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TLR4, BTN1A1, and *Pit-1* polymorphisms and their associations with the estimated breeding values of growth traits of Brahman cattle in Thailand

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Abstract

The molecular analysis of genes is considered an acceptable tool for accelerating the genetic improvement of production performance in cattle. Single nucleotide polymorphisms (SNPs) in *Toll-like receptor 4 (TLR4)* and *Butyrophilin subfamily 1 member A1 (BTN1A1*) genes are related to immunity and may affect all aspects of cattle production. *Pituitary-specific positive transcription factor 1 (Pit-1*) is related to expression of the growth hormone-releasing factor gene. There were used in this study to examine the associations with estimated breeding values (EBVs) of growth traits [birth weight (BW), weaning weight (WW), and yearling weight (YW)] of Brahman cattle. Two hundred sixty-five cattle blood samples were obtained for DNA extraction, and gene polymorphisms were analyzed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The *TLR4* gene was associated with the EBV of WW, which was significantly higher with the AB genotype than with the AA genotype ($p \leq 0.05$) in cattle. This gene was associated with the EBV of YW, which was significantly higher in the cattle with the AB genotype than in those with the AA genotype ($p \leq 0.05$). Cattle with the genotypes AA and AB had a higher ($p \leq 0.05$) EBV of WW than cattle with the BB genotype of the *BTN1A1* gene, which was also significant. The *Pit-1* gene was associated with the EBV of BW, which was significantly lower in cattle with the genotype BB than in those with the genotype AA ($p \leq 0.05$). The possibility of selecting growth traits using these three genetic markers is therefore interesting.

Keywords: Thai Brahman cattle, *TLR4*, *BTN1A1, Pit-1,* Growth traits, EBV

1. Introduction

As of 2021, Thailand's total beef cattle population was approximately 6.2 million head [1]. In recent years, the country has experienced a surge in beef consumption, which cannot be met by current cattle production. Thailand's annual beef consumption is approximately 170,000 tons, with an average per capita consumption of 1.8 kilograms. In 2020, Thailand imported 42,940 tons of beef, which accounted for approximately 25% of its total domestic consumption [2]. Therefore, cattle farmers in Thailand must increase their cattle productivity to satisfy domestic demand. Brahman cattle are among the most popular breeds in Thailand and have been raised since 1954. Compared to Brahman in other nations, Thai Brahman calves have lower birth weights (BW) and weaning weights (WW) (28.51 kg and 142.8 kg, respectively) [3]. Hence, rearing Brahman cattle is a critical problem in Thailand since many factors, including genetics, can affect their growth, meat and reproduction. Genetic markers obtained through candidate genes that are linked to known physiological functions associated with desired traits can aid in the genetic selection process to improve the growth of Brahman cattle breeds in Thailand. Diseases can have detrimental effects on all aspects of cattle production, including growth and reproduction. When the animals' immune systems are performing sufficiently, the cattle are in good health. Genes related to health are important for animal selection to improve growth traits [4]. In general, genetic variants in the *TLR4* gene have been

demonstrated to affect pathogen recognition and the innate immune response of the host [5]. Based on expected haplotypes for innate immune genes, the specific *Bos taurus taurus* beef and dairy breeds could not be differentiated from one another [6]. The Toll-like receptor (TLR) family is crucial for innate immunity, with *TLR4* being a significant subfamily that stimulates the immune system by recognizing pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include lipopolysaccharides found in the outer membrane of gram-negative bacteria and lipoteichoic acid present in the walls of some gram-positive bacteria [7]. Studies suggest that *TLR4* may also play a role in resistance to mastitis, a common disease that affects dairy cows. For instance, an association analysis with G+265C in the 5'-UTR loci (GenBank acc. no. AC_000165) of the *TLR4* gene, as well as genotyping with *Msp*I enzymes, revealed an association between *TLR4* genotypes and milk production traits and somatic cell scores [7]. Butyrophilins (BTNs) are proteins located in the epithelial cells of mammary glands, and *Butyrophilin subfamily 1 member A1* (*BTN1A1*) is a crucial gene associated with milk yield and composition, specifically fat content [8]. Studies have indicated that the administration of recombinant BTN1A1 can reduce T-cell activation and hinder the development of autoimmune diseases, such as experimental autoimmune encephalomyelitis in rat models [9]. Expression studies have also shown the significance of *BTN1A1* in growth traits and milk yield in Saanen goats [9]. *BTN1A1* gene in ruminant mammary cells of bovine is critical for formation of lipid droplets. *BTN1A1* exerts some degree of control on transcription of lipogenic genes [10]. Although the *TLR4* and *BTN1A1* genes are related to quality milk and health traits, there are not directly related to growth traits, they may have an indirect physiological relationship that can significantly enhance a correlated response in growth traits. The pituitary transcription factor Pit-1, encoded by the *Pit-1* gene, is essential for the expression of the growth hormone-releasing factor gene and plays a significant role in growth [11]. A study on Canchim cattle demonstrated the effect of *Pit-1* on the standardized weaning weight (W240) and average daily weight gain from birth to weaning [12]. Genetic Group 1 (GG1) included 232 Canchim animals with an average of 5/8 Charolais and 3/8 Zebu breeds (Nelore, Guzerá, and Indubrasil) [13], while Genetic Group 2 (GG2) had 277 animals with an average of 21/32 Charolais and 11/32 Nelore breeds [14]. Given that the *TLR4*, *BTN1A1*, and *Pit-1* genes are associated with growth traits, they can be used as genetic markers in breeding programs for Brahman cattle in Thailand to promote growth traits.

