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Research Article

Restriction Mapping of *MC4R* Gene on Bali Cattle (*Bos sondaicus*) as Genetic Marker for Breeding Program in Compared to *Bos taurus* and *Bos indicus*

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ABSTRACT
MC4R is a gene that has potential effects on growth traits such as body weight and feed intake. The usage of single nucleotide polymorphism (SNPs) in *melanocortin-4 receptor* (*MC4R*) as selection markers could help achieve effectiveness in the breeding program. This study aimed to analyze the restriction mapping based on SNPs in the *MC4R* gene for Bali cattle (*Bos sondaicus*) compared to various breeds of cattle. Partial *MC4R* gene was amplified using a primer (F: 5'-ACC AAT GTC AGT GAG TCC CC- 3' and R: 5'-CTT CAT GTT GGC GCC CTG-3') with a polymerase chain reaction (PCR) method. Genotype and allele frequencies were calculated using Chi-Square test and analyzed with Hardy–Weinberg law. Restriction enzyme was analyzed using Nebcutter V.2 to see the association between SNPs and the recognition site of restriction enzyme. The result showed four SNPs g.554 T>C, g.634 G>T, g.673 C>T, and g.742 G>A were found in the exon region. SNP g.742 G>A was found as a heterozygote genotype and the rest are SNP g.554 T>C, g.634 G>T, and g.673 C>T were found as homozygote genotypes. All SNPs were synonymous which did not change the amino acid translated. Three restriction enzymes were identified as *MmeI*, *TspRI*, and *BsrI* which attach to SNPs g.554 T>C, g.634 G>T, and g.742 G>A respectively. SNPs found notably g. 742 G>A can be used as genetic markers associated with growth traits for further research on Bali cattle.

INTRODUCTION
Melanocortin receptor is a family of five 7-transmembrane G protein-coupled melanocortin receptors (Yang 2011). It is consists of five subtype genes, those are *MC1R*, *MC2R*, *MC3R*, *MC4R*, and *MC5R* which are located on autosomes. The *MC4R* gene has been studied extensively due to its function in regards to energy control. The association of human *MC4R* to obesity initiated studies on the relationship between fat tissue accumulation in livestock (Switonski et al. 2013).

MC4R gene has been proven to have an important role in regulating feed intake, energy homeostasis, obesity, and controlled eating behaviour (Kurniawati et al. 2021) which plays in sympathetic nerve activity, adrenal and thyroid function, as well as mediates the duty of leptin on energy

homeostasis. A study has been conducted to see the affiliation between polymorphism in the *MC4R* gene and some aspects involved in growth traits such as feed intake, feed conversion ratio, average daily gain, and weaning weight. It shows a significant association between SNP found and the weaning weight, weaning body length, digestibility matter (DM), organic matter (OM), and total digestible nutrient (TDN) of Bligon goats (Latifah et al. 2020).

Single Nucleotide Polymorphism (SNP) is a difference in unit of a base in DNA chain found in unalike individuals. Usage of SNP in order to mark the specific base differences in genes has been used widely to determine gene markers concerning improvement in the livestock selection program. SNP's position could be obtained by comparing with reference and DNA sequencing, while SNP emergence can cause changes in proteins translated (Albakri & Hartatik 2021). SNPs exist throughout the entire genome both within coding regions as well as outside of coding sequences. Those within coding regions are categorized into synonymous SNP and nonsynonymous SNP. Nonsynonymous SNP is a coding-region SNP that alters the transcribed codon such that different amino acid is incorporated. Synonymous SNP within coding regions do not lead to a change in the amino acid incorporated at the site of their occurrence (Hunt et al. 2009).

Restriction enzyme usage for genotyping is a cost-effective method and it is a fundamental way of how PCR-RFLP works. RFLP is a technique in which individual SNPs are differentiated using analysis of patterns derived from cleavage of their amplified DNA. Sample with different nucleotides in the same site of SNP would differ in distance between sites of cleavage of a particular restriction endonuclease which makes the length of fragments produced would differ. Restriction endonuclease is an enzyme that cleaves DNA molecules when specific nucleotide sequences are recognized. Sites of recognition are usually four to six base pairs in length (Chuang et al. 2008). Therefore the aim of this study was to identify SNP found and related to the restriction enzymes based on *MC4R* gene sequences which could be utilized for further research related to a growth trait potential gene on Bali cattle.

