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Investigation of polymorphism and expression of the tyrosinase (TYR) gene as a gene controlling coat color in Bali cattle

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ABSTRACT

Cattle coat color is governed by numerous genes, notably the tyrosinase gene (TYR). This study analyzed coat color anomalies like albinism and white spotting in Bali cattle. It aims to discern the TYR gene's diversity, expression patterns, and correlation with coat color abnormalities. The research encompassed 189 cattle, including those with standard coat color (n=53), white-spotted (n=11), and albino (n=17) Bali cattle, as well as Simmental (n=37), Limousin (n=14), Madura (n=21), and Peranakan Ongole (PO) cattle (n=36). Total DNA was extracted and the TYR gene in exon 1 was amplified using forward and reverse primers with a target amplicon length of 994 bp. Direct sequencing unveiled TYR gene diversity, analyzed using BioEdit and MEGA6 software to identify SNPs. PCR-RFLP was used for SNP genotyping, while qPCR analyzed TYR gene expression. Two mutations (SNP g. 939A>G and SNP g. 887C>T) were discovered in Bali cattle TYR exon 1. SNP g. 939A>G exhibited polymorphism, with the highest GG genotype frequency in standard Bali cattle, indicating a high G allele frequency. Conversely, Madura, Simmental, Limousin, and PO cattle had the lowest allele frequency. Chi-square (χ^2 test) results showed non-significance across all cattle types. TYR gene expression differed significantly between standard Bali cattle and albinos ($p<0.05$).

Keywords: *Bali cattle, Coat color, PCR-RFLP, SNP, TYR gene expression*

INTRODUCTION

Coat color is one of the distinctive phenotypes in cattle (Senczuk *et al.,* 2020) and is generally controlled by genetic factors (Gao *et al.,* 2017). Cattle worldwide exhibit variations in coat color, such as the presence of brown color in newborn calves that later change to black as they mature, as well as differences in coat color between male and female cattle. Some cattle have a white base pigment (Charolais cattle) (Gutiérrez-Gil *et al.,* 2007) while others have red and black pigments (Angus cattle) (He *et al.,* 2022). Cattle colors such as spotting, dilution, roan, and brindle are determined by different genes that interact with the base coat color genes (Kunene *et al.*, 2022). Pigmentation is crucial in productivity and adaptability to the environment,

making coat color a significant factor in livestock breeding (Goud *et al.,* 2021). Tyrosinase (TYR) is a monooxygenase involved in melanin biosynthesis, catalyzing the o-hydroxylation of tyrosine (monophenol) into 3,4-dihydroxy-L-phenylalanine or L-DOPA (o-diphenol), and the oxidation of L-DOPA into dopaquinone (o-quinone), which can be further converted into melanin pigments through enzymatic and non-enzymatic reactions (Nunes dan Vogel 2018). Tyrosinase also plays a crucial role in the biosynthesis of melanin, the most important pigment synthesized through melanogenesis in melanocytes, serving as the primary determinant of coat and skin color (Kim dan Uyama 2005).

Several genes such as melanocortin 1 receptor (MC1R) (extension), KIT (dominant white), ASIP (agouti), TYRP1 (brown), KITLG (roan), MITF (white-spotted), and TYR (albinism) have been identified and found to influence coat color pigmentation (Schmutz 2012). The genes KIT and MC1R have been reported by (Jakaria *et al.,* 2023) to have Single Nucleotide Polymorphisms (SNPs) in the partial intron 2 exon 3 region of the KIT gene in Bali cattle. However, these SNPs do not indicate standard, white-spotted, or albino coat colors in Bali cattle. On the other hand, the MC1R gene is monomorphic. The Tyrosinase gene has been extensively studied in various cattle breeds and other animals, including Brown Swiss cattle (Schmutz *et al.,* 2004). Korean native cattle (Kim *et al*., 2006) Fleckvieh cattle (Stein *et al.,* 2011) male Deoni cattle (Dongre *et al.,* 2023), water buffalo (Cecília *et al.,* 2012), deer (Chen *et al.,* 2023) and albino cattle and buffalo (Putra 2019) It has also been studied in Tianzu white yak (*Bos grunniens*) (Zhang *et al.,* 2012). Mutations in the tyrosinase gene have been identified as determinants of oculocutaneous albinism (OCA) in humans and animals (Tripathi *et al.,* 1992). In Bali cattle (*Bos javanicus*), standard males have a black coat color, while females have a reddish-brown coat. Their lower legs are white, resembling the appearance of wearing socks, and they have a crescent-shaped white patch on their rear (BSN, 2020). However, recently, abstandardities in the coat color of Bali cattle, such as albinism, have been observed that do not conform to the typical criteria for Bali cattle breeding.

