# Restriction Mapping of *Melanocortin 4 Receptor* in *Bos taurus* and *Bos indicus* Based on GenBank Data

**Albakri M.W. and Hartatik T.***
Department of Animal Breeding and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, INDONESIA
*tety@ugm.ac.id

***Abstract***
*Bos taurus and Bos indicus are two species of cattle that are widely used as livestock or draught animals. Genetic selection can be conducted to increase the productivity of both species. Melanocortin 4 receptor is a gene that controls feed intake, metabolism regulation, and body weight in cattle; this gene can be used as a selection marker to increase the productivity of B. taurus and B. indicus via polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). This study aimed to identify potential restriction enzymes in the MC4R genes of B. taurus and B. indicus based on GenBank data and crossbreeds from both types of cattle. Eleven genomic DNAs of the MC4R gene were obtained from the NCBI GenBank database, and partial genomic sequences of the MC4R genes of B. taurus and B. indicus were compared by using BioEdit software to identify variations in these genes. Restriction enzyme mapping was established by using the NEBCutter program.*

*The results revealed 16 restriction enzymes in the MC4R gene sequences, six of which could be used to genotype specific single nucleotide polymorphisms (SNPs). Restriction enzymes HpyCH4IV and BfaI appeared to be useful for genotyping SNPs 1133C>S and 1266G>R respectively. The results of this study improve the current knowledge on potential genetic markers for future research on the MC4R gene in B. taurus, B. indicus, local cattle, and crossbred beef cattle in Indonesia.*

**Keywords:** *Bos taurus, Bos indicus*, MC4R, PCR–RFLP, Restriction enzyme.

## Introduction

*Bos taurus* and *Bos indicus* are two species of cattle that are widely used as livestock or draught animals. Selection is needed to optimize the productivity of these species. Genetic markers have helped accelerate livestock selection over the past 50 years. Selection using genetic markers can produce superior livestock that may be employed as breeders for the next generation without needing to wait for information about the phenotype to be obtained[15]. An association between phenotype and genotype data is necessary to apply selection based on genetic markers. Genotypes can be determined by using differences in base polymorphisms which are known as single nucleotide polymorphisms (SNPs), of a gene encoding a certain trait. One gene that encodes growth properties in cattle is the *melanocortin 4 receptor* (MC4R) gene. This gene is in chromosome 24 and contains one exon.

MC4R expresses proteins released by the hypothalamus and affects appetite, metabolic regulation, and body weight[2]. SNPs in the MC4R gene have been associated with appetite and growth in several animal species. El Sabrout[3] found that the MC4R gene plays an important role in behavior and growth in rabbits. Moreover, SNPs of the MC4R gene have been associated with carcass quality in cattle[9,17] and broilers[14].

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is a selection technique based on genetic markers that allows easy, low-cost analysis. However, the technology also presents a number of disadvantages including a lack of consideration for SNPs that do not have restriction endonuclease-recognizing sites for genotyping[16]. The present study maps restriction enzymes in the MC4R genes of *B. taurus* and B. *indicus* on the basis of GenBank data. The results of this work may help facilitate further research on SNPs and restriction enzymes that could be used as genetic markers.

## Material and Methods

**Sample Collection.** Eleven MC4R nucleotide sequences were obtained from NCBI GenBank database (Accession Nos. EU366350, DQ665825, EU366351, EU366349, FJ430565, AF265221, BC148892 NM174110.1, ACB14851, ACB14850, and ACB14852). Ten blood samples from local cattle (*B. indicus*, Sumba Ongole) and crossbred beef cattle were obtained from PT WMP Klaten. Genomic DNA was extracted from 10 blood samples of crossbred beef cattle using a SYNCTM DNA Extraction Kit (Geneaid, Biotech Ltd., Taiwan).