MATERIALS AND METHODS

Sample Collection

Blood samples used in this study were obtained using Vacutainer with EDTA as anticoagulant from 10 Bali cattle (*Bos sondaicus*) which collected from BPTU HPT Denpasar with ID number 3B2013-50, 3B2013-61, 3B2013-77, 3B2013-113, 3B2013-126, 3B2013-150, 3B2013-153, 3B2013-159, 3B14-8, and 3B16-3; 3 Belgian Blue cross cattle (*Bos taurus*) Id number 3B630, 3P648, and 3B635; a Brahman cross cattle (*Bos indicus*) Id number 3116; and 2 Wagyu cross cattle (*Bos taurus*) ID number 3P637 and 3W507 respectively through intravenous injection. Belgian Blue cross, Wagyu cross, and Brahman cross blood samples were obtained from PT. Widodo Makmur Perkasa. All blood samples were collected in August 2019. Blood samples were then isolated

and extracted using SYNC™ DNA Extraction Kit (Geneaid, Taiwan). An *MC4R* gene reference was acquired from the NCBI GenBank nucleotide database, with accession numbers EU366351.1.

DNA amplification

PCR was performed on a total of 16 samples using target primers *MC4R*.III (F: 5'ACC AAT GTC AGT GAG TCC C 3'; R: 5'CTT CAT GTT GGC GCC CTG 3') with reagent set up in a 25 µL reaction volume containing 2 µL of DNA with total 20 ng, 0.5 µL both forward and reverse primers with the concentration of 10 pmol/µL, 12.5 µL of PCR kit (KAPA BIOSYSTEMS, USA), and 9.5 µL Double Distilled Water (DDW). The result of PCR was 654 bp in length covering coding section (exon) of the *MC4R* gene in cattle. The procedure was conducted as follows, pre-denaturation phase at 94°C for 3 minutes, denaturation at 94 °C for 30 seconds, annealing at 63 °C for 30 seconds, elongation at 72 °C for 30 seconds, and post elongation at 72 °C for 10 minutes. The PCR processing were repeated for 35 cycles.

DNA sequencing and SNP identification

The 25 µL samples of PCR product and 10 µL of primers forward were sent to LPPT UGM, Yogyakarta, Indonesia for sequencing. Sixteen samples were aligned along with GenBank reference EU366351.1 using Bioedit v.7.2.5 software. Clustal W Multiple alignment was applied to all samples with 1000 bootstrap NJ Tree and Full multiple alignment. The alignment result was then identified as the SNPs emerged. Amino acid changes were identified in sites where SNPs were found.

Genotype and allele analyzing

The electroforegram for each sample was analyzed using Bioedit v.7.2.5 to recognize if SNPs found were include homozygote or heterozygote form of genotype. Electroforegram on each SNP result was identified by its double peak. Allele frequencies were calculated using Chi-square test and reevaluated with Hardy-Weinberg equilibrium law.

Restriction enzyme

Restriction mapping was conducted using the NebCutter V2.0 website. Enzymes used were all available from NEB (New England Biolabs). An enzyme detected around or at the cutting site where SNPs occur was analyzed for its specificity, type of enzyme, and site where cutting occurs. The amount of cutting sites displayed was analyzed with 1, 2, and 3 cutters for samples and GenBank reference respectively.

RESULTS AND DISCUSSION

Results

A total of sixteen samples were compared along with GenBank reference (Accession no. EU366351.1) for alignment sequence analysis of *MC4R* gene

in the whole coding sequence (exon) section. Coding sequence (CDS) of *MC4R* EU366351.1 starts at 278 bp up to 1276 bp with sizes of 999 bp. PCR result using a primer in this study has sizes of 654 bp which can be found in EU366351.1 sequence, located in 350 bp until 1003 bp within an exon. The result showed 4 identified SNPs which are shown in Figure 1 with clear electroforegram interpretation, those are g.554 T>C, g.634 G>T, g. 673 C>T, and g. 742 G>A. All SNPs were found in Bali cattle (*Bos sondaicus*) samples. Out of 4 SNPs found, one was a heterozygote genotype and the rest were homozygote genotypes. At SNP g. 742 G>A which is a heterozygote, two alleles were identified with GG, GA, AA genotypes. The Chi-square analysis as represented in Table 1 shows that SNP g.554 T>C, g.634 G>T, and g. 673 C>T have X^2 count > X^2 table result, which indicates that these SNPs deviate from the Hardy-Weinberg law. Conversely, SNP g. 742 G>A has X^2 count < X^2 table result which indicates that it has fits with the Hardy-Weinberg law. Five genotypes of Bali cattle determined via sequence analysis were submitted to NCBI's GenBank with ID number 2521876 (Genbank access. number OL623708-OL623717).

