ISSN 1819-1878

Asian Journal of Animal Sciences

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OPEN ACCESS Asian Journal of Animal Sciences

ISSN 1819-1878
DOI: 10.3923/ajas.2017.40.46

Research Article
Sequence Analysis and Identification of Allele Distribution of Melanocortin 1 Receptor (MC1R) Gene in Indonesian Cattle (*Bos sondaicus*×*Bos indicus*)

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Abstract

Background and Objective: The melanocortin-1-receptor (MC1R) gene is an important candidate gene for the coat color trait. Since, Indonesia has the diversity of local cattle with various type of colour, it is very interest to investigate the sequence of MC1R gene. The aim of this study was to identify polymorphism of melanocortin-1-receptor gene in Indonesian cattle based on sequence analysis. This study was conducted at Laboratory of Animal Genetic and Breeding, Department of Animal Breeding and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, between August, 2012 and December, 2016. **Materials and Methods:** The total number of 164 cattle were used in this study that consist of Aceh cattle (n = 25), Filial Ongole cattle (n = 6), Limousin × Madura (Limura, n = 11), Madura cattle (n = 7), Brahman cattle (n = 60), Pesisir cattle (n = 9) and Bali cattle from Kupang (n = 46). A fragment of 296 base pair of melanocortin 1 receptor (MC1R) gene was amplified by forward primer: 5′-GGA CCC TGA GAG CAA GCA C-3′, reverse primer: 5′-CTC ACC TTC AGG GAT GGT CTA-3′. Polymerase Chain Reaction (PCR) products were digested with *Msp*I enzyme for identifying of restriction pattern (PCR-RFLP result). Sequencing of the representative sample was conducted base on PCR-RFLP result with different type of polymorphism. **Results:** The PCR-RFLP analysis showed three pattern of restriction, there are EE genotype, Ee genotype and undigested sample with ee genotype. Sequence analysis show point mutation along the 296 bp of MC1R gene with two type of mutation (substitution 147C→T and deletion/insertion 162indelG). Bali cattle (*Bos soandaicus*) and Aceh cattle (*Bos indicus*) revealed the monomorphic with EE genotype. The others (PO, Limura, Madura, Pesisir and Brahman) showed the polymorphic with equilibrium in chi-square analysis except Limura (*Bos soandaicus* × *Bos indicus* × *Bos taurus*) with $\chi^2 = 7.64$ for Limura population. **Conclusion:** Local cattle such as Bali and Aceh cattle have higher E allele distribution compare to that of the other local cattle. Indeed, the homozygosity of MC1R gene (EE) in local cattle also higher than that of the crossbred cattle. The MC1R gene can be used to identify the genetic variation of local cattle and showed heterogeneity in crossed breed cattle.

Key words: MC1R gene, sequencing, polymorphism, genotype, PCR-RFLP, homozygote, heterozygote, chi-square, phylogenetic tree

Received: August 09, 2016 Accepted: November 14, 2016 Published: December 15, 2016

Citation: Tety Hartatik, 2017. Sequence analysis and identification of allele distribution of melanocortin 1 receptor (MC1R) gene in indonesian cattle (*Bos sondaicus* × *Bos indicus*). Asian J. Anim. Sci., 11: 40-46.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

A large number of coat colour have been described in different mammalian species. This diversity is due to the presence, distribution and biochemical activity of the melanocytes in which two types of melanin pigment. Melanins are pigments with a different molecular weight and formed by enzymatic oxidation of the tyrosine amino acid from which two kind of pigments derive: Eumelanins and pheomelanins. Eumelanins produced black/brown colors whereas pheomelanin produced red/yellow colors. Pigmentation is essentially determined by the distribution of the two pigments respectively producing a black/brown and a yellow/red colour. The extension locus encodes for melanocortin 1 receptor (MC1R), a 7 trans-membrane domain receptor. In cattle, the MC1R gene is located on chromosome 18 and consists of a single exon 954 bp long[1]. Coat colour in animals is controlled by ratio and amount of black-brown eumelanin and yellow-reddish phaeomelanin[2]. Dominant alleles at the extention locus induce black pigmentation, whereas recessive alleles extend the production of pheomelanins, determining red/yellow/pale pigmentation. Mutations at the agouti locus caused apposite action i.e., dominant alleles determine pheomelanin phenotypes whereas recessive alleles cause black coat color with a few expectations[3].