**DNA Amplification and Sequencing.** Ten genomic DNA samples from crossbred beef cattle were amplified by using the PCR method with a Perkin Elmer Thermal Cycler PCR system[9]. The target sequences of the MC4R gene were amplified by using a pair of primers (forward, F: 5′-GTCGGGCGTCTTGTTCATC-3′; reverse, R: 5′-GCTTGTGTTTAGCATCGCGT-3′) and the PCR product size was 493 bp. The PCR reaction conditions were as follows: 5 min at 94 °C for pre-denaturation, 35 cycles of 30 s at 94 °C for denaturation, 30 s at 58 °C for annealing, 30 s at 72 °C for extension, and 10 min at 72 °C for a final extension. The target sequence was developed by LPPT UGM.

**Comparative Analysis.** SNP identification, sequence comparison, and amino acid change identification were conducted by using Bioedit software. All sample sequences were compared by using clustalW multiple alignment. Sample sequence alignment was carried out with a bootstrap value of 1000 [4]. An SNP was determined when the result of sequence alignment showed a different nucleotide. GenBank accession no. EU366350 was used as a reference to observe the amino acid chain of each sample. Changes in amino acids were determined when the SNP contained a missense mutation.

**Restriction Enzyme Determination.** The restriction enzymes were obtained from NEBCutter V2. The individual target sequences of the MC4R genes of the cattle were entered in NEBCutter V2 with all other parameters in their default settings. The program calculated the positions of all restriction enzymes and then displayed all restriction enzymes recognizing the target sequence[13]. Specific restriction enzymes that could recognize the target were determined by the appearance of a red line under a sequence.

## Results and Discussion

A total of 11 sequences of *B. taurus* (GenBank accession no. EU366350, DQ665825, EU366351, EU366349, FJ430565, AF265221, and BC148892) and *B. indicus* (GenBank accession no. NM174110.1, ACB14851, ACB14850, and ACB14852) of the MC4R gene were obtained for alignment analysis against that of crossbred beef cattle. According to the alignment sequences obtained, 11 SNPs are present in the MC4R genes of all samples when compared with GenBank accession no. EU366350 as a template. The SNP identification results are presented in (Table 1). The position of SNPs of crossbred cattle is shown in (Figure 1). Four genotypes of crossbred beef cattle determined via sequence analysis were submitted to GenBank with accession numbers of MN692245, MN692246, MN692247, and MN692248 for BBWAGYUP630, BX392, BBWAGYUP648 and BB637 respectively.

The position of an SNP can be obtained by comparison with a reference and DNA sequencing. In this study, the SNPs of *B. taurus*, *B. indicus*, and crossbred cattle identified not only homozygotic but also heterozygotic mutations. Eight out of 11 SNP positions changed from heterozygotic to homozygotic or vice versa (Table 1). Comparative studies on the MC4R gene report that SNPs in four species of cattle, buffalo, sheep, and goat may be found at 110 site of the gene. Specifically, 12 variations at the 5'UTR, 72 located in exons, and 26 at the 3'UTR could be observed. A total of 19 out of the 72 SNPs in the exon region can change amino acids[6]. In the leptin gene of the same sample, SNPs were found in eight locations[1].

SNPs can cause changes in proteins, specifically silent and missense mutations. A silent mutation occurs if the SNP only changes bases in the DNA sequence but does not change its amino acid. Missense mutations occur if the SNP changes the amino acid. The results of amino acid analysis of the coding sequence (CDS) are presented in (Table 2). In the leptin gene of the same sample, for example, four out of eight SNPs can change amino acids. These SNPs are located at 1120C>T, 1130G>A, 1180C>G, and 1181G>A[1].

