (PCR) was performed in a 25 µL volume containing 2 µL genomic DNA, 9.5 µL of double-distilled water, 0.5 µL of each primer (forward and reverse), and 12.5 µL of 2x My Taq HS Red Mix gSYNCTMPCR Kit (Bioline, London). The PCR conditions were 94^{o}C for 5 min and 35 cycles of 30 s at 94^{o}C, 30 s at 58^{o}C for annealing, 30 s at 72^{o}C for extention, and a final step of 10 min at 72^{o}C using a thermal cycler (Infinigen, USA). The PCR products were visualized in 1.5% standard agarose gels stained with ethidium bromide (Figure 1). The PCR fragments were purified and sequenced by 1st BASE DNA Sequencing Division (www.base-asia.com). The DNA sequences were analyzed with the BioEdit program ver. 7.00 (Tom Hall, Ibis Therapeutics, USA) and the single nucleotide polymorphisms (SNPs) were confirmed based on the electrophoregram results.

Table 1. Primers for PCR Amplification and Restriction Enzyme Information for Genotyping of MC4R Gene

Gene	GenBank Accession No.	Sequence (5' to 3')	Annealing Temperature (^{o}C)	PCR Product Size (bp)	Restriction Enzyme
MC4R	EU366350.1	F : GTCGGGCGTCTTGTTCATC R : GCTTGTGTTTAGCATCGCGT	58	493	*HpyCH4*IV

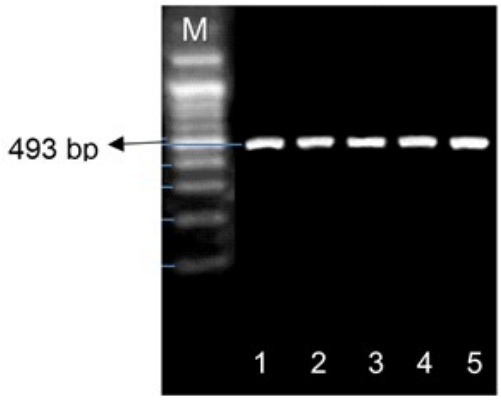

Figure 1. Agarose Gel Electrophoresis to Detect PCR Amplified Products (493 bp) of MC4R Gene in Madura Cattle Population M: 100 - 1500 bp Size Marker

Genotyping Animals

The SNP located at g.1133C>G of MC4R gene (GenBank accession no. EU366350.1) within exon 1 was used for animal genotyping using PCR-RFLP method. The PCR product was digested by *HpyCH4*IV restriction enzyme at 37^{o}C for at least 5 h. The digested DNA fragments were separated on 3% agarose gel by electrophoresis with 1x TBE buffer. The gel was stained with ethidium bromide and the fragments were visualized using a UV transilluminator (UVP TEM-40, USA).

Statistical Analysis

To identify the distribution of MC4R gene in the population, allele and genotype frequencies were calculated by simple allele counting method. Hardy-Weinberg equilibrium (HWE) in examined population was tested by comparing expected and observed genotype frequencies using a Chi-square test. The effects of MC4R genotypes on growth traits were tested using the one way-ANOVA in the SPSS ver. 17.0 program (SPSS, USA). Significant differences among mean values of different genotypes were calculated using Duncan's multiple range test. The following model was used to test the association of the genotype and growth traits:

$$Y_{ij} = \mu + \alpha i + \varepsilon ij$$

Where, Y is the phenotypic data (growth trait) of sample i, μ is the overall mean, αi is the genotype effect of sample i and ε is a random error.

RESULTS

Growth Trait Performance

Descriptive statistics for growth traits, including BW, CC, BL and SH in different ages (birth, weaning and yearling) are presented in Table 2.

SNP identification and genotyping

In this study, two SNPs (g. 1108 C>T and g.1133 C>G) were identified in exon 1 of MC4R gene (Figure 2). SNP g.1133 C>G was used to genotype all investigated animals. An approximately 493 bp of MC4R gene was digested using *HpyCH4*IV restriction enzyme. Based on PCR-RFLP analysis, animals with a single fragment (493 bp) were defined as homozygous CC. Animals were determined as homozygous GG when 2 fragments, 173 and 318 bp were produced. In additon, CG heterozygous animals were defined as they have 3 fragments, 173, 318 and 493 bp (Figure 3).

Allelic and genotypic frequencies are presented in Table 3. In this study, the frequency of G allele was higher than that of C allele. GG genotype had a higher frequency than CC

genotype. Person's Chi-square test indicated that the genotype distribution of MC4R gene in the studied population was in accordance to HWE.