Xi *et al.*[4] investigated the relationship of polymorphism of the MC1R with coat colour in the Chinese yakow, the coding sequence (CDS) and the flanking region of MC1R. The sequence target of the Chinese yakow MC1R is 1134 bp sequences including the full CDS (954 bp) and parts of the 5' and 3'-untranslated regions (162 and 18 bp, respectively). They found 13 single nucleotide polymorphisms (SNPs) including 4 SNPs (T-129C, A-127C, C-106T and G-1A) in the 5 -untranslated region and 9 SNPs (C201T, T206C, C340A, C375T, T663C, G714C, C870T, G871A and T890C) in the CDS. In another research of melanocortin 1 receptor gene, three alleles (E^D, E+ and e) were found in Japanese, Korean and Chinese native cattle. Black cattle had two different alleles (E^D and E+) that induce black pigment synthesis[5,6]. Mutations of the gene coding for MC1R have been shown to affect coat colour in many mammals.

Indonesian local cattle have variation of color such as black, white and red to yellow. Variations of color are of great interest for investigation. Bali cattle have black color for male and yellow color for female. Madura cattle have red to yellow colour whereas Filial Ongole has white colour. Up to now, study on MC1R gene in local Indonesian cattle has not been reported. Therefore, the objective of this study was to identify polymorphism of melanocortin-1-receptor gene based on sequence analysis to gather important information on the variation genetics of local cattle in Indonesia.

MATERIALS AND METHODS

Samples: A total of 164 cattle were used in this study which consisted of Aceh cattle (n = 25), Filial Ongole cattle from Yogyakarta (n = 6), Limousin × Madura (Limura) from Madura island (n = 11), Madura cattle from Sapudi, Madura island (n = 7), Brahman cattle from Lampung (n = 60), Pesisir cattle from West Sumatera (n = 9) and Bali cattle from Kupang (n = 46). Especially for Bali Cattle, we used the previous sample data[7]. Approximately 3 mL whole blood was withdrawn from jugular vein of each animal using vacuum tubes containing K_3EDTA as an anticoagulant. Blood samples were stored at -20°C until DNA extraction.

DNA extraction and Polymerase Chain Reaction (PCR): Whole blood was used to extract the genomic DNA in this study. The DNA extraction used GeneSYNC™DNA Extraction Kit (Genetika Science). The extracted DNA samples were stored at -20°C and used later as a substrate for PCR reaction. To amplify DNA fragments of MC1R gene, we used the primers based on Li *et al.*[8] as follow MC1R forward: 5′-GGA CCC TGA GAG CAA GCA C-3' and MC1R reverse: 5′-CTC ACC TTC AGG GAT GGT CTA-3′.

The PCR reactions were performed in a 30 µL reaction mixture containing 1 µL of DNA (10-100 ng), 1.5 µL of each primer (10 pmol μL^{-1}), 15 µL PCR kit (KappaFast2G, Biosystem, USA) and 12.5 µL aquabidest. The 296 bp MCIR gene fragment was amplified using 30 amplification cycles (Peqlab, Germany) under the following conditions: Initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 10 min[8]. Each amplification product was analyzed by electrophoresis on a 1% agarose gel in TBE buffer. The DNA bands were stained with ethidium bromide to visualization by Ultra Violet (UV) light.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP): The polymorphism was analyzed using the PCR-RFLP method with *Msp*I enzyme. A total volume of 15 µL containing 4 µL of PCR product, 0.1 µL *Msp*I enzyme (0.2 U), 1.5 µL buffer tango and 9.4 µL aquabidest were incubated at 37°C for 4 h. The resulting products were separated by agarose gel 2.5% and visualized by UV light.

Sequencing analysis: Total volume of 30 µL PCR for each PCR products and 10 µL MC1R primer (10 pmol μL^{-1}) was prepared for sequencing. The DNA sequence of PCR product was performed by PT. Genetika Science. DNA sequences were aligned by using BioEdit version 7.7 for identification of the single nucleotide polymorphism.