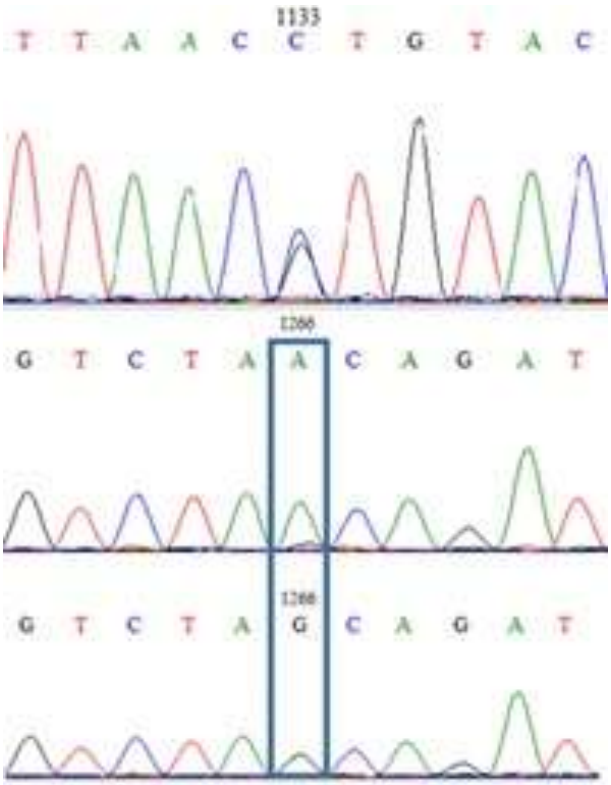


**Fig. 1: Chromatogram of the sequence analysis of SNPs at 1133 G>S and 1266 G>R in crossbred cattle**

**Table 1**
**Alignment of MC4R Genes of *Bos Taurus* And *Bos Indicus***

| SNP (EU366350) | *B. taurus* | | | | | | | *B. indicus* | | | | Cross bred cattle |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 1 | 2 | 3 | 4 | |
| | EU366350.1 | DQ665835 | EU366351 | EU366349 | FJ430562 | AF265221 | BC148912 | NM174101 | ACCB18548 | ACCB18550 | ACCB18552 | |
| 86K>T | K | NA | T | T | T | T | NA | T | NA | NA | NA | NA |
| 118A>T | A | NA | A | A | A | A | T | A | NA | NA | NA | NA |
| 149R>A | R | NA | A | A | A | A | A | A | NA | NA | NA | NA |
| 316Y>C | Y | NA | C | C | C | C | C | C | Y | C | C | NA |
| 811G>R | G | G | G | R | G | G | G | G | G | R | G | NA |
| 1133C>S | C | C | C | C | S | C | C | C | C | C | C | S |
| 1266G>R | G | NA | G | G | R | G | G | G | G | G | G | A |
| 1454Y>T>C | Y | NA | T | T | T | C | T | T | NA | NA | NA | NA |
| 1634M>C | M | NA | C | C | C | C | C | C | NA | NA | NA | NA |
| 1850C>Y | C | NA | C | Y | C | C | C | C | NA | NA | NA | NA |
| 1859A>C | A | NA | A | A | A | A | C | A | NA | NA | NA | NA |

Note: Nucleotides are according to International Union of Biochemistry group codes
A = adenine, C = cytosine, G = guanine, T = thymine, K = G or T, R = A or G, Y= C or T, S = G or C, M = A or C, NA = not available

**Table 2**
**Amino Acid Analysis in The Coding Sequences Region**

| SNP | Codon | Amino acid | Mutation |
|---|---|---|---|
| 316Y>C | CTY | Leucine/ Valine | Missense |
| | CTC | Leucine | |
| | CTT | Valine | |
| 811G>R | ACG | Threonine | Silent |
| | ACR | Threonine | |
| | ACA | Threonine | |
| 1133C>S | CTG | Leucine | Missense |
| | STG | Leucine/Alanine | |
| | GCG | Alanine | |
| 1266G>R | AGC | Serine | Missense |
| | ARC | Serine/Asparagine | |
| | AAC | Asparagine | |

The CDS is a DNA sequence that codes a protein, and the protein obtained can affect an individual's phenotypic appearance. Identification of the CDS region is very important because mutations of this region may result in protein changes. Some protein changes may affect the phenotype of an organism. The coding sequence of the MC4R gene in EU366350 starts at 278 bp and ends at 1276 bp.

Liu et al[8] reported that the SNP at 1069*C>G* in the MC4R gene is associated with body weight and carcass weight in Qinchuan cattle. Other research reported marbling scores in Korean brown and brindle cattle[7]. A previous study was reported SNP at 1133*C>G* in the Kebumen Ongole Grade cattle, the SNP has association with body length of birth[9] and in the Madura cattles, the SNP has association with growth traits and feed intake[11]. Therefore, the SNP at 1133C>S in the present study contributes to future research on the association between genotype and phenotype in crossbred beef cattle.