Cases of albinism in Bali cattle have been reported at a rate of 7.63%, and these were found in Kupang (Tabun *et al.,* 2016). Albino Bali cattle are also referred to as "Sapi Putih Taro" and were discovered in their original breeding area, which is the village of Taro, Tegallalang District, Gianyar Regency, Bali Province, Indonesia. The population of these white cattle is quite limited, numbering around 33 individuals, and their habitat is confined to the Taro Village forest, as reported by (Heryani *et al.,* 2018). The uniqueness of these white cattle includes their entirely white coat, and they are considered sacred, revered, and respected by the community in Taro Village. There are specific designations for the females, referred to as "Dayu Biang," and the males, referred to as "Ida Bagus." In their care, they are not to be subjected to harsh language, they are treated with politeness, and they are not utilized for plowing fields or for commercial purposes (Tabun *et al.,* 2016). They are only used for religious ceremonies such as "memukur" (Atma wedana), "Tri Buana," and "Eka Dasa Rudra," and they are not to be sold or slaughtered except for religious rituals. Furthermore, it is forbidden to pierce the nostrils of these white cattle. The Taro white cattle are highly revered by the Balinese Hindu community (Heryani *et al.,* 2018). The TYR gene, which encodes the enzyme tyrosinase for melanin synthesis(Anello *et al.,* 2019), has been used to identify coat color abnormalities, such as albinism and white spotted, in cattle. Genetic markers, specifically the TYR gene, are crucial for identifying albinism in Bali cattle. These cattle exhibit a genetic anomaly that deviates from the criteria set in the Indonesian National Standard (SNI) for Bali cattle breeding. Based on previous research, the identification of the TYR gene in Bali cattle has never been reported. Therefore, this research aims to identify genetic variations in the tyrosinase gene and its expression in normal and albino Bali cattle, within the coding region of exon 1 of the TYR gene, and to analyze its association with coat color abnormalities in Bali cattle.

MATERIALS AND METHODS

Study Period and Location

The research was conducted from June to September 2023. The study was carried out at several locations, including genetic analysis in the Molecular Genetics Laboratory, Division of Animal Breeding and Genetics, Faculty of Ani-

mal Science, IPB University. Blood sample collection from Bali cattle with standard and albino coat colors was performed in the village of Taro and the Livestock Centre in Sobangan, Bali Province. The research was conducted from June to September 2023.

Sample Collection and DNA Extraction

A total of 189 blood samples from cattle were used in the study, including: standard-coated Bali cattle (n=53); white spotted Bali cattle (n=11); albino Bali cattle (n=17); Simmental cattle (n=37); Limousin cattle (n=14); Madura cattle (n=21); and Peranakan Ongole (PO) cattle (n=36) as a comparative group. The standard-coated Bali cattle samples were collected from the Sobangan livestock center, and the 17 blood samples from albino Bali cattle were collected from the village of Taro, Bali. The remaining blood samples were obtained from the Molecular Genetics Laboratory of Animal Science, Faculty of Animal Science, IPB University. Blood samples were collected using a venoject needle from the Jugular vein in the neck area of the cattle. The venoject needle was connected to a vacutainer tube containing EDTA. Approximately 5 mL of blood was drawn, and the blood was stored in a refrigerator for further analysis. DNA extraction was performed using the Geneaid™ protocol (Geneaid Biotech Ltd., New Taipei City, Taiwan).