Data analysis: Analysis of genotype and allele frequencies was done based on the pattern the PCR-RFLP result and the observation of DNA sequence result. Three pattern (genotypes) were produced as the result of *Msp*I restriction site (C'CGG) such as 160, 136 bp (EE), 296, 160, 136 bp (Ee) and 296 bp (ee) of DNA band pattern could be distinguished on agarose gel 2.5%, which are the products of two alleles (E and e). The expected allele frequencies were calculated according to the Hardy-Weinberg equilibrium:

$$p^E = \frac{2\,EE + Ee}{2N}$$

and:

$$q^e = \frac{2\,ee + Ee}{2N}$$

Where:

N = The No. of genotyped animals
p^E and q^e = The expected frequencies of E and e alleles
EE, Ee and ee = The No. of animal with different genotypes
EE = $n \times p^2$
Ee = $2n \times p \times q$
ee = $n \times q^2$

RESULTS AND DISCUSSION

Genotype and allelle frequency: The PCR product of MC1R gene was 296 bp. The PCR-RFLP using *Msp*I restriction enzyme (with recognition site CCGG) produced three genotypes (EE, Ee and ee). The EE genotype had fragment size of 160 and 136 bp. The Ee had fragment size of 296, 160 and 136 bp and the ee has 296 bp only. Figure 1 shows the DNA fragments of local cattle in Indonesia. There are two type of restriction fragment, two DNA bands in line 1, 3 and 4 with EE genotypes (160 and 136 bp) and three DNA bands in line 2, 5 and 6 with Ee genotypes (296, 160 and 136 bp). The results in Table 1 showed the black Bali cattle have dominant genotype (EE) in each color group (>98% EE). Aceh cattle have 100% genotype EE. The same results were found in Aceh cattle (*Bos indicus*) and were also shown in red and white Bali cattle (*Bos sondaicus*). However Bali cattle phenotypically had white color but the results of PCR-RFLP MC1R gene showed the genotypes EE (the same as red and black color). There was no evidence that particular SNPs were associated with coat colour in Indonesian cattle. Similar result in the Chinese yakow MC1R gene study, indicating that coat colour in this species could be affected by other genes or factors[4]. Coat colour could be a complex trait which is linked to many genes, pathways or networks. It was known that more than 150 genes and 300 genetic loci are related to the pigmentation and coat colours of animals[9].

Colour pigmentation in cattle is influenced by Melanocyte Stimulating Hormone Receptor (MSHR) or melanocortin 1 receptor (MC1R) genes. The pigmentation in the color of cattle may be affected by the MC1R gene that plays a role in the formation of melanocytes that stimulates tyrosinase to produce eumelamin that produces brown to black color.

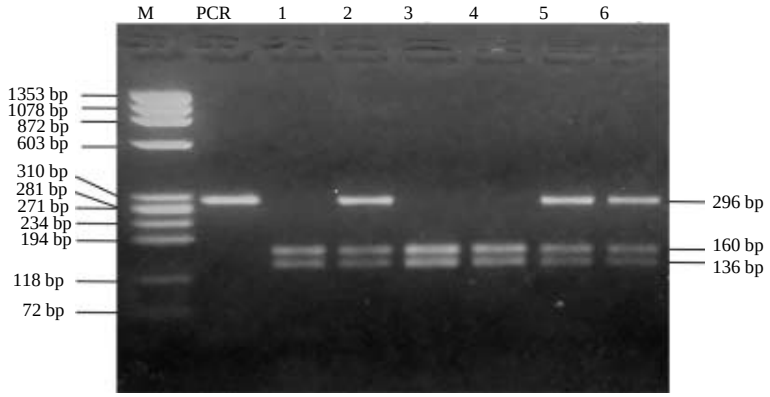

Fig. 1: *Msp*I digestion of PCR product. M: Marker ϕX174 DNA/BsuRI (*Hae*III), lanes 1, 3 and 4: EE genotype, lanes 2, 5 and 6: Ee genotype and lane 11: PCR product (296 bp)

Table 1: Frequencies of genotypes and alleles of the MC1R gene with different coat color