According to table 2., three out of four SNPs in the CDS region of the MC4R gene, namely, 316Y>C, 1133C>S and 1266G>R, cause a missense mutation. The first SNP at

316Y>C changes an amino acid from leucine to valine. The second SNP at 1133C>S changes an amino acid from leucine to alanine. The third SNP at 1266G>R changes an amino acid from serine to asparagine.

Changes in amino acids may affect the phenotypic appearance of an organism. SNPs at 316Y>C, 1133C>S and 1266G>R are recommended as genetic markers for future research. These three SNPs have been proven to be located at the CDS region and change amino acids (proteins).

Genotyping of the MC4R gene can be carried out via PCR–RFLP[9,10]. In this technique, genotyping can be achieved with one or more DNA bands depending on the cutting site of a restriction enzyme. A restriction enzyme cuts the recognition site and produces two or more DNA bands. These DNA bands can differentiate between homozygotic and heterozygotic samples. The PCR–RFLP method allows easy analysis at low cost[16]. Mapping of restriction enzymes is very important as a guidance for PCR–RFLP. Figure 2 shows the results of the restriction enzyme search using NEBCutter.

Restriction enzymes that can recognize SNP positions can be identified by using NEBCutter. NEBCutter is useful for finding enzymes that can excise the desired region. As shown in figure 2. All enzymes that can cut the sequence site are displayed when fewer than 60 nucleotides are inputted into the text box of NEBcutter. Specific sequence bases forming parts of a certain restriction enzyme are highlighted when the mouse is moved over an enzyme name. Then, a box shows the enzyme name, sequence site recognition, and number of cutting sites (Figure 2).

Nucleotide K (Keto) has heterozygote genotype G or T. The SNP at 86*K*>*T* can be recognized by the MnlI and SfaNI restriction enzymes which can identify the sequences GAGG and GATGC respectively. The results of restriction enzyme mapping are shown in table 3.

Table 3 reveals that 11 SNPs (Table 1) can be recognized by 16 restriction enzymes. However, two SNPs (118 A > T and 1634 M > C) cannot be identified by the restriction enzymes because their sequences do not have specific recognition sites. Five (86K>T, 316Y>C, 811G>R, 1133C>S, 1454Y>T>C) of these 11 SNPs can be cut by several restriction enzymes (Table 3).

Missense mutation is a polymorphism that occurs in the exon region which causes changes in amino acid. The SNP position can be identified by restriction enzymes. Nine restriction enzymes were found in the SNP positions of 316Y>C, 811G>R, 1133C>S and 1266G>R (Table 3). The restriction enzymes in SNPs at 1133C>S and 1266G>R are recommended for genotyping of crossbred beef cattle.