Amplification and Sequencing

The primer design for sequencing analysis was carried out using Primer3 and subsequently analyzed using the Multiple Primer Analyzer and Primer Stat programs. The primer design for sequencing analysis was based on sequence data obtained from GenBank NCBI (National Center for Biotechnology Information) with the accession number NC_037356.1. The target amplicon length was 994 bp, and it was derived from exon 1 of the TYR gene. The primer design was initiated with the forward primer sequence 5'-GGA GCT GGA AAG GGA AGA GT-'3 and the reverse primer sequence 5'-GGC AGG AGA ATA ACA GAC GG-'3. Amplification of the TYR gene was conducted through the PCR technique, utilizing the AB System PCR machine. The gene amplification process involved a mixture composed of 0.3 µL each of the forward and reverse primers, 12.5 µL of MyTaq HS RedMix, and 9.9 µL of nuclease-free water (NFW), which was carefully transferred to a 1.5 mL tube. This prepared mixture was then evenly distributed among the DNA samples, previously extracted from 2 µL of blood and transferred into 0.2 mL tubes. The PCR procedure consisted of several key steps, commencing with predenaturation at 95°C for 5 minutes. This was followed by denaturation at 95°C for 10 seconds, annealing at 59°C for 20 seconds, extension at 72°C for 30 seconds, and concluding with a final extension at 72°C for 5 minutes. These conditions facilitated the specific amplification of the TYR gene, a critical aspect of the research. Subsequently, PCR product electrophoresis was carried out using 3 µL of the PCR product on a 1.5% agarose gel for 35 minutes. The agarose gel was prepared by mixing 0.45 g of agarose powder with 30 mL of 0.5x TBE. The agarose solution was heated in a microwave for approximately 4 minutes. It was then cooled down using a magnetic stirrer at a speed of 50 rpm for about 2 minutes. Subse-

Table 1. The primer sequences for the TYR gene and housekeeping genes (GAPDH and β-actin) for gene expression analysis

Gene	Primer Sequences	Amplicon Length (bp)	Annealing Temperature (°C)	GenBank Code
TYR**	F: 5'-ACAAGATGGTGAAGGTCGGA-3'	115	52	ENSBTAG00000011813
	R: 5'-CATTGATGGCGACGATGTCC-3'			
GAPDH*	F: 5'-ATCTACTCAGCCCAGCATCC-3'			
	R: 5'-CGCAGTAATGGTCCCTCAGA -3'	101	52	ENSBTAG00000014731
β-actin*	F:5-ATGATATTGCTGCGCTCGTG-3'			
	R: 5-GTGCTCAATGGGGTACTTGA-3'	212	54	-

**= target gene; *= housekeeping gene

quently, 1 µL of PeqGreen was added and homogenized for approximately 2 minutes before being placed into a mold. The gel solidified over approximately 30 minutes at room temperature. The prepared agarose gel was then placed in an electrophoresis tank filled with 0.5x TBE buffer. A 3 µL volume of the amplicon was loaded into the wells along with a 100 bp marker, and electrophoresis was conducted at a voltage of 100 V (± 35 minutes). The agarose gel, following electrophoresis, was examined for the length of DNA bands using a UV Transilluminator. The analysis of PCR product sequencing of 4 samples of normal and albino Bali cattle was conducted using the laboratory services of 1st Base in Selangor, Malaysia, through PT Genetika Science Indonesia.

Genotyping (PCR-RFLP)

The PCR products were dispensed into 0.5 mL Eppendorf tubes, with 5 µL in each tube. To these PCR products, a mixture of 2 µL was added, which consisted of 0.3 µL of restriction enzyme, 0.7 µL of 10x buffer R, and 1 µL of nuclease-free water (NFW) in a 1.5 mL tube. Based on the results of the enzyme determination using NEBcutter (https://nc3.neb.com/NEBcutter/), the restriction enzyme used was *Bst*XI (5'-CCANNNNN/NTGG-3'). The reaction mixture was incubated for 4 hours at 37°C. After digestion with the restriction enzyme, the DNA samples were then subjected to electrophoresis on a 2% agarose gel at a voltage of 100 V for 40 minutes. The final results were visualized under a UV Transilluminator machine. The DNA bands that appeared at this stage were compared to a marker to determine their length. Genotype determination was based on the length of the DNA bands.