			Genotypes			Allele (%)	
Breed	Coat color	n	EE	Ee	ee	E	e
Bali (*B. sondaicus*)	Black	21	20	1	0	0.98	0.02
Bali (*B. sondaicus*)	Red	17	17	0	0	100	0.00
Bali (*B. sondaicus*)	White	8	8	0	0	100	0.00
Aceh (*B. sondaicus*× *B. indicus*)	Red	25	25	0	0	1.00	0.00
Filial Ongole cattle (*B. indicus*)	White	6	3	3	0	0.50	0.50
Madura (*B. sondaicus*× *B. indicus*)	Red	7	6	1	0	0.93	0.07
Limura (*B. indicus*× *B. taurus*)	Dark red	11	1	10	0	0.55	0.45
Brahman (*B. indicus*)	White	60	46	13	1	0.88	0.13
Pesisir (*B. indicus*)	Red	9	6	3	0	0.83	0.17
Total		164	132	31	1	0.90	0.10

Table 2: Analysis chi-square of 7 local cattle in Indonesia

		Genotype (observed)			Genotype frequencies (%)			Allele frequencies		Genotype (expected)			
Breed	N	EE	Ee	ee	EE	Ee	ee	E	e	EE	Ee	ee	χ^2
Bali	46	45	1	0	97.78	2.22	0.00	0.99	0.01	44.01	0.99	0.01	0.01
Aceh	25	25	0	0	100.00	0.00	0.00	1.00	0.00	25.00	0.00	0.00	-
PO	6	3	3	0	50.00	50.00	0.00	0.75	0.25	3.38	2.25	0.38	0.67
Limura	11	1	10	0	9.09	90.91	0.00	0.55	0.45	3.27	5.45	2.27	7.64*
Madura	7	6	1	0	85.71	14.29	0.00	0.93	0.07	6.04	0.93	0.04	0.04
Brahman	60	46	13	1	76.67	21.67	1.67	0.88	0.13	45.94	13.13	0.94	0.01
Pesisir	9	6	3	0	66.67	33.33	0.00	0.83	0.17	6.25	2.50	0.25	0.36
Total	164	132	31	1	80.49	18.90	0.61	0.90	0.10				

*$\chi^2 = 5.99$

Melanocyte Stimulating Hormone (MSH) plays an important role in determining the response of the skin to ultraviolet radiation and can affect the development of melanoma[10]. The MC1R gene mutations in some mammals produces dark or black coat color, whereas the loss of function in mutations of those genes causes red-yellow coat color or white[3].

The alleles frequency of Aceh cattle were 100% and Bali cattle were E = 0.99 and e = 0.01 (Table 2). This results showed that Aceh cattle and Bali cattle in this study is monomorphic with the proportion of homosigosity loci 0.99 (99%). Based on the research results reported that the E allele frequency of MC1R/*Msp*I locus was higher among Indonesia local cattle. The low frequency of MC1R/*Msp*I e allele of cattle population in this study can be due to low actual e allele frequency in population or the effect of severse natural selection at this locus.

Polymorphism of MC1R gene base on sequence analysis: Based on the sequence alignment analysis the changes of sequence order occur at position 310/311 (Fig. 2a). GenBank accession number GU982927 and accession number Y19103 were used as a control[4]. Based on sequence result from MC1R 296 bp, deletion of G (162delG) for individual cattle were found in Brahman cattle (*Bos indicus*) number Br8 but not in Brahman cattle number Br41. The nucleotide at position 147 shifted from G to T at the following sequence (originally position 148) which produce homozygote ee were also identified in Brahman cattle (*Bos indicus*) number Br8 (Fig. 2a, b). The recessive allele (e) is characterized by a frameshift mutation at position 310 and encodes a truncated protein with only three putative transmembrane domains. Animals homozygous for this allele show red/white coat color phenotypes across a large number of cattle breeds[11].

This study provides new knowledge on Indonesian cattle (*Bos sondaicus*× *Bos indicus*) breeds. The result of ClustalW multiple alignment using BioEdit program at position 140-150 bp performed the sequence 140-GTCATGCCGCT-150 for Bali cattle and the sequence 140-GTCATGCTGCT-150 for Aceh cattle. Furthermore, the sequence result of MC1R gene at position 147 clearly showed that there are two types genotype: Heterozygote (T/C) for Bali cattle (Fig. 3a) and homozygote (T/T) for Aceh cattle (Fig. 3b). Thus, the mutation T instead of C was called as subsitution 147C→T. There were also indel mutation as shown in Fig. 3c and d (162indelG). Bali cattle (*Bos soandaicus*) and Aceh cattle (*Bos indicus*) revealed the monomorphic pattern. The others (PO/*Bos indicus*, Limura/*Bos soandaicus*× *Bos indicus*× *Bos taurus*, Madura/*Bos indicus*, Pesisir/*Bos indicus*, Brahman/*Bos indicus*) showed the polymorphic pattern with equilibrium in chi-square analysis except Limura cattle (*Bos sondaicus*× *Bos indicus*× *Bos taurus*) with χ^2 = 7.64 (Table 2). Since the