Association of Gene-specific SNP Marker with Growth Traits

Effects of genotype on growth traits showed that SNP g.1133 C>G was significantly associated ($P<0.05$) with SH at yearling. Animals with GG genotype had a higher SH (110.35±6.40 cm) than those with CC (102.00 ± 8.00 cm) and CG genotype (105.96±6.23 cm) as presented in Table 4. No significant association was detected between SNP g.1133 C>G and BW, CC and BL. ADG was different as showed by different genotype at weaning weight and yealing weight (Table 5).

Table 2. Descriptive Statistics of Growth Traits in Madura Cattle Population (n=198)

Traits	Mean	SDs
Birth Age		
BW(kg)	19.30	2.35
CC (cm)	56.35	4.71
BL (cm)	50.74	7.02
SH (cm)	62.69	4.55
Weaning Age		
BW(kg)	87.89	14.66
CC (cm)	96.19	11.26
BL (cm)	82.65	11.53
SH (cm)	89.46	9.05
Yearling Age		
BW(kg)	158.35	35.02
CC (cm)	124.07	9.60
BL (cm)	106.95	9.48
SH (cm)	108.33	6.80

BW: Body weight; CC: Chest circumference; BL: Body length; and SH: Shoulder height; SDs: standard deviations

DISCUSSION

In this study, in order to determine whether there was a relationship between MC4R polymorphism and growth traits, an exonic region, approximately 493 bp of MC4R gene was tested as target sequences gene for Madura cattle. The results showed that the targeted PCR product of the bovine MC4R gene was successfully amplified in all animals studied (Table 1). A 100 bp-DNA ladder was used to ensure the size of PCR product. As shown in Figure 1, the PCR products were in good specifity and fell across the predicted size on a 1.5% gel electrophoresis. In order to genotype all investigated animals, PCR products were firstly subjected to DNA pool sequencing. Two SNPs, g.1133C>G and g.1108C>T were found (Figure 2). SNP g.1133C>G was further used to genotype all individuals investigated by PCR-RFLP method using *HpyCH4*IV restriction enzyme.

The PCR-RFLP analysis showed two alleles, namely C and G were identified, with frequencies of 0.23 and 0.77, respectively (Table 3). Furthermore, genotype frequencies of CC, CG and GG were 0.05, 0.36 and 0.59, respectively. Allele and genotype frequencies resulted in this study was comparable with those reported by Liu *et al*. (2010), Seong *et al*. (2012) and Du *et al*. (2013). In a previous study, a higher CG genotype frequency (0.54) was observed than CC (0.28) and GG (0.18) genotypes frequencies in Qinchuan cattle (Liu *et al*., 2010). Moreover, Seong *et al.* (2012) reported genotype the frequencies of 0.42 for CG, 0.19 for CC and 0.39 for GG genotypes in

Table 3. Allele and Genotype Frequencies for g.1133C>G SNP of the MC4R Gene in Madura Cattle

SNP Marker	Number of Animal	Genotype Frequency			Allele Frequency		He	HWE	
		CC	CG	GG	C	G		X^2	P-value
g.1133C>T	198	0.05	0.36	0.59	0.23	0.77	0.354	0.513	0.85