This study aimed to examine the association between candidate genes and the EBV of growth traits in Thai Brahman cattle.

2. Materials and methods

2.1 Animal

For this study, data from 8,728 Thai Brahman cattle, including birth weight (6,574 records), weaning weight (5,011 records), and yearling weight (720 records), between 1993 and 2018 were obtained from the Beef Cattle Research and Development Center in Lop Buri Province, Thailand (Table 1). The YW data was less because not all data was collected by staff.

Table 1 Characteristics of the data structure analysis record of Thai Brahman cattle.

Traits	N	MEAN	MIN	MAX	SD
Animals with pedigrees	8,728	-	-	-	-
Birth weight records (kg)	6,574	28.74	20	45	3.52
Weaning weight records (kg)	5,011	168.43	120	239	26.37
Yearling weight records (kg)	720	255.97	160	338	39.95

N: number of data, MIN: minimum, MAX: maximum, SD: standard deviation.

2.2 Extraction of DNA

At the Beef Cattle Research and Development Center, blood samples (5 mL/animal) were drawn from Thai Brahman cattle through the jugular vein and collected in tubes containing Ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The genomic DNA was then extracted from the buffy coat using a guanidine hydrochloride protocol that was modified according to the method described by [15]. The DNA of each sample was quantified using a NanoDrop 2000 spectrophotometer.

2.3 PCR-RFLP

Primers for PCR were carried out using a 10 µl reaction mixture comprising approximately 1 µl (20-50 ng) of genomic DNA, 1.5 µl of 10×PCR buffer, 1 µl of 2.5 µM primer for each candidate gene, 1 µl of 1 mM each dNTP (Promega, USA), 0.8 µl of 25 mM MgCl2, and 0.1 µl (5 units) of Taq DNA polymerase (Thermo Scientific). The cycling conditions for PCR were carried out by thermal cycling (Biometra® TProfessional machine). The thermal

cycling conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 95°C for 30 sec, annealing at a temperature specified in Table 2 for 30 sec, and extension at 72°C for 7 min, followed by a final extension step at 72°C for 5 min. The presence of polymorphisms was determined by PCR-RFLP (restriction fragment length polymorphism) analysis. PCR-RFLP analyses were performed for *TLR4/MspI, BTN1A1/Hae*III, and *Pit-1/Hinf*I in 256, 253, and 261 DNA samples obtained from Thai Brahman cattle, respectively. The amplified fragments were digested overnight with 5 units of the respective restriction enzymes. PCR-RFLP visualization was carried out with 8% polyacrylamide gel electrophoresis (*TLR4/Msp*I*)*, 10% polyacrylamide gel electrophoresis (*BTN1A1/HaeIII)* and 2% agarose gel electrophoresis *(Pit-1/Hinf*I) stained with GelStar™ Nucleic Acid Gel Stain and visualized under UV light.

Table 2 Summary of the genes, primers, annealing temperatures, PCR product sizes, digestion product sizes, and enzymes used for the PCR-RFLP analysis of the blood samples of Thai Brahman cattle.