A study conducted by Albakri and Hartatik (2021) reported that SNPs found in the CDS of the *MC4R* gene were g.316Y>C, g.811G>R, g.1133C>S and, g.1266G>R. A previous study was reported that two SNPs g.1108 C>T and g.1133 C>G in Madura cattle which have significant associate with shoulder height at yearling age (Prihandini et al. 2019). Another research by Maharani et al. (2018) showed SNP g. 1133 C>G found in Kebumen Ongole cattle has affected high birth body length with GG genotype. Meanwhile, research conducted by Fathoni et al. (2020) shows that SNP g. 1133 C>G has no significant association with growth traits in Sumba Ongole cattle. Liu et al. (2009) found in their research that 2 SNPs g.-129

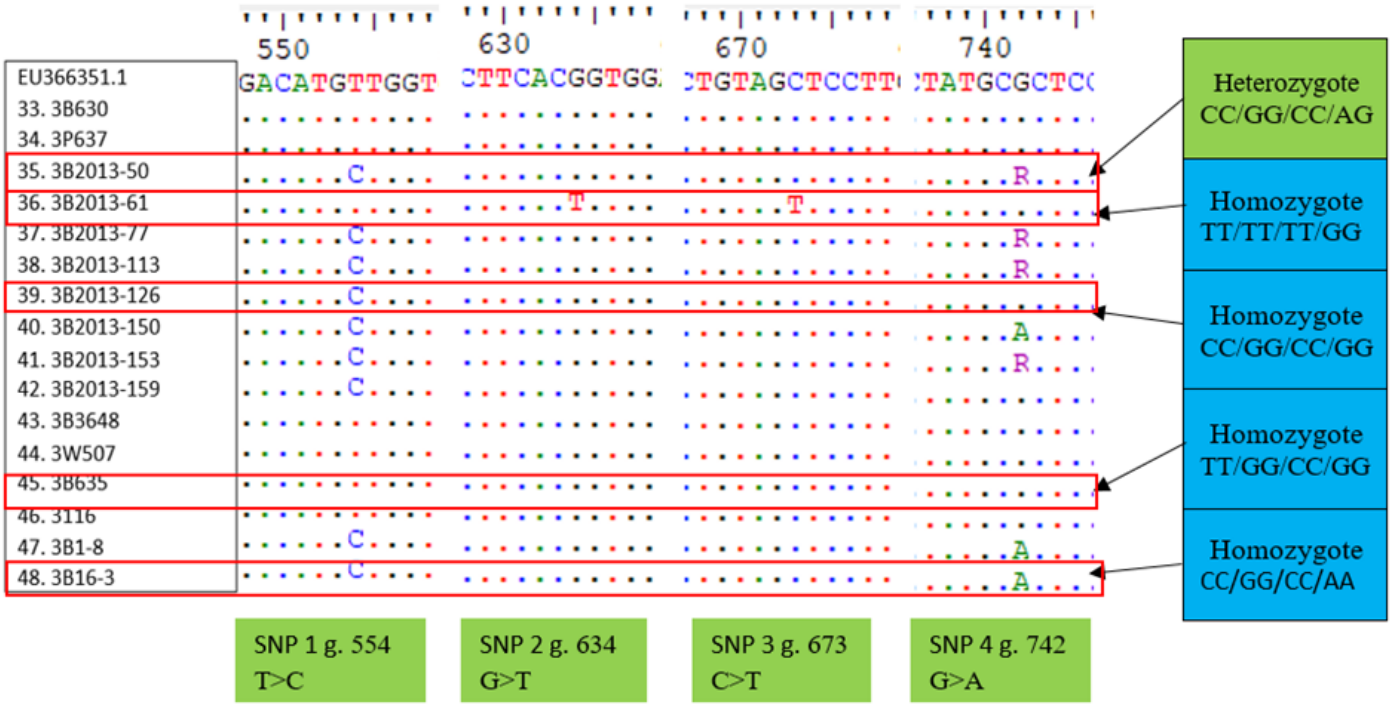

Figure 1. Alignment recap on SNPs at g.554 T>C, g.634 G>T, g. 673 C>T, g. 742 G>A.

A>G which has a significant effect on live weight and g.1069 C>G has significantly associated with live weight, carcass weight, backfat thickness, and marbling score in cattle.

Table 1. Chi-Square analysis result on Bali cattle (*Bos sondaicus*).