He:heterozygosity; HWE:Hardy-Weinberg equilibrium

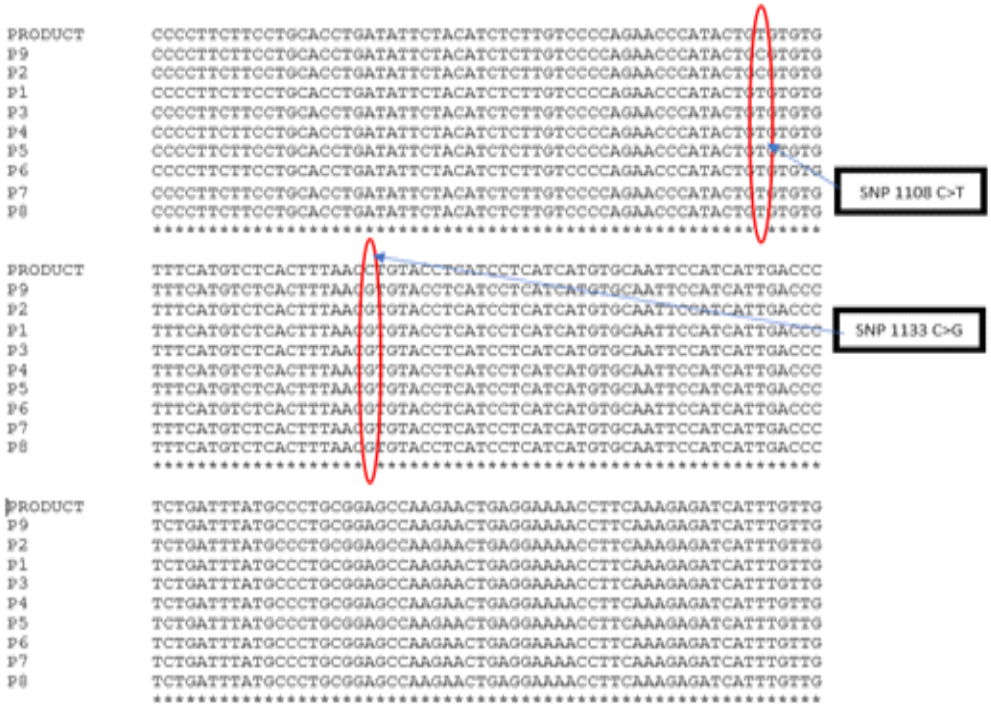

Figure 2. Detection of the SNP g. 1108 C>T and g.1133 C>G based on Sample Sequence Alignment using Clustal W Alignment Method

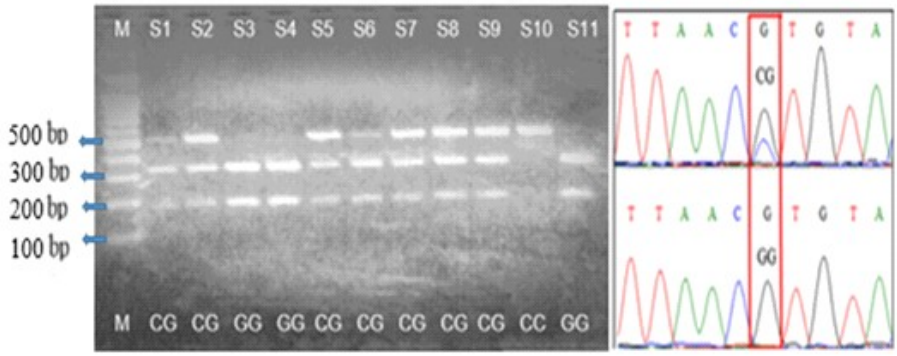

Figure 3. PCR-RFLP partern and parcial sequence chromatograms of CG (heterozygotes) and GG (homozygotes) genotypes of the MC4R gene in Madura cattle population. Lines 3, 4 and 11 represent GG genotype. Lines 1, 2, 5, 6, 7, 8, and 9 represent CG genotype. Lines 10 represent CC genotype. M: 100 - 1500 bp size marker

Korean cattle. In this study, frequency of GG genotype was the highest in Madura cattle population, which was similiar with reports in PO Kebumen cattle (Maharani *et al.*, 2018).

The bovine MC4R genotypes had only a significant effect on shoulder height at yearling age. SNP g.1133 C>G was identified to change amino acid from valine to leucine (Figure 4). A change of amino acid from valine (Val) to methionine (Met) was reported in the MC4R gene in Qinchuan cattle (Seong *et al*., 2012). In pig, identified SNP of MC4R gene changed amino acid from leucine to valine, which further affected growth traits and meat quality (Huang *et al*, 2010). Any mutation in the exonic region of a gene can change the gene expression, which in

Table 4. Association Analysis between the Different g.1133C>G of MC4R Gene and Growth traits in Madura Cattle Population

Trait	Genotype			P-value
	CC (n=5)	CG (n=82)	GG (n=111)	
Birth Age				
BW (kg)	19.50± 0.71	18.50± 3.39	19.60± 2.03	0.640
CC (cm)	59.00± 2.83	53.17± 3.31	57.27± 4.91	0.138
BL (cm)	55.00± 7.07	47.50± 6.19	51.47± 7.25	0.353
SH (cm)	65.00± 4.24	61.50± 5.47	62.87± 4.37	0.643
Weaning Age				
BW(kg)	82.74± 6.74	84.68±13.85	90.58±15.02	0.069
CC (cm)	100.00± 2.83	95.97± 7.93	96.25±13.48	0.883
BL (cm)	87.50± 0.71	83.09±11.13	82.17±12.03	0.759
SH (cm)	90.00± 5.66	87.46± 9.49	91.03± 8.53	0.086
Yearling Age				
BW(kg)	178.67±37.00	147.00±22.96	164.20±39.72	0.087
CC (cm)	127.67± 9.50	124.21±10.20	123.71± 9.41	0.627
BL (cm)	108.33±16.07	108.25±10.52	106.00± 8.37	0.913
SH (cm)	102.00± 8.00[a]	105.96± 6.23[ab]	110.35± 6.40[b]	0.008*

BW: Body weight; CC: Chest circumference; BL: Body length; SH: Shoulder height; and n: number of animals

*Effect was significant at P<0.05;

[a'b] Within a row, means with different superscript letter indicates different (P<0.05).