Fig. 2(a-d): Sequence analysis using BioEdit program, (a) Individual sequence alignment of EE (Br41), Ee (PO18) and ee (Br8), (b) ABI chromatogram of homozygote deletion (ee genotype, Br8), (c) ABI chromatogram homozygote (EE genotype, Br41) and (d) ABI chromatogram heterozygote (Ee genotype, PO18)

crossbreeding program by artificial insemination using *Bos taurus* semen is spread widely in entire the country, so the genetic marker of MC1R gene can be used to protect Bali and Aceh Cattle. The new finding of this study that the standard colour of Bali and Aceh cattle is red colour with the genotype EE. This seems that deletion of 162G (or 310G at related references) doesn't occur in both cattle. For breed authentication, the optimal situation would be if an allele is present (or absent) only in the breed of interest[12]. The use of the polymorphic site in case of authentication should be evaluated according epistatic mechanism that contributes to the constitution of the breeds (Bottlenecks, reproductive isolation, crossbreeding, introgression, etc.).

Guastella *et al.*[13] studied the genetic polymorphism at MC1R locus in three endangered Italian cattle breeds which revealing a notable genetic variation associated to coat colour. The missense substitution T296C that characterised allele E^D was confirmed in Cinisara cattle breed, as well as the G310 del in e allele in red coated breeds. The insertion of 12 bp GGCATTGCCCGG starting from nucleotide 669 was established in all the carriers of E^1 allele. Moreover the sequence analysis revealed a nonsynonymous substitution (C667T, accession number GU982927) already described by Maudet and Taberlet[14] in some Italian breeds and now named E^2. Actually, a great variation between breeds expressing different coat colours was shown by MC1R allele frequencies. Klungland and Vage[15] reported MC1R allele frequencies in multicoloured breeds like Icelandic cattle or in Dolafe, the allele E^+ is one of the most represented whereas E^1 allele was observed at high frequency in Aubrac, Gasconne[16], Rendena and especially, in Italian Brown[17].

Phylogenetic tree analysis: In order to investigate the genetic relationship between local cattle in Indonesia, 31 individual samples which were representative of Bali, Aceh, Madura, Limura, Pesisir, PO and Brahman cattle were aligned by using BioEdit software. The results of phylogenetic tree by Neighbor-Joining/UPGMA method version 3.6a2.1 was shown in Fig. 4. Homozygote and heterozygote cattle were separate clearly in this tree.