No	SNP	Data	Genotype Frequency	Allele Frequency	Chi-square test value	
					X^2 Count	X^2 Table
1	554 T>C (Exon)	TT	0.1	T = 0.1 C = 0.9	10	3.84
		TC	0			
		CC	0.9			
2	634 G>T (Exon)	GG	0.9	G = 0.9 T = 0.1	10	3.84
		GT	0			
		TT	0.1			
3	673 C>T (Exon)	CC	0.9	C = 0.9 T = 0.1	10	3.84
		CT	0			
		TT	0.1			
4	742 G>A (Exon)	GG	0.3	G = 0.5 A = 0.5	0.4	5.99
		GA	0.4			
		AA	0.3			

SNPs are base nucleotide differences found within DNA sequence which could affect the translation of amino acid. Thymine (T) transform into Uracil (U) in the mRNA which goes down into the process of amino acid translation. The occurrence of SNPs could cause a change in a codon which could change the type of amino acid, this type of mutation is called nonsynonymous SNP. On the other hand, SNPs occur and cause a change in a codon, but do not change the type of amino acid and still have the same translation protein called synonymous SNP. A change of amino acids in CDS could deliver a sufficient impact on the phenotype. As shown in Table 2, all SNPs found in this study show a synonymous SNP type, which shows no change in amino acid translated in CDS. Synonymous SNP may be not related directly to the protein translation that occurs in the DNA, but it might have an impact on DNA and RNA regulatory and replication. Hunt et al. (2009) stated that synonymous SNPs have influences in generating ectopic mRNA splicing, silence the effects of a deleterious mutation, intricateness of infectious disease, and impact mRNA stability and translation. A study conducted by Koren et al. (2012) shows there was a strong association between DNA replication timing and the specific types of point mutation (SNPs) observed such as transitions and transversions mutation. Transition mutations is a change of base nucleotide from pyrimidine (C or U) to another pyrimidine or from purine (A or G) to another purine, whilst transversion mutation is a change of base nucleotide from purine to pyrimidine or vice versa. The result shows g.554 T>C, g.742 G>A, and g.673 C>T belonged to transitions mutation type and g.634 G>T was included in transversions mutation type.

Table 2. Mutation type and Amino acid changes.

SNP	Codon	Amino acid	Mutation
g.554 T>C	UUG	Leucine	Synonymous
	CUG	Leucine	
g.634 G>T	ACG	Threonine	Synonymous
	ACU	Threonine	
g.673 C>T	AGC	Serine	Synonymous
	AGU	Serine	
g.742 G>A	GCG	Alanine	Synonymous
	GCA	Alanine	

Restriction mapping is a method used to analyze if there are restriction enzymes related to SNPs found which could be used as a tool in order to utilized for genetic marking. Characteristic of restriction enzyme is cut at the specific site of the DNA alignment. Results displayed in Table 3 present 3 enzymes that have the cutting site in or around a point where SNPs emerge. *MmeI* cuts in g. 554 T>C, *TspRI* in g. 634 G>T, and *BsrI* in g. 742 G>A. *MmeI* and *TspRI* are 3 cutter types whilst *BsrI* is 1 cutter type. The amount of cut type could be used as consideration when using a restriction enzyme for genetic marking as 1 cutter type enzymes could have more relevant cut than 2 or 3 cutters. Based on Table 3, *TspRI* has the shortest fragment size which is 15 bp and *MmeI* has the longest fragment size which is 415 bp.

Determining restriction enzyme that could be used as a recommended gene marker and be used for genotyping are based on two criteria, first the range of enzyme sites must not too numerous and not too short, at least more than 100 bp. Another criterion for enzyme selection is the price of the enzyme (Kurniawati et al. 2021). *BsrI* has moderate fragment size compared to *MmeI* and *TspRI*, moreover, *BsrI* has 1 cutters site which can be more specific.

CONCLUSION

A total of three restriction enzymes were found in three different SNPs *MmeI* in g. 554 T>C, *TspRI* in g. 634 G>T, and *BsrI* in g. 742 G>A. The *BsrI* enzyme is recommended as a tool for genotyping for further cattle selection programs, especially on Bali cattle. The SNP g. 742 G>A which is recognized by *BsrI* enzyme can be utilized as marker candidate for growth traits. Hence, further research should be addressed to investigate the association between the SNP g. 742 G>A and growth traits on Bali cattle.

Table 3. Restriction mapping related to 3 SNPs in this study.

SNP	Enzyme	Recognizing site	Site on CDS (bp)	Amount of cut	Site of cut	Fragment Size
g. 554 T>C	*MmeI*	TCCRAC	277	3 cutters	275, 158, 311	86, 117, 36, 415
g. 634 G>T	*TspRI*	CASTG	357	3 cutters	357, 87, 576	15, 270, 219, 150
g. 742 G>A	*BsrI*	ACTGG	465	1 cutters	466	393, 261

AUTHORS CONTRIBUTION

T.H. designed the research, collected samples, and supervised all the processes, Y.C.P. analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

We certify that there is no conflict of interest with any organization or third party regarding the material discussed in this research.

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