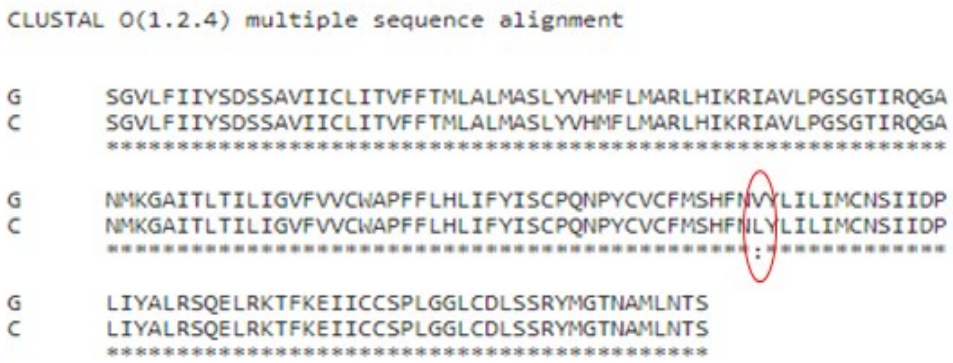

Figure 4. The Amino Acid Change in SNP g.1133 C>G (missense mutation of Leusin to Valin)

turn a different protein with different characterizes is created as a result of amino acids change.

The MC4R gene is responsible for the regulation of feeding and satiety, which occurs in the hypothalamus. Feeding signals (AgRP) released from agouti-related protein neurons and satiety signals (α-MSH) released from pro-opiomelanocortin neurons are ligands for the MC4R gene (Buch *et al.,* 2009). Because it receives both feeding and satiety signals, the MC4R critically regulates feeding behavior and

Table 5. Association Analysis between the SNP g.1133C>G of MC4R Gene and Feed Intake as well as ADG in Madura Cattle Population

Traits	Genotype			P-value
	CC (n)	CG (n)	GG (n)	
ADG weaning age (kg/day)	0.28± 0 (1)	0.21±0.13 (14)	0.20±0.09 (26)	0.735
ADG yearling age (kg/day)	0.22±0.06 (2)	0.36±0.27 (25)	0.32±0.14 (33)	0.537
Feed intake (kg/day)	29.81± 0 (1)	34.08±3.34 (8)	33.55±4.19 (21)	0.606

ADG: Average Daily Gain; n: Number of animal

energy homeostasis. The amino acid change in MC4R gene may affect the function of a particular protein. It is possible that variation in amino acids may cause a variation in the function of MC4R gene in controlling feed intake and body weight, as well as the shoulder height in cattle.

Although numerical differences were observed in the growth traits among different genotypes, the effects of MC4R genotypes were not statistically significant on BW, CC and BL at birth, weaning and yearling age, as well as SH at birth and weaning age. Many published studies demonstrated that the bovine MC4R genotypes had a significant association with growth traits, meat quality and carcass composition (Kim *et al*., 2000; Haegeman *et al*., 2001; Bruun *et al*., 2006; Zhang *et al*., 2009; Liu *et al*., 2010; Maharani *et al*., 2018; Huang *et al*., 2010). Dvorakova *et al*. (2011) found that a missense mutation Asp298Asn in swine MC4R gene did not affect the growth traits and feed intake, but it significantly effect on fatness traits. It is clear that the effect of the bovine MC4R genotype on growth traits resultes various outcomes. In the present study, only SH at yearling age was significantly affected by the MC4R genotypes, while the remaining traits were not significantly affected.

Molecular mechanism of the MC4R gene has been identified in both animals and humans. Its expression is varying since various SNPs are presence in the MC4R gene for each individual. Shoulder height is important trait in the selection program of Madura cattle, as it is a considerable trait in selecting Madura cattle for *Sonok* contest. Since growth traits are controlled by multiple genes, identifying for several SNPs in the MC4R genes and understanding the interaction between several SNPs is recommended for the best explanation regarding the molecular mechanism in growth traits.

CONCLUSION

The SNP g.1133C>G of MC4R gene had a significant association with shoulder height at yearling age. Hence, it may be used as marker-assisted selection for SH trait in Madura cattle. Moreover, further attempt should be addressed to investigate the association between several SNPs in the bovine MC4R and growth traits with expanded sample size in order to get clearer explanations.

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