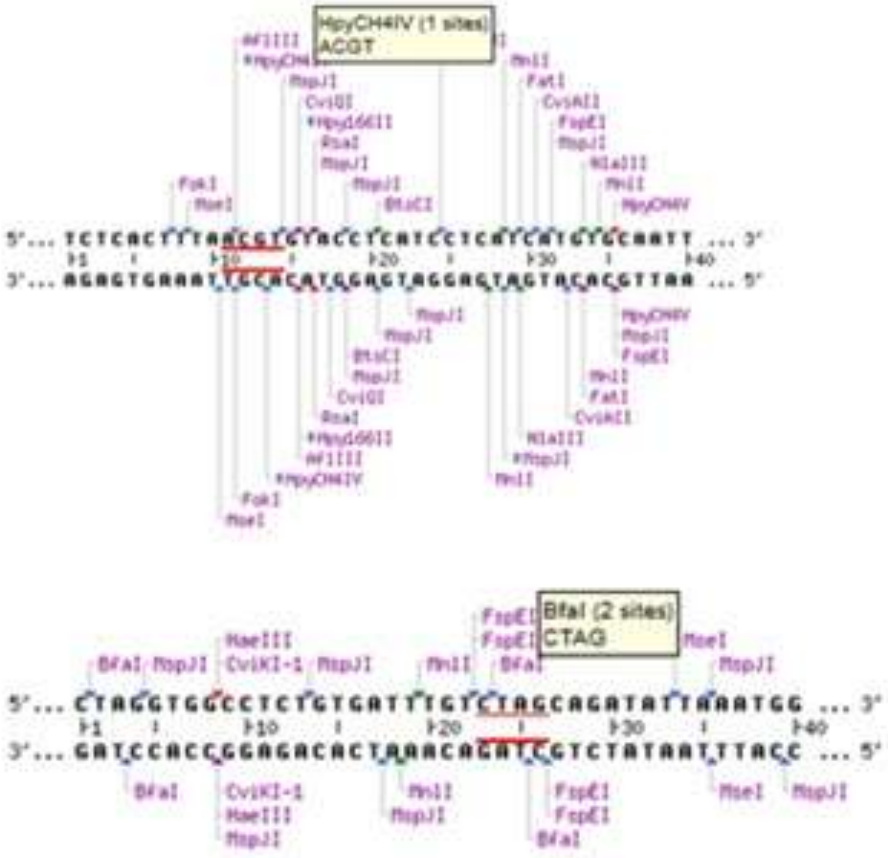


**Fig. 2: Restriction enzymes HpyCH4IV in SNP 1133C>S (top) and BfaI in SNP 1266G>R (bottom)**


**Fig. 3: Illustration of two recommended restriction enzymes HpyCH4IV in SNP 1133C>S and BfaI in SNP 1266G>R based on target sequence**

**Table 3**
**Mapping of Restriction Enzymes in MC4R Gene**

| SNP (EU366350) | Nucleotide | Restriction enzyme | Recognition sequence | location |
|---|---|---|---|---|
| 86K>T | G | MnlI | GAGG | 5′ UTR |
| | T | SfaNI | GATGC | |
| 118A>T | - | - | - | 5′ UTR |
| 149R>A | G | HpyCH4IV | ACGT | 5′ UTR |
| 316Y>C | T | MboII, EarI | TCTTC, CTCTTC | CDS |
| 811G>R | A | TspRI, BtsI, MutI | CASTG, CAGTG | CDS |
| 1133C>S | C | LpnPI | CCDG | CDS |
| | G | HpyCH4IV, Hpy166II | ACGT, GTNNAC | |
| 1266G>R | G | BfaI | CTAG | CDS |
| 1454Y>T>C | C | BsmAI, BcoDI | GTCTC, GTCTC | 3′ UTR |
| 1634M>C | - | - | - | 3′ UTR |
| 1850C>Y | C | HpyCH4III | ACNGT | 3′ UTR |
| 1859A>C | A | MseI | TTAA | 3′ UTR |

Note: N is any base, D is A or G or T

The illustration of recommended restriction enzyme in the target sequence of MC4R gene is shown in figure 3. Prihandini et al[11] and Maharani et al[9] genotyped the MC4R genes of Madura and PO Kebumen cattle by using the HpyCH4IV restriction enzyme in the SNP position 1133C>S.

Other studies on crossbred beef cattle (Brahman cross, Belgian Blue bull cross, Wagyu bull cross) determined the genotype of IGFBP3 using the PvuII restriction enzyme[5,12]. The results revealed four genotypes (i.e. xx, yy, xy, and zy). The different genotypes of crossbred beef cattle obtained from the present study of restriction enzymes (HpyCH4IV and BfaI) have been registered in GenBank with accession numbers MN692245, MN692246, MN692247, and MN692248.

## Conclusion

Sixteen restriction enzymes can be used to recognize in the SNPs of MC4R gene sequence. Six restriction enzymes in the CDS sequence can be used to genotype the MC4R gene. The restriction enzymes HpyCH4IV in SNP 1133C>S and BfaI in SNP 1266G>R are recommended as a tool for genotyping in crossbred beef cattle.

## Acknowledgement

This study was supported by a research grant from Rekognisi Tugas Akhir (RTA) 2020 contract no. 2488/UN1.P.III/DIT-LIT/PT/2020. The authors thank Latifah for providing helpful discussions during the writing of this paper and Retno Setyawati for technical assistance for this research.