Genes	Primers (5'→3')	Annealing temperatures (°C)	PCR product sizes (bp)	Digestion product sizes (bp)	Enzymes	References
TLR4	F-GGGTATTTTGTTATGGCTGG R-CCATCATCCTGGCATTTT	60*	477	CC(AA) - 245, 125 GG(BB) - 370, 65, 42	*Msp*I	[7]
BTN1A1	F-TCCCGAGAATGGGTTCTG R-ACTGCCTGAGTTCACCTCA	59*	893	AA - 371, 231, 185 BB - 338, 83	*Hae*III	[16]
Pit-1	F-GAGCCTACATGAGACAAGCATC R-AAATGTACAATGTGCCTTCTGA	64*	611	AA- 611 BB - 367, 244	*Hinf*I	[17]

*For this study.

2.4 Statistical analyses

To estimate the variance components and breeding values for birth weight (BW), weaning weight at 200 days of age (WW), and weight at 400 days of age (YW), EM-REML and BLUP were used with single-trait animal models in REMLF90 and BLUPF90 software [18]. The model used was Equation 1:

$$y = X\beta \times Z_a a + Z_m m + W_p + \varepsilon \quad (1)$$

In this equation, y represents the vector of observations for BW, WW, and YW. The vector β contains the solution for fixed effects such as contemporary group (herd-year-season), sex, age of dam, and animal. The vectors a, m, and p correspond to the direct additive genetic effects, maternal genetic effects, and permanent environmental effects of the dams, respectively. The vector ε is the residual effect, and the incidence matrices X, Za, Zm, and W are associated with the fixed effects, random direct additive genetic effects, random maternal genetic effects, and random permanent environmental effects, respectively.

The frequencies of genes and genotypes were determined following the method described in [19]. The Hardy-Weinberg equilibrium was assessed using the Chi-square test by comparing the observed and expected genotype frequencies. To determine the relationship between the genotypes of candidate genes and their corresponding traits, the PROC GLM procedure of SAS [20] was utilized. The linear model used in the analysis was as follows:

$$Y_{ijkl} = \mu + YS_i + P_j + G_k + e_{ijkl} \quad (2)$$

where Y_{ijkl} represents the estimated breeding value observation for the traits, μ is the overall mean for each trait, YS_i represents the effect of the i_{th} year and season of calving, Pj represents the effect of the j_{th} parity, G_k represents the fixed effect of the k_{th} genotype, and e_{ijkl} represents the random residual effect.

3. Results

The results of the variance components and genetic parameters for the growth traits BW, WW, and YW. Moderate heritability estimates of BW (0.27±0.03), WW (0.34±0.02), and YW (0.14±0.06). Maternal heritability estimates of BW (0.07±0.03), WW (0.12±0.002), and YW (0.01±0.001) were obtained (Table 3).

Table 3 Variance components of the birth weight (BW), weaning weight (WW), and yearling weight (YW) of the Thai Brahman cattle.

Variance components and genetic parameters	Traits		
	BW	WW	YW
Additive variance	3.28	195.4	122.6
Maternal variance	0.94	69.77	12.38
Permanent environmental variance	0.21	41.24	8.25
Residual variance	7.43	260.9	719.52
Heritability (h^2)	0.27±0.03	0.34±0.02	0.14±0.06
Maternal heritability (m^2)	0.07±0.03	0.12±0.002	0.01±0.001

± Standard errors.

PCR-RFLP analyses were performed for *TLR4/Msp*I*, BTN1A1/Hae*III, and *Pit-1/Hinf*I in 256, 253, and 261 DNA samples obtained from Thai Brahman cattle, respectively. PCR-RFLP visualization was carried out with polyacrylamide gel electrophoresis (8%). The targeted sequence of *TLR4* (477 bp) was digested with the restriction enzyme *Msp*I and showed three cleavage types on G+265C in the 5'-UTR (GenBank acc. no. AC_000165) [7] (Figure 1). Meanwhile, the sequence targeted for *BTN1A1* (893 bp) underwent digestion with *Hae*III restriction enzymes, which resulted in three types of cleavage at position 6804-6807 (GenBank acc. no. Z93323) [16] (Figure 2). *Pit-1* (611 bp) was digested with the restriction enzyme *Hinf*I and showed three cleavage types on chromosome 1 between the regions of TGLA57 and RM95 [17] (Figure 3).

