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Sequencing and phylogenetic melanocortin-1-receptor (MC1R) gene of bali cows in kupang district, nusa tenggara timur provincy of Indonesia

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Abstract
Bali cattle is a type of beef cattle that must be preserved and developed because Bali cattle are Indonesian germplasm that belong to the genetic resources of local beef cattle. The research objectives is to determine to study the nucleotide sequence and phylogenetic tree melanocortin 1 receptor (MC1R) gene of different coat color in Bali cows of Kupang. Blood samples were taken from seventeen sorrel, eighteen black, and eight white of Bali cows, respectively. The method used research was PCR and DNA sequencing. The amplification of the PCR product to the MC1R-gene of Bali cows in Kupang was at 296bp. Nucleotide change were c.52C>T, c.223C>A in cattle (sorrel, and white Bali cows) and c.147C>T in cattle (black; sorrel and white Bali cows). Nucleotide C plays an important role in the formation of black pigment, changes nucleotide C to nucleotide T causes a change in coat color at sorrel and white Bali Cows. It can be concluded that the melanocortin 1 receptor gene play a role in the formation of coat color in Bali cow Kupang with a percentage of nucleotide similarity were 99.662% at black Bali cow, Sorrel and white Bali cows were 98.986%.

Keywords: Bali cows, MC1R-gene, coat color, Single nucleotide polymorphism

Introduction
Bali cattle are native cattle of Indonesian which has the superiority is adaptability to high temperature, high fertility rate, high carcasses percentage, and being able to eat low-quality feed (Warmadewi *et al*., 2019) [16]. Bali cattle have diverse characteristics, namely when the calf, male and female was sorrel color, then change the black color on the bulls as an adult while the cows remain sorrel (Bidura, 2019) [2].
Bali cattle experiencing a color deviation from normally traits such as deviation coat color in Bali cow from sorrel color to black color (*Injin* cattle), white (*Albino* cattle) and spotted (*Poleng* cattle). Handiwirawan and Subandrio (2004) [5] stated that the Bali cattle are experiencing deviations color of sorrel/brown/black in the legs by 17%, spotted Bali cattle (0.6%) and *Injin* cattle (0.3%).
The differences in the coat color of the cattle result from the pigment that is influenced by the Melanocortin-1-Receptor (MC1R) genes expressed on melanocytes surface. Garcia-Barron *et al.* (2005) [3] suggested that melanin was biopolymer polymorphous and multifunctional consisting of eumelanin (brown-black), pheomelanin (red-yellow), the mixture of melanin (eumelanin and pheomelanin), and neuromelanin. Klungland and Vage (2003) [6] states that the amino acid substitutions in the MC1R gene in cattle and sheep affect the dominance of black and decrease pigmentation MC1R gene domination will be more real with no have been affected by agouti gene.
Coat color variation in the Bali cows can be caused by the melanocortin 1 receptor gene (MC1R). This research was to determine the nucleotide sequence and phylogenetic tree different coat color of Bali cows at Kupang District, Nusa Tenggara Timur Provincy of Indonesia.

Materials and Methods
DNA extraction
The blood samples were collected from Bali cattle raised in the village of Sulamu and Kupang Timur District, Kupang Regency, Indonesia. The Total Samples were 46 blood of cattle are

17 of sorrel, 18 black, 8 white Bali cows and 3 of Bali bull. Blood samples were collected from jugular vein by using vacutainer containing EDTA which was preserved under -20 ^{0}C. DNA was extracted from blood samples by using standard SDS/Proteinase K extraction (Sambrook *et al.*, 1989) [11]. The analysis of DNA samples was conducted on the Laboratory of Animal Breeding, Faculty of Animal Science, Gadjah Mada University, Indonesia.

PCR

MC1R gene amplification using primer pairs based by instructions Li *et al*. (2008) [8] that the MC1R (Forward: 5'-GGACCCTGAGAGCAAGCAC-3'; Reverse: 5'-CTCACCTTCAGGGA TGGTCTA-3'). The DNA amplification process is carried out with a total volume of as much as 10 µl consisted of 0.5 µl DNA, PCR kit 5 µl, 10pmol, and 3.5 µl DDW. Amplification of specific DNA fragments in the MC1R gene was 296bp using Thermal Cycler machine with predenaturation conditions at 95 ^{0}C for 5 min, denaturation at 94 ^{0}C for 30 sec, Annealing at 57°C for 30 seconds and elongation (extension) at 72 ^{0}C for 30 seconds repeated 30 cycles, and final extension at 72 ^{0}C for 10 minutes.