RNA extraction and reverse transcriptase (cDNA)

RNA extraction was performed using the Geneaid™ Protocol (Geneaid Biotech Ltd., New Taipei City, Taiwan). Reverse transcriptase cDNA synthesis was conducted following the kit's protocol for cDNA synthesis (Toyobo).

qRT-PCR and data analysis

Complementary DNA (cDNA) was utilized for gene TYR expression quantification through quantitative real-time PCR (qRT-PCR) conducted on an Analytik Jena AG qTower 4-channel instrument in Germany. The real-time PCR reaction involved the use of the SYBR Green Select Master Kit (Applied Biosystem, USA). The reaction mixture comprised 5 µL of SYBR Green Select Master Kit, 0.5 µL of each forward and reverse primer (refer to Table 1), 1 µL of cDNA from the samples, and 3 µL of nuclease-free water (NFW). The PCR conditions were as follows: an initial denaturation stage at 95°C for 5 minutes, denaturation at 95°C for 10 seconds, followed by an annealing stage at 54°C for 20 seconds, an extension stage at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes, repeated for 40 cycles. The target gene TYR sequences were analyzed using FinchTV (https://www.softpedia.com/get/Science-CAD/FinchTV.shtml)andBioEdit7.2 (https://bioedit.software.informer.com/). The identification of mutations or single-nucleotide polymorphisms was conducted using Clustal W in MEGA software version 10 (https://www.megasoftware.net/). Genotype frequency, allele frequency, observed heterozygosity, expected heterozygosity, and Hardy-Weinberg equilibrium (HWE) balance were determined using POPGEN version 1.32 (https://sites.ualberta.ca/~fyeh/popgene_download.html). The data from qRT-PCR were analyzed using the 2^-ΔΔCT method (Livak and Schmittgen, 2001) to determine the relative expression changes of the TYR gene in albino cattle compared to standard coat color cattle, relative to the expression of the housekeeping gene (Table 1). In this method, ΔΔCT is calculated as follows: ΔΔCT = (average Ct_{target} in the target group - average $Ct_{housekeeping}$ in the target group) - (average Ct_{target} in the control group - average $Ct_{housekeeping}$ in the control group). The genotypes with +/+ and the standard coat color phenotype were grouped into the control group. The difference in TYR gene expression between albino and standard coat color Bali cattle was tested using a *t*-test, where $p<0.05$ was considered statistically significant. The *t*-test was conducted using SPSS software version 29.

RESULTS AND DISCUSSION

Discovery Single Nucleotide Polymorphism of TYR Gene

The TYR gene was successfully amplified at annealing temperature of 59°C, resulting in a