## References

1. Anugratama L.E. and Hartatik T., Short communication: Identification of leptin gene in crossbred beef cattle, *Biodiversitas*, **21(1)**, 226-230 **(2020)**

2. Chun-yu L. and Hui L., Association of MC4R gene polymorphisms with growth and body composition traits in chicken, *Asian-Australas J. Anim. Sci*., **19**, 763-768 **(2006)**

3. El-sabrout K., Associations between single-nucleotide polymorphisms of melanocortin gene and sexual desire behavior in rabbit (Oryctolagus cuniculus), *J. Vet. Behav*., **19**, 69-71 **(2017)**

4. Hartatik T., Practical Approach: Polymorphism DNA of Aceh Cattle, Gadjah Mada University Press, Yogyakarta, 118-134 **(2016)**

5. Hartatik T. et al, Association of IGFBP-3 gene polymorphism g.3.930 G>A with birth size and birth weightin crossbred beef cattle, *J. Indonesian Trop. Anim. Agric*, **44(4)**, 356-363 **(2019)**

6. Latifah, Hartatik T., Maharani D. and Kunstantinah, Comparison study of melanocortin 4 receptor in cattle, buffalo, sheep and goat based on genbank data, presented at the Conf. 1st International Conference on Bioinformatics, Biotechnology and Biomedical Engineering (Biomic) Yogyakarta, Indonesia **(2016)**

7. Lee Y., Park S., Kim H., Lee S.K., Kim ·J.W., Lee H.K., Jeong D.K. and Lee S.J., A C1069G SNP of the MC4R gene and its

association with economic traits in Korean native cattle (brown, brindle, and black), *Electron. J. Biotech*., **16(50)**, 1-5 **(2013)**

8. Liu H., Tian W., Zan L., Wang H. and Cui H., Mutations of MC4R gene and its association with economic traits in Qinchuan cattle, *Mol. Biol. Rep*., **37**, 535-540 **(2009)**

9. Maharani D., Fathoni A., Sumadi, Hartatik T. and Khusnudin M., Identification of MC4R gene and its association with body weight and body size in Kebumen Ongole Grade cattle, *J. Indonesian Trop. Anim. Agric*, **43(2)**, 87-93 **(2018)**

10. Pourzand C. and Cerutti P., Genotypic mutation analysis by RFLP/PCR, *Mutat. Res-Fund Mol. M*., **288(1)**, 113-121 **(1993)**

11. Prihandini P.W., Sumadi, Budisatria I.G.S. and Maharani D., Identification single nucleotide polimorphism of Melanocortin 4 receptor gene in Madura Cattle, In Conf. The 7th International Seminar on Tropical Animal Production, Yogyakarta, Indonesia, 691-695 **(2017)**

12. Priyadi D.A., Panjono, Bintara S. and Hartatik T., Genotype of Brahman and Brahman Cross Cattle based on SNP in Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) gene sequences, *Biodiversitas*, **18(2)**, 795-800 **(2017)**

13. Vincze T., Posfai J. and Roberts R.J., NEB cutter: a program to cleave DNA with restriction enzymes, *Nucleic Acids Res*, **31(13)**, 3688-3691 **(2003)**

14. Wang Y., Yi S., Xiaosong J., Yiping L., Xiaocheng L., Zengrong Z., Huarui D. and Qing Z., Study on association of single nucleotide polymorphism of MC3R and MC4R genes with carcass and meat quality traits in chicken, *J. Poult. Sci*., **46**, 180-187 **(2009)**

15. Wolc A., Understanding genomic selection in poultry breeding, *Worlds Poult. Sci. J*., **70**, 309-314 **(2014)**

16. Xiao J., Xin X., Luan X., Wei D. and Yang S., A modified simple RFLP-PCR method for single nucleotide polymorphism (SNP) typing, *Genet. Mol. Biol*, **29(3)**, 562-565 (**2006)**

17. Zhang C.L., Wang Y.H., Chen H., Lan X.Y., Lei C.Z. and Fang X.T., Association between variants in the 5'-untranslated region of the bovine MC4R gene and two growth traits in Nanyang cattle, *Mol. Biol. Rep*., **36**, 1839-1843 **(2009)**.

(Received 20th June 2020, accepted 25th August 2020)