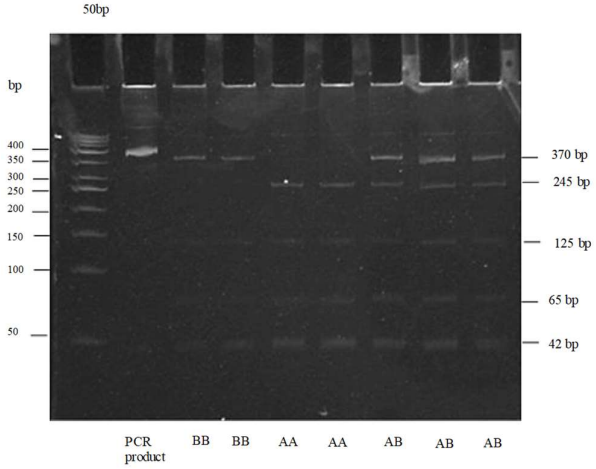

Figure 1 The *TLR4* gene was genotyped through PCR-RFLP utilizing *Msp*I restriction enzyme on an 8% polyacrylamide gel electrophoresis, which also included a 50 bp marker. The genotypes were identified as follows: AA = 245+125 bp, BB = 370+65+42 bp, and AB = 370+245+125+65+42 bp.

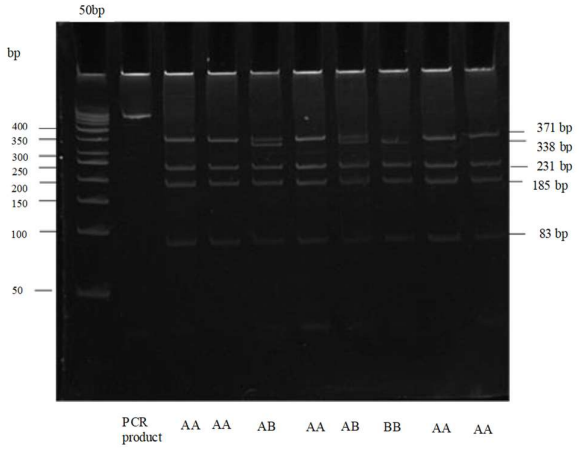

Figure 2 The *BTN1A1* gene genotype was determined by performing PCR-RFLP with the *Hae*III restriction enzyme on 10% polyacrylamide gel electrophoresis, with a 50 bp marker. Genotypes: AA=371+231+185 bp, BB=338+83 bp, and AB=338+371+231+185+83 bp.

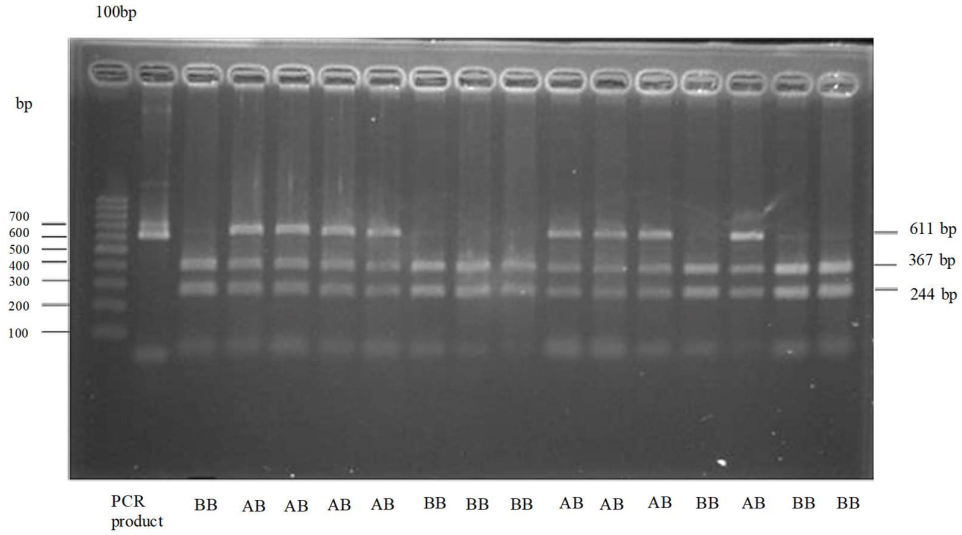

Figure 3 The *Pit-1* gene was genotyped using PCR-RFLP with the *Hinf*I restriction enzyme in 2% agarose gel electrophoresis along with a 100 bp marker. Genotypes: BB=367+244 bp and AB=661+367+244 bp.