Sequencing and phylogenetic *tree*.

Total volume reaction of the amplification MC1R gene by 30 µl consisted of 1.5 µl DNA, PCR kit 15 µl, 10pmol primers are 3 µl, and 3.5 ml DDW. The DNA sequencing of the PCR product in Korea. The DNA sequencing was aligned, single nucleotide polymorphism by *Bio Edit* version 7.7 and the phylogenetic tree of different coat color Bali cows by using Mega7.

Results and Discussion

PCR MC1R Gene Bali Cows in Kupang

Amplification of DNA fragments amounting 296 base pairs (bp). The position of DNA fragments that are recognized by the PCR primers can be seen in Figure 1

Visualization result electrophoresis PCR product MC1R gene at Bali cows of Kupang with 0,8% agarose gel and using a marker (*ΦX174 DNA/suRI/HaeIII*) as well as marker measurement from band DNA Bali cows of Kupang on fragment amounting 296bp. The band measurement result of DNA amplification in accordance with the instructions Li *et al*. (2008) [8] state that MC1R gene amplification fragment at the white color and red-white color china Holstein cattle amounting 296bp.

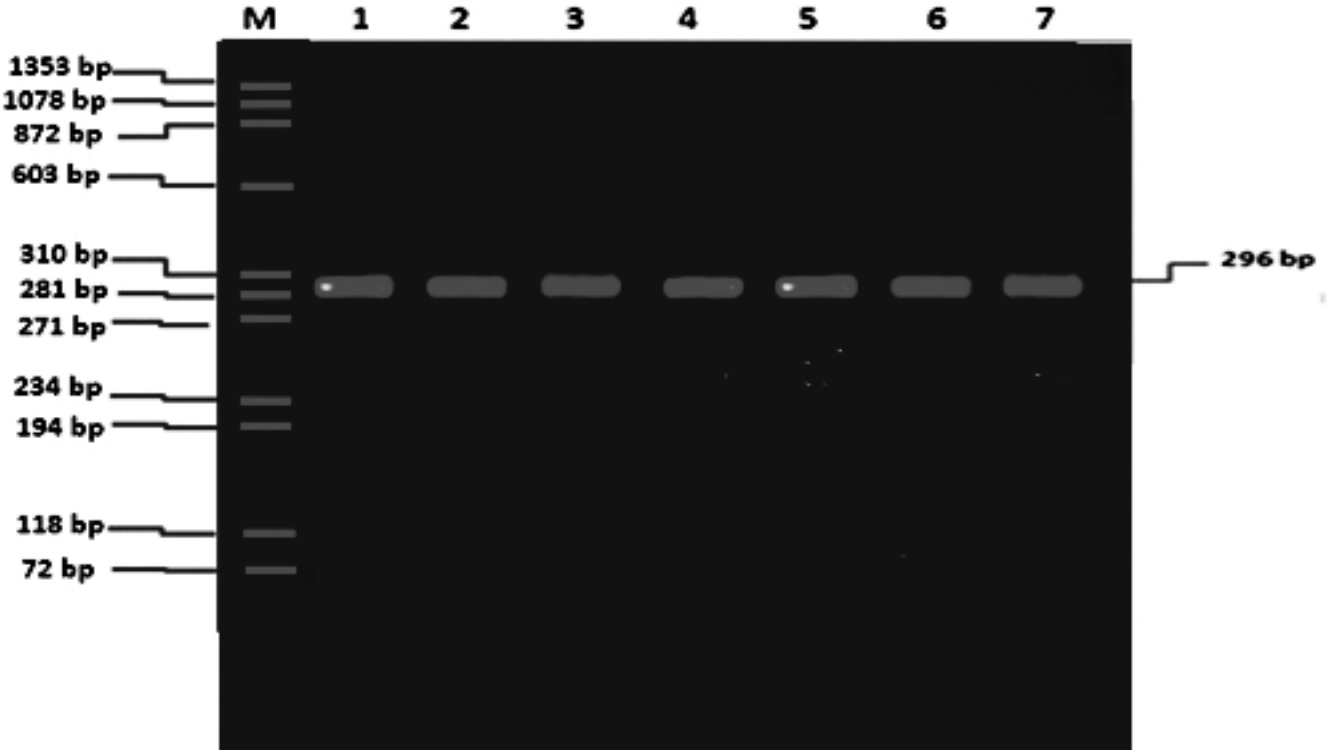

Fig 1: PCR Electrophoresis result of MC1R gene at Sorrel-, Black-, White- Bali Cow and Bali Bull. Line M:M: Marker, Line 1, 2, 3, 4, 5, 6, 7: PCR product (296bp)