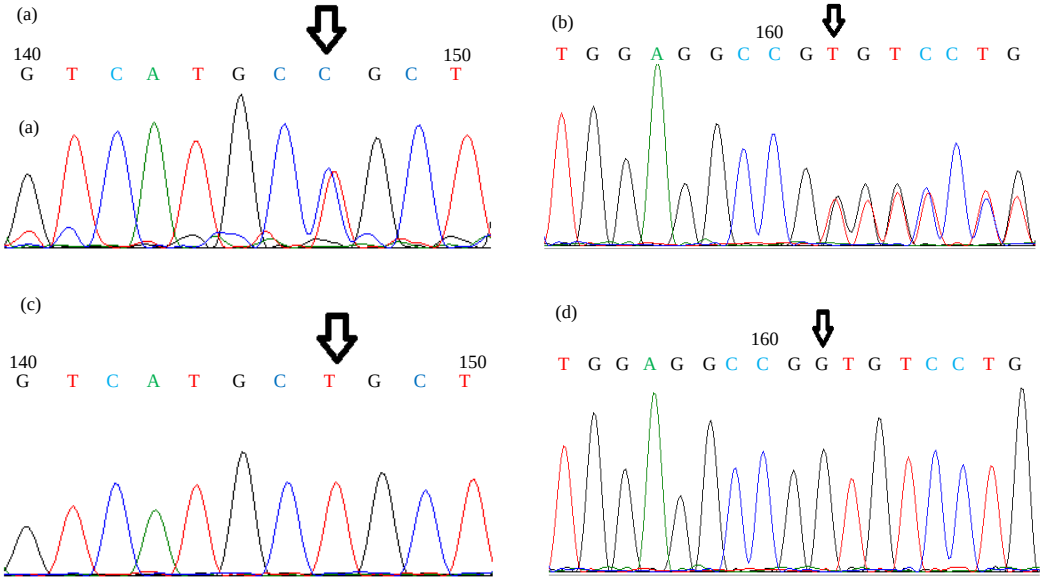

Fig. 3(a-d): Mutation of MC1R gene with substitusion (147C→T) and insertion/deletion (162IndelG), (a) Heterozygote (147TC) in Bali cattle, (b) Homozygote (147TT) in Aceh cattle, (c) Heterozygote individual with deletion (162DelG) in PO cattle and (d) Homozygote individual in Aceh cattle with insertion (162InG)

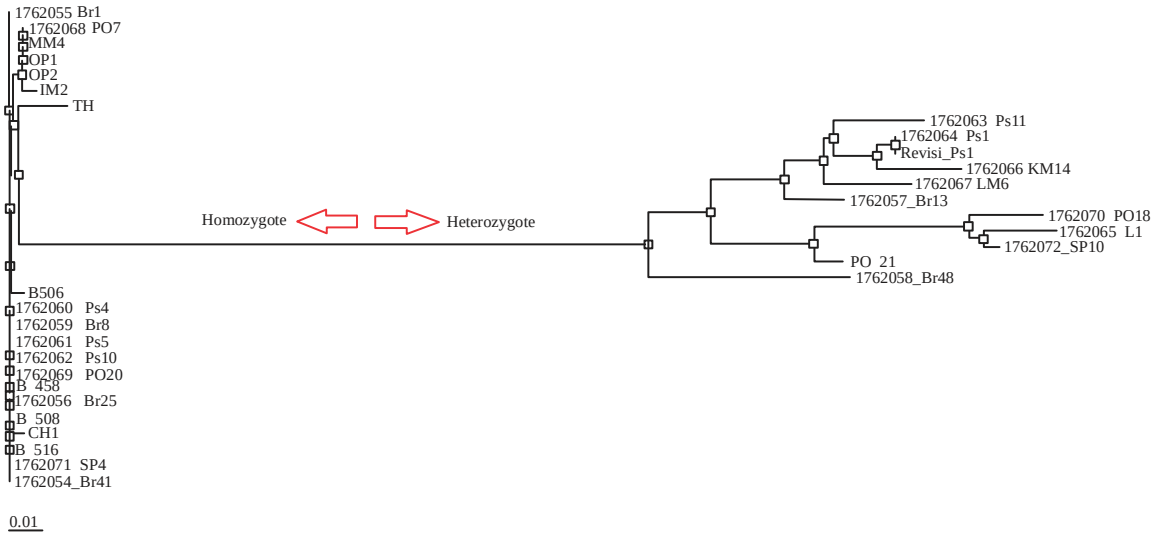

Fig. 4: Phylogenetic tree by Neighbor-Joining/UPGMA method version 3.6a2.1, base on sequence result of 31 individual samples

CONCLUSION

The MC1R gene can be used to identify the genetic variation of local cattle with 5 point mutation and showed heterogeneity in crossed breed cattle with $\chi^2 = 7.64$. Local cattle such as Bali (*Bos sondaicus*) and Aceh (*Bos Indicus*) cattle have higher E allele distribution compare to that of the other local cattle. Indeed, the homozygosity of MC1R gene (EE) in local cattle also higher than that of the crossbred cattle.

SIGNIFICANCE STATEMENTS

- Polymorphism of specific/partial gene is useful for DNA marker

- Phylogenetic tree illustrated the group of cattle breed and distinguished the homozygote/heterozygote of individual cattle which is useful for monitoring of breeding program
- The information of molecular genetics marker can contribute in breeding policy so that give benefit to the country in preventing the extinction of local genetics resources

ACKNOWLEDGMENTS

This study was partly supported by UPR-LPPM UGM year 2013. We would like to thank A. Rastosari, D.E. Putra, W.B. Pintaka Putra, Arnold C.H. Tabun for their contribution in collecting and preparing the DNA samples and technical assistance in Laboratory of Animal Genetic and Breeding, Faculty of Animal Science.

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