The RFLP-PCR technique was utilized to distinguish the various genotypes in the study. The *Msp*I enzyme digestion of *TLR4* PCR products yielded three genotypes (AA, AB, and BB) located at nucleotide position G+265C, with respective frequencies of 77%, 22%, and 1%. The allele frequencies were A (88%) and B (12%). The *Hae*III enzyme produced 3 genotypes after digesting the PCR products of *BTN1A1*, which were located at nucleotide accession number Z93323. The AA, AB, and BB genotypes were produced with frequencies of 72%, 24%, and 4%, respectively. The allele frequencies at 6804-6807 produced by the A and B genotypes were 84% and 16%, respectively. The *Hinf*I enzyme produced 2 genotypes after digesting the PCR products of *Pit-1*, and these genotypes were located in the region between TGLA57 and RM95. The frequencies produced by the AB and BB genotypes were 12% and 88%, respectively. The A and B genotypes had allele frequencies of 6% and 94%, respectively. The distribution of genotypes for all three genes in the population sample was found to be consistent with Hardy-Weinberg equilibrium (Table 4). A genotype is not considered an association if the observation value is less than 5%.

The study findings revealed significant associations between genotype and EBV but no associations between growth traits (p >0.05) (Table 5). The BB genotype of *TLR4* was observed at less than 5%, so the *TLR4* gene was associated with the EBV of weaning weight, which was significantly higher in cattle with the AB genotype (8.78 ± 1.43) than in cattle with the AA genotype (3.96 ± 0.76) (p≤0.05). The *TLR4* gene was associated with the EBV of yearling weight, which was significantly higher in cattle with the AB genotype (11.03 ± 1.98) than in those with the AA genotype (5.04 ± 1.06) (p ≤0.05). The *BTN1A1* gene was associated with the EBV of weaning weight, which was significantly higher in cattle with the AB genotype (5.54±1.42) than in those with the BB genotype (-2.60 ± 0.3.15) (p ≤0.05). The *Pit-1* gene was associated with the EBV of birth weight, which was significantly lower in cattle with genotype BB (0.16 ± 0.07) than in those with genotype AA (0.59 ± 0.19) (p ≤0.05) (Table 5).

Table 4 Genetic equilibrium base for *TLR4, BTN1A1,* and *Pit-1* in Brahman cattle.

Genes		Genotype frequency			Allele frequency		χ^2 test
		AA	AB	BB	A	B	*p value*
TLR4	Observed	196	57	3	0.88	0.12	0.88
	Genotype frequency	0.77	0.22	0.01			
BTN1A1	Observed	182	60	11	0.84	0.16	0.13
	Genotype frequency	0.72	0.24	0.04			
Pit-1	Observed	-	31	230	0.06	0.94	0.59
	Genotype frequency	0	0.12	0.88			

χ^2 value < $\chi^2_{0.05:1}$ (3.841) = consistent with Hardy-Weinberg equilibrium.

4. Discussion

Heritability was determined to be moderate for BW (0.27±0.03), WW (0.34±0.02), and YW (0.14±0.06). The heritability estimates obtained in this study were close to the values reported by [21] (BW (0.30) and WW (0.28). The heritability of YW was 0.22, which is similar to [22] (0.25) in Brahman cattle from Mexico.

The polymorphism of 3 genes was not found associated with the traits, because traits or phenotypes are varies from environmental effect. The analysis revealed that *TLR4* was associated with the EBV of growth traits (WW and YW), which indicated that the health of an animal could be related to its growth traits. If animals are in good health, they will grow well. *TLR4* plays a role in the innate immune system and serves as the primary recognition receptor for lipopolysaccharide (LPS) [23], an endotoxin that is generated by the breakdown of dead gram-negative bacteria [24]. One of the many signaling channels that react to inflammatory stimuli is the nuclear factor kappa B pathway, and it does so in response to LPS [25]. The level of LPS exerts strong control on *TLR4*, the gene encoding the LPS signaling receptor. *TLR4* is probably involved in macrophage immunological responses [26]. The SNP of *TLR4* in this study was similar to that in a study by [7], in which the locus of the SNP in the 5'-UTR was detected. Additionally, a genetic association analysis was conducted on Chinese Holstein cows, and the statistical results showed that the SNP was significantly correlated with 305-day milk yield ($p \leq 0.05$) and somatic cell score (SCS) ($p \leq 0.01$). Consequently, *TLR4* may be a genetic marker in cattle for growth and maternity.

Table 5 Association analyses of the *TLR4*, *BTN1A1*, and *Pit-1* genes with the traits birth weight (BW), weaning weight (WW), and yearling weight (YW) and the EBV of these traits in the analyzed Brahman cattle (least squares, means, and standard errors).