DNA Sequencing

The sequencing MC1R gene of Bali cow of Kupang different coat color constitutes a PCR product amounting 296bp. Results of multiple alignments (*ClustalW Multiple Alignment*) of MC1R gene Bali cow Kupang with sequences species standard that taken from *Gen Bank*, and performed analysis of nucleotide diversity and amino acid composition of the compare with *Bos taurus* (NM_174108). The analysis showed that the nucleotide diversity among sampled Bali cow of Kupang different colors with *Bos taurus* found several single nucleotide polymorphisms (*SNPs*) that are different in Figures 2 and 3.

	0	1	1	1	1	1	2	2
	5	3	4	6	6	8	2	6
	2	5	7	0	2	9	3	7
Bos taurus (AF4456411)	C	C	C	C	G	T	C	C
B.GruniesxTaurus (JN123363)	.	.	T	.	.	.	.	.
BreedBrindel (AF547663)	.	.	T	.	.	.	.	.
Brown Swiss	.	.	T	.	.	.	.	.
BSSB (AJ291701)	.	.	T	–	.	.	.	.
Bos taurus (U39469)	.	.	.	.	.	.	.	.
Bos taurus (AF547663)	.	Y	Y	.	.	K	.	Y
Bos taurus (NM_174108)	.	.	.	.	.	.	.	.
Bos taurus (Y13957)	.	.	T	.	.	.	.	.
FH wildtype(Y19103)	.	.	T	.	.	.	.	.
Hanwoo Cattle	.	.	T	.	–	.	.	.
Black Bali Cow	.	.	T	.	.	.	.	.
Black Bali Cow	.	.	T	.	.	.	.	.
Sorrel Bali Cow	T	.	T	.	.	.	A	.
Sorrel Bali Cow	T	.	T	.	.	.	A	.
White Bali Cow	T	.	T	.	.	.	A	.
White Bali Cow	T	.	T	.	.	.	A	.

Fig 2: Multiple Alignment result of Mc1R gen sequen (296 bp) at sorrel, black, and white Bali cows with standard sequen from NCBI GenBank (Note: Identical to the base on top (...), identical to C/T (Y); and identical to G/T (K)