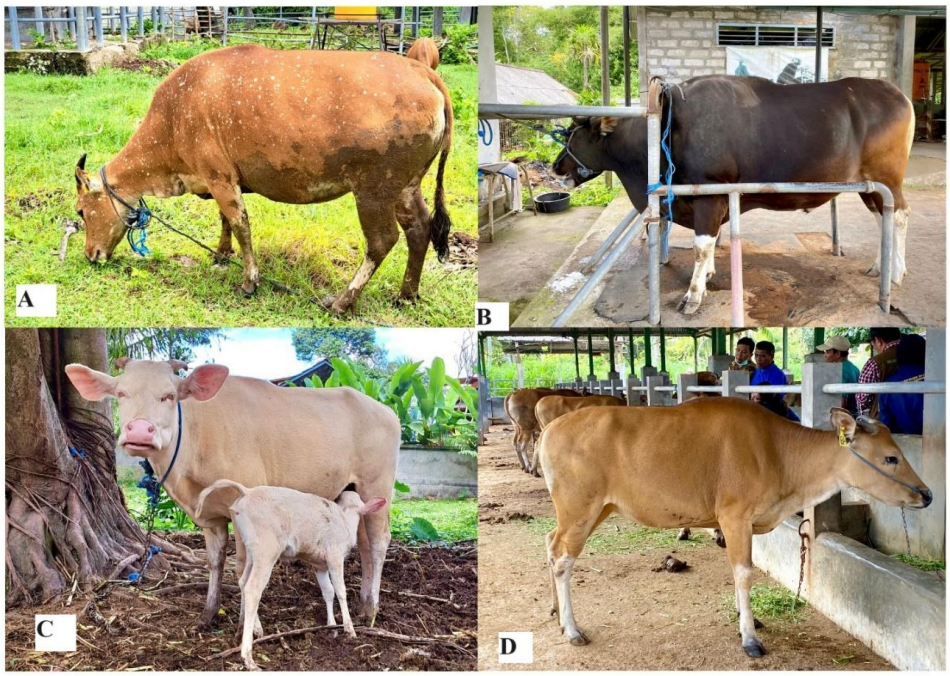

Figure 1. Coat color variations in Bali cattle, including: (B, D) standard, (A) white spotted, C) albino.

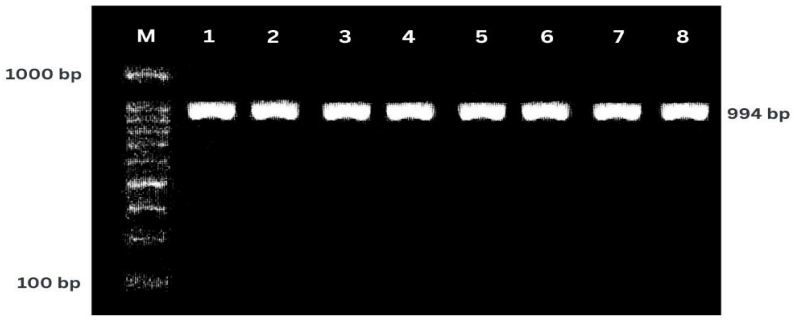

Figure 2. Electrophoresis results from the polymerase chain reaction (PCR) products of the tyrosinase genes at exon-1 (M = 100 bp marker; lines 1–8 represent samples)

target amplicon length of 994 bp (Figure 2), in Bali cattle with both standard and albino phenotypes (Figure 1). Two mutations (SNP g. 939A>G and SNP g. 887C>T) were identified within the coding region of the TYR gene, as indicated in (Figure 3). The SNP g.939A>G is a nonsynonymous SNP, while the SNP g.887C>T is synonymous. Synonymous SNPs do not alter the amino acid sequence, while nonsynonymous SNPs cause changes in the amino acids. Based on previous research, it's generally believed that synonymous SNPs do not have a significant impact because they primarily maintain the protein's primary sequence (Hunt *et al.,* 2009).

The success and purity of the PCR product produced are influenced by several parameters, one of which is the annealing temperature (Rychlik *et al.,* 1991). This process can have an impact on the success of DNA amplification using the PCR method. Figure 2 shows that the analyzed sample was successfully amplified at the optimized temperature of 59°C with a target amplicon length of 994 bp.

Allele and Genotype Frequencies and Chi-Square Test of SNP g.939A>G

Table 2. The frequency of genotype and allele in SNP g. 939A>G in exon 1 of the TYR gene using PCR-RFLP technique