Genes	Genotype	Traits			Traits (EBV)		
		BW	WW	YW	BW	WW	YW
TLR4	AA(196)	27.37±0.36	154.49±2.33	218.50±5.81	0.28±0.08	3.96^{a}±0.76	5.04^{a}±1.06
	AB(57)	26.10±0.65	156.20±4.33	208.87±11.23	0.08±0.15	8.78^{b}±1.43	11.03^{b}±1.98
	BB(3)	-	-	-	-	-	-
p value		0.0932	0.7342	0.4521	0.2673	0.0104	0.0220
BTN1A1	AA(182)	27.15±0.37	155.27±2.42	214.84±6.24	0.25±0.08	5.31^{a}±0.77	6.96±1.08
	AB(60)	26.80±0.66	152.40±4.32	216.29±9.53	0.08±0.16	5.54^{a}±1.42	6.43±1.99
	BB(11)	28.55±1.64	153.00±10.25	240.50±30.89	0.01±0.35	-2.60^{b}±3.15	-1.96±4.41
p value		0.6148	0.8437	0.7224	0.5352	0.0492	0.1476
Pit-1	AA(-)	-	-	-	-	-	-
	AB(31)	27.45±0.89	155.40±5.83	226.18±13.01	0.59^{a}±0.19	7.53±1.88	9.51±2.63
	BB(230)	27.02±0.33	154.61±2.19	214.29±0.5.48	0.16^{b}±0.07	4.84±0.71	6.21±0.99
p value		0.6623	0.9012	0.4022	0.0377	0.1828	0.2427

Values with different superscripts within the same column differed significantly at $^{a, b}$ $p \leq 0.05$.

The results of this study showed an association between SNPs in the *BTN1A1* gene and the EBV of WW. The highest EBV of WW was found in Brahman cattle that presented genotype AB. Cattle *BTN1A1* was only found in the mammary gland; it was not found in the liver, kidney, heart, or intestine [27]. BTN is a key protein associated with milk fat droplets that can trigger an inflammatory response in the central nervous system (CNS), leading to the formation of scattered meningeal and perivascular infiltrates of T cells and macrophages [8]. BTN1A1 controls five biological pathways, including apoptosis, cytokine–cytokine receptor interaction, influenza A, measles infection, and natural killer cell-mediated cytotoxicity [10]. *BTN1A1* transcripts are not limited to lactating mammary tissue but have also been identified in virgin mammary tissue, spleen tissue, and thymus tissue. Additionally, the BTN1A1 protein was found in thymic epithelial cells [28], indicating a possible relationship between *BTN1A1* and the immune system as well as growth traits. The maternal-effect is often evident when the calf is weaned. Milk quality genes *TLR4* and *BTN1A1* affect calf weaning weight. The gene may be passed from the mother to the calf. Future research on this topic should be done.

The results of the *Pit-1* polymorphism showed that the *Hinf*I enzyme produced 2 types of genotypes (AB and BB) after the digestion of PCR products, with the BB genotype corresponding to lower birth weight. Due to the finding of two genotypes, it is possible that semen from sources outside the farm was used to artificially inseminate the farm's cattle. The *Pit-1* gene sequence was identified as polymorphic at the recognition site for the *Hinf*I restriction enzyme. The absence of allele A (BB genotype) in cows resulted in a higher milk yield [17]. The association of *Pit-1* with the EBV of BW revealed that *Pit-1* may regulate the transcription of the *bGH* gene in early bovine embryos. *Pit-1* transcripts were identified in oocytes and all embryonic stages [29]. Additionally, *Pit-1* was found to play a dominant role in growth and development as well as the expression of growth hormone [30].

5. Conclusion

In summary, this study is the first to analyze the effects of three genes including *TLR4, BTN1A1*, and *Pit-1* on Thai Brahman cattle, with the following findings obtained: 1) *TLR4* was associated with the EBV of WW and YW, 2) *BTN1A1* was associated with the EBV of WW, and 3) *Pit-1* was associated with the EBV of BW. Although the *TLR4* and *BTN1A1* formed an indirect relationship with growth traits, they were associated with the EBV of

growth traits. Thus, the findings revealed that *TLR4, BTN1A1*, and *Pit-1* may conceivably be genetic markers for molecular breeding programs because they can affect the growth traits of Thai Brahman cattle and potentially improve these traits. For research to be conducted using more animals, phenotypic data, and genotypic data to support this potential relationship.

6. Ethical approval

This study was reviewed and approved by the institutional animal care department based on the Ethics for Animal Experimentation of the National Research Council of Thailand (No. IACUC-KKU-100/62).

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