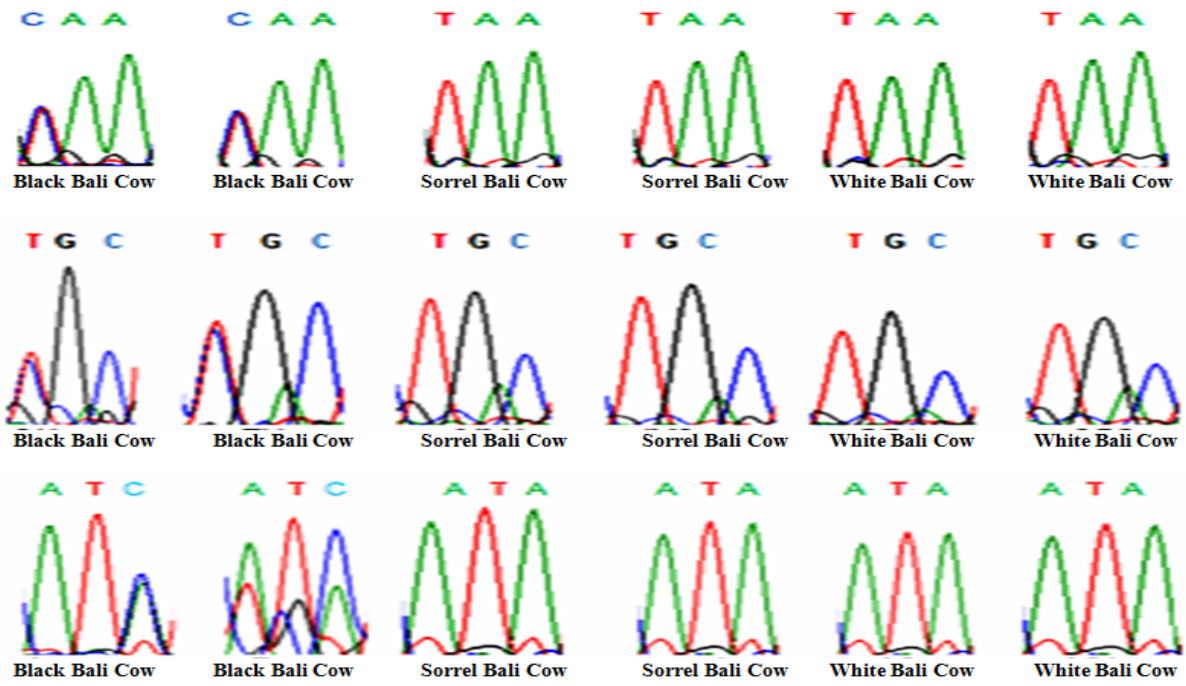

Fig 3. SNP identification of MC1R gene at different coat color Bali Cow of Kupang.

Genetic Distance

Substitutions base per site of the sequence are shown analyzed using the Maximum Likelihood Model Composite (Tamura *et al.*, 2004[14] and Tamura *et al.*, 2011[15]) with a software program MEGA5, showed that the genetic distance between Bali cow with *Bos taurus* can be seen in Tables 1 and 2.

Cattle type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Bos taurus (AF4456411)																	
Bos grunie (JN123363)	0,00																
BreedBrindel (AF547663)	0,00	0,00															
Brown Swiss	0,00	0,00	0,00														
BSSB (AJ291701)	0,00	0,00	0,00	0,00													
Bos taurus (U39469)	0,00	0,00	0,00	0,00	0,00												
Bos taurus (AF547663)	0,00	0,00	0,00	0,00	0,00	0,00											
Bos taurus (NM_174108)	0,00	0,00	0,00	0,00	0,00	0,00	0,00										
Bos taurus (Y13957)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00									
FH-wildtype (Y19103)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00								
Hanwoo cattle	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00							
Black Bali cow	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00						
Black Bali cow	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00					
Sorrel Bali cow	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01				
Sorrel Bali cow	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00			
White Bali cow	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00	0,00		
White Bali cow	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	

Table 1: The genetic distance Analysis of different coat colors Bali cows of Kupang with *Bos taurus* using Maximum Likelihood Model Composite (Tamura *et al.*, 2004 [14] and Tamura *et al.*, [15]).

Cattle type	Total Nucleotide	Total SNP	Total Nucleotide Similarity	Persentage Nucleotide Similarity (%)	Total Nucleotide Diversity (SNP)	Persentage Nucleotide Diversity (%)
Bos taurus (NM_174108)	296	0	296	100,000	0	0,000
Bos taurus (AF4456411)	296	0	296	100,000	0	0,000
Bos taurus-MSHR (U39469)	296	0	296	100,000	0	0,000
Bos taurus (AF547663)	296	0	296	100,000	0	0,000
B.Grunies x Taurus (JN123363)	296	1	295	99,662	1	0,338
BreedBrindel (JQ004019.1)	296	1	295	99,662	1	0,338
Brown Swiss	296	1	295	99,662	1	0,338
Bos taurus (Y13957)	296	1	295	99,662	1	0,338
Fhwildtype(Y19103)	296	1	295	99,662	1	0,338
Black Bali cow	296	1	295	99,662	1	0,338
Black Bali cow	296	1	295	99,662	1	0,338
BSSB (AJ291701)	296	2	294	99,324	2	0,676
Hanwoo cattle	296	2	294	99,324	2	0,676
Sorrel Bali cow	296	3	293	98,986	3	1,014
Sorrel Bali cow	296	3	293	98,986	3	1,014
White Bali cow	296	3	293	98,986	3	1,014
White Bali cow	296	3	293	98,986	3	1,014
Average			294,647	99,543	1,353	0,457
Standar Deviation			1,115	0,377	1,115	0,377

Table 2: Percentage of similarity and diversity nucleotide (SNP) MC1R gene in the Bali cow of Kupang compared with *Bos taurus* (AF4456411) with ClustalW

Phylogenetic tree

The phylogenetic relationship of a species or between species can be identified by genetic distance and determination of suspect origin breeds. These predictions can be done with the preparation of the phylogenetic tree (Phylogenetic tree) some species/group. MC1R gene analysis of genetic distances by using a fragment of 296 bp can be seen in Figure 4.