Population	N	Genotype Frequency			Allele Frequency		He	Ho	$\chi 2$ Test
Bali (normal)	53	AA (3)	AG (24)	GG (26)	A	G	0,45	0,41	ns
		0,06	0,45	0,49	0,28	0,72			
Bali (albino)	17	AA (5)	AG (6)	GG (6)	A	G	0,35	0,5	ns
		0,29	0,35	0,35	0,47	0,72			
Bali *(white spotted)*	11	AA (1)	AG (5)	GG (5)	A	G	0,45	0,43	ns
		0,45	0,45	0,09	0,32	0,68			
Madura	21	AA (21)	AG (0)	GG (0)	A	G	0,00	0,00	nc
		1,00	0,00	0,00	1,00	0,00			
Limosin	14	AA (14)	AG (0)	GG (0)	A	G	0,00	0,00	nc
		1,00	0,00	0,00	1,00	0,00			
Simmental	37	AA (37)	AG (0)	GG (0)	A	G	0,00	0,00	nc
		1,00	0,00	0,00	1,00	0,00			
Pranakan ongole (PO)	36	AA (36)	AG (0)	GG (0)	A	G	0,00	0,00	nc
		1,00	0,00	0,00	1,00	0,00			

SNP= single nucleotide polymorphisms; N= sample size; ns= not significant; nc=not calculated

Table 3. The mean values of Cycle Threshold (CT), ΔCT, ΔΔCT, 2^(-ΔΔCT), and t-test for the tyrosinase (TYR) gene using GAPDH and β-Actin housekeeping genes in Bali cattle

Phenotype	HK Gene	CT	ΔCT	ΔΔCT	2 ^-ΔΔCT	Uji-T (P-*value*)
Standard (n=15)	GAPDH	31,48 ± 0,86	8,25 ± 1,06	7,11 ± 1,06	59,97 ± 47,88	s
Albino (n=19)		32,30 ± 0.48	8,22 ± 0,54	0,02 ± 0,54	3,06 ± 1,23	
Standard (n=3)	β-Actin	31,61 ± 1.20	4,53 ± 1,18	0,00 ± 1,18	2,01 ± 1,57	ns
Albino (n=6)		30,38 ± 0.95	5,38 ± 1,34	0,85 ± 1,34	2,23 ± 1,13	

n=number of samples; HK=housekeeping gene; ns=not significant (p>0.05); s=significant (p<0.05)

The new SNP g.939A>G is found exclusively in Bali cattle and differs from the known SNP g.887C>T found in other cattle breeds, as reported in the GenBank data (Ensembl: ENSBTAG00000011813) under the code (rs449429955). Moreover, SNP g.939A>G is a potential candidate as a genetic marker due to its ability to cause an amino acid change, specifically, the alteration from serine (AGC) to glycine (GGC). Both amino acids belong to the non-essential category. However, a deficiency in the amino acid glycine could harm melanocytes and lead to vitiligo, a condition where the skin is unable to produce melanin effectively (Marzabani *et al.,* 2021).

The allele and genotype frequencies of seven cattle populations are presented in Table 2. In Bali cattle, three genotypes were identified, whereas in other cattle populations, including Madura, Simmental, Limousin, and Peranakan Ongole (PO), only a single genotype was observed at the SNP g.939A>G locus. The allele frequencies in the three Bali cattle subpopulations (white-spotted, albino, and standard) exhibit values ≤ 0.99. This observation underscores the polymorphic nature of the TYR gene in Bali cattle, while in the Madura, Simmental, Limousin, and PO populations, the gene remains monomorphic. According to Allendorf *et al.,* (2010) a population is considered polymorphic if the allele frequency within a large population falls within the range of ≤ 0.99 to ≥ 0.01, and when more than one allele is identified.

Following the chi-square test, the SNP g.939A>G in all Bali cattle populations is in Hardy-Weinberg equilibrium (P>0.05), whereas in

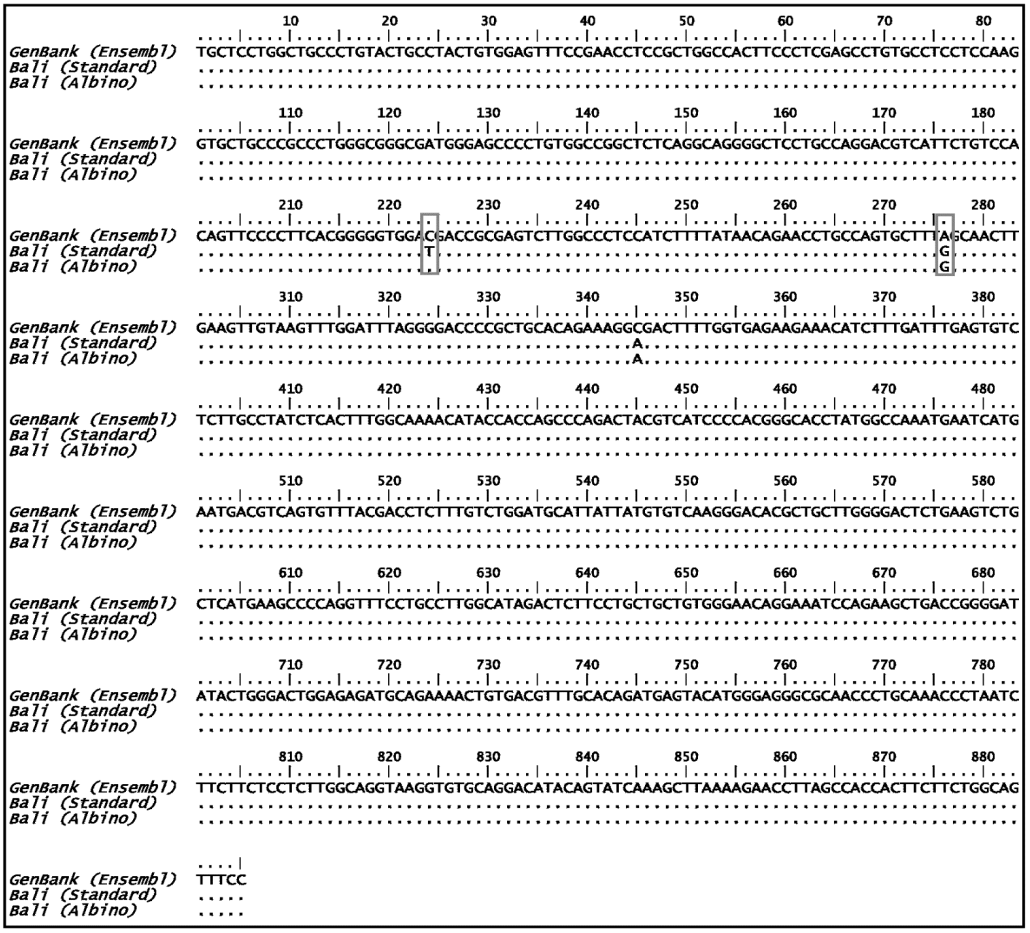

Figure 3. Nucleotide sequence alignment of the tyrosinase (TYR) gene exon 1 in Bali cattle, including standard and albino coat color.

the Madura, Simmental, Limousin, and Peranakan Ongole (PO) cattle populations, it could not be analyzed due to their monomorphic nature. As per Wu *et al.,* (2004), the disequilibrium of SNP polymorphism is attributed to factors such as non-random mating, mutation, and selection within the population.

The Expression of TYR gene in Bali cattle

The results of TYR gene expression analysis in standard coat color and albino phenotypes of Bali cattle are shown in Figure 5. Higher TYR gene expression levels increase melanin production, resulting in darker color expression, whereas low or almost absent melanin production results in white or lighter color expression (Munyard, 2011).

Table 3 presents the expression values (2^-ΔΔCT) of the TYR gene in Bali cattle with standard coat color, showing higher expression levels compared to other phenotypes. Based on the qRT-PCR analysis, the TYR gene expression values for both phenotypes indicate differences. Statistical analysis (t-test) shows significant differences (p<0.05) in TYR gene expression between Bali cattle with standard coat and albino phenotypes on the GAPDH housekeeping gene, as presented in Table 3.

TYR gene expression can distinguish between the albino and standard coat color populations of Bali cattle, however, TYR gene expression in standard coat color and albino Bali cattle on the β-actin housekeeping gene shows nonsignificant results (p>0.05). This may be due to the sample factor used for gene expression analysis being blood samples, resulting in the target gene not being maximally expressed in blood.