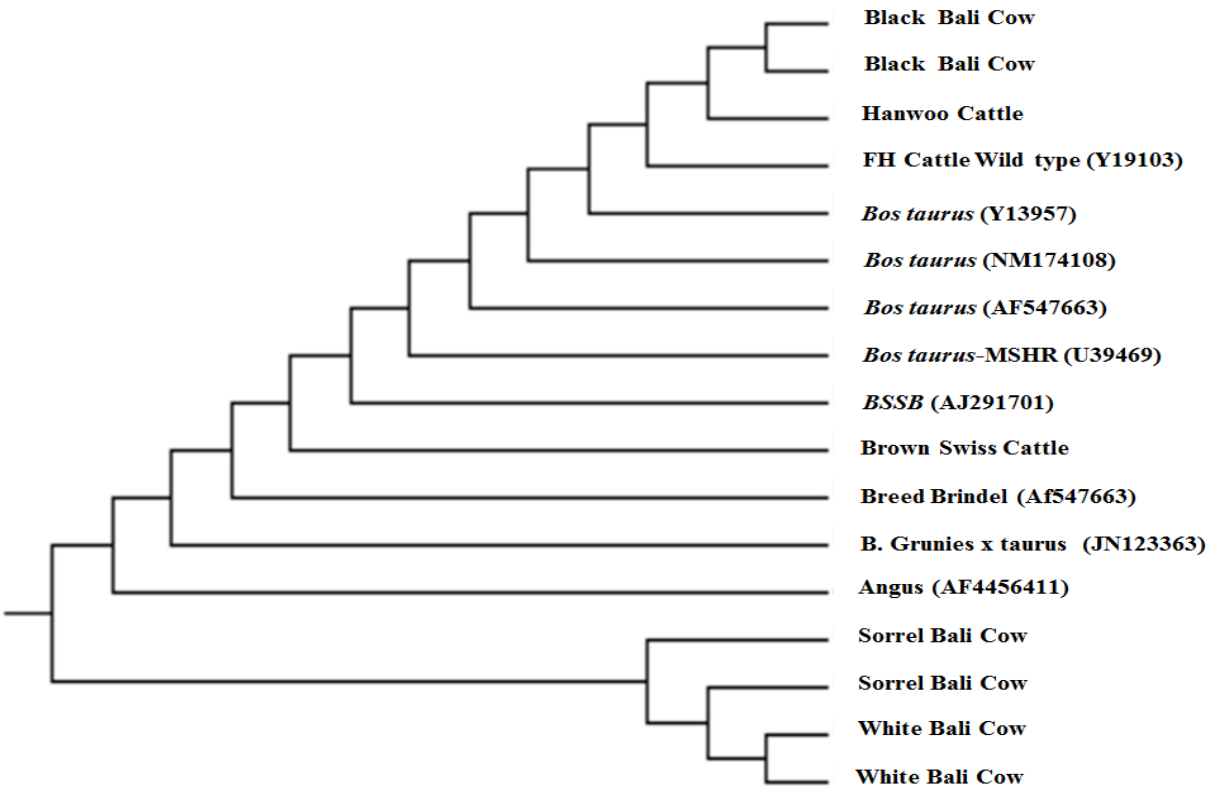

Fig 4: Phylogeny Tree Bali cows of Kupang with compared *Bos taurus*, Hanwoo cattle (*GenBank*)

Discussion

Identify of Single Nucleotide Polymorphism (SNP) of the MC1R gene sequencing results in Bali cow of Kupang found three SNPs were nucleotide changes compared with SNPs MC1R gene of *Bos taurus* from *Gen Bank* (*NCBI*) contained one SNP (G/T). Nucleotide change is c.52C>T, c.223C>A in cattle (Sorrel and white cattle) and c.147C>T in cattle (Black; sorrel, white cattle). Changes Single Nucleotide Polymorphism (SNP) resulting in a change in the genetic code. Nucleotide changes in Figure 5 changes (c.52C>T; c.223C>A) causes changes in the genetic code (CGC>CGT/Arginine and ATC>ATA/isoleucine) but this does not lead to change its amino acid composition (Appendix 4), whereas p147C>T genetic code changes cause changes in the amino acid alanine (CCG) to Leucine (CTG).

SNP identification results on c.52C>T changes experience C to T nucleotide. Nucleotide C plays an important role in the formation of black pigment, nucleotide C to T change causes a change in coat color in sorrel and white Bali cow. When the concentration of cAMP in the cell increases will enable nucleotide C and increases tyrosinase synthesis, causing the increased synthesis of eumelanin and pheomelanin synthesis reduction, thus causing the formation of black leather (Garcia-Borron *et al*., 2005) [3]. Substitution nucleotide T to C at Angus cattle amounting 296 bp carrying the dominant black allele responsible for the black phenotype (Kungland *et al*., 1995) [7]. Hanwoo cattle is not a change nucleotide T to C causes the total eumelanin is low melanin compared with Angus cattle (Mohantry *et al*., 2008) [10]. A black dominant E allele is present in cattle, due to a T296C substitution, as previously observed in Holstein Italian and in other black cattle. A black dominant E allele is present in cattle, due to a T296C substitution, as previously observed in Holstein Italian and in other black cattle. This gain of function mutation is located, as is the bovine “e” allele, in the 2dtransmembrane domain, like 2 out of 3 mutations responsible for black pigmentation in mice. The melanocortin 1 receptor (MC1R) was found to play a major role in pigmentation in many species through regulating the synthesis of two major pigment components, Eumelanin and phaeomelanin (Schmutz *et al*., 2007 [13]; Anderson *et al*., 2009 [1] Miao *et al*., 2010 [9]). Stimulation of MC1R by endogenous agonist α-melanocyte-stimulating hormone (α-MSH) leads to production of eumelanin through activating tyrosinase, and gives black coat color. Under the low level of tyrosinase without MC1R stimulation, either caused by a non-functional MC1R or inhibited by antagonist agouti protein, leads to the dilution of eumelanin and gives light coat color, such as brown or red (Gutierrez *et al*., 2007) [4].

Table 4 shows that the overall average and standard deviation of percentage nucleotide similarity Bali cows with some standard nucleotide sequences at *Gen Bank* compared with the Bos taurus sequence (NM_174108) was 99.543 ± 0.377, and percentage nucleotide diversity was 0,457 ± 0,377. Comparison of nucleotide similarity Bali cow of Kupang with the *Bos taurus* is a black Bali cow (99.66%), sorrel and white Bali cows are (98.99%). The highest percentage of nucleotide diversity in sorrel and white Bali cows are (1.01%), then the black Bali cow is (0.34%).

Thus it can be stated that the black Bali cows have MC1R gene sequence similarity with *Bos taurus* in producing eumelanin to produce a black color. Klungland and Vage (2003) [6] suggested that in cattle and sheep, amino acid substitutions in the MC1R affect the dominance of black pigmentation decrease. MC1R is a member of the G-protein-coupled receptors (GPCR) superfamily. It expresses in melanocyte of the epidermis and hair follicle. MC1R can be activated by the adrenal cortical hormone and the -melanocyte stimulating hormone (MSH), and participates in the coupling of the cAMP signal, in stimulating the synthesis of eumelanin,

and causing a black coat or skin phonotype. A-MSH cannot bind with MC1R, which stimulates the synthesis of phaeomelanin, causing the deposit of red or yellow pigment. The coded MC1R protein allele performs the adenylate cyclase function. When the concentration of CAMP in the cell increased, c gene will get activated and increases the tyrosinase synthesis, causing the increase of eumelanin synthesis and reduction of phaeomelanin synthesis, thus causing the black coat color formation (García-Borrón *et al*., 2005) [3]. Sánchez-Más *et al.* (2005) [12] reported that Thr314, Cys315, and Trp317 were the key amino acids in the binding of MC1R and -MSH.

The phylogenetic tree of the MC1R gene is 296 bp using composite model maximum likelihood method (Tamura *et al.*, 2004 [14] and Tamura *et al.*, 2011 [15]) with compared *Bos taurus* species. Phylogenic tree Bali cattle based on MC1R gene sequences after comparing with several other *Boss taurus* speises, it can be seen that black Bali cattle are in the group approaching *Bos taurus* with a percentage of nucleutide similarity of 99.668% and nucleutide diversity of 0.34% while Bali cattle are sorrel and white coat color bali cows genetic distance 99.99% and nucleutide diversity as much as 1.01% of *Bos taurus.*

Conclusion

We conclude that the melanocortin 1 receptor (MC1R) gene play a role in the formation of coat color in Bali cow in Kupang with a percentage of nucleotide similarity were 99.662% at black Bali cow, Sorrel and white Bali cows were 98.986%.

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