The use of the GAPDH housekeeping gene in this study tends to have higher sensitivity compared to the β-actin housekeeping gene. This can be observed in Figure 5, where the expression values or fold change of the TYR gene using the

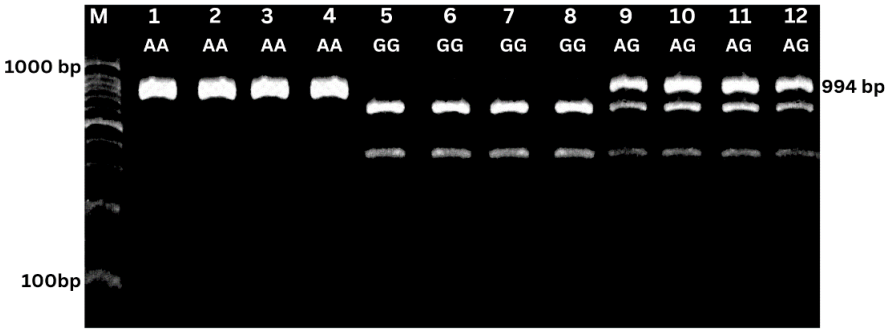

Figure 4. The Genotyping results using PCR-RFLP for SNP g. 939A>G (M=marker; 1-4=Madura, Simmental, Limousin, and PO; 5 & 10= Bali (standard); 6 & 11= Bali (albino); 7, 9 & 12= Bali (white spotted); AA, GG, and AG=genotype).

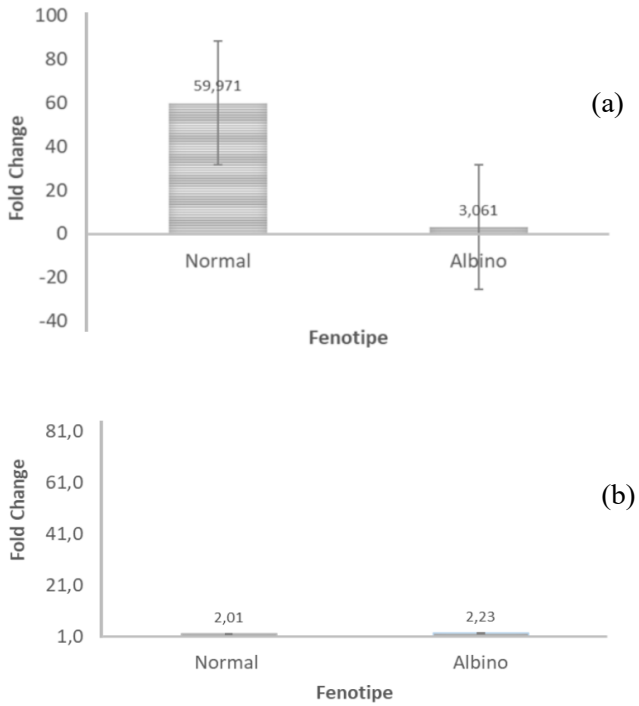

Figure 5. Expression of TYR gene in Bali cattle standard and albino coat color: expression using GADPH housekeeping gene (a); expression using β-Actin housekeeping gene (b).

GAPDH housekeeping gene reached 59.971, while the β-actin housekeeping gene only reached a fold change of 2.23. In the β-actin housekeeping gene, the TYR gene expression in albino Bali cattle is higher compared to those with standard coat color. However, the GAPDH housekeeping gene shows the opposite expression pattern, with albino coat color exhibiting lower TYR gene expression compared to standard coat color.

CONCLUSION

Analysis shows two mutations (SNP g. 939A>G and SNP g. 887C>T) in Bali cattle. These SNPs don't mark coat color. Yet, SNP g. 939A>G is unique to Bali cattle, absent in other breeds. It's polymorphic in Bali but monomorphic in Madura, Simmental, Limousin, and Peranakan Ongole (PO) cattle. TYR gene expression, using GAPDH, distinguishes standard and albino coats in Bali cattle. However, β-actin can't differentiate.

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