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Genotype of Brahman and Brahman Cross Cattle based on SNP in Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) gene sequences

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Abstract. *Priyadi DA, Panjono, Bintara S, Hartatik T. 2017. The genotype of Brahman and Brahman Cross Cattle based on SNP in Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) gene sequences. Biodiversitas 18: 795-800.* The IGFBP-3 gene is a potential marker of cattle growth, which is related to the functions that influence the growth, energy metabolism, reproduction and immunity. The genome was isolated from whole blood samples of 10 Brahman cattle and 16 Brahman Cross cattle. Cattle IGFBP-3 gene that targeted in this study was located in the part of intron 2, exon 3 and part of intron 3. The gene targets were amplified using specific primers by Polymerase Chain Reaction (PCR) technique, resulting 563 bp amplification product. As data comparison to reveal the SNP, the GenBank sequences (n = 14) from various breed and countries were used. The objective of this study was to reveal the SNP on the Brahman and Brahman Cross IGFBP-3 gene. The results obtained 3 genotypes from one SNP that spread in the sample population. The SNP was located in intron 2 at position 3,930 (G→A). The Polymorphism could be recognized by PvuII restriction enzymes. There was not enough evidence to associate the SNP with the phenotype (at pre-weaning age) of Brahman and Brahman Cross cattle. There was a genetic diversity in the population studied. Knowledgeable SNP could be used as genetic markers for further research on Brahman and Brahman Cross cattle.

Keywords: Brahman cattle, Brahman Cross cattle, genetic marker, Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3), Single Nucleotide Polymorphism (SNP)

INTRODUCTION

Increased productivity of beef cattle is one of the solutions to answer the meet the increasing demand for beef in Indonesia. Production performance cannot be separated from cattle genetic factor. One of the gene candidates on productivity is Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3). The IGFBP-3 gene in cattle is located on chromosome four, consist of 4 introns and 5 exons (Kim et al. 2005), and the gene is approximately 8.407 bp length (GenBank; www.ncbi.nlm.nih.gov). Growth trait in mammals are affected by various hormones, one of them is Insulin-Like Growth Factor system (IGFs) (Zhang and Steinle 2014). IGFs hormone functions and capabilities are modulated by its binding protein, called Insulin-Like Growth Factor Binding Protein (IGFBP). The IGFBP family consists of six types of protein (IGFBP 1 to 6) that has four important functions, namely, to regulate metabolism of IGF system, to indicate the path to location of the specific tissue or an organ that will be addressed by IGFs, to regulate the interaction between IGFs and their receptors that would control the biological action, and to act as a transport protein in the blood plasma IGFs which regulates the discharge flow into the bloodstream (Clemmons 2016).

The hormonal component of the mammalian growth system include Growth Hormone (GH), Insulin-Like Growth Factor I and II (IGF-I and II), and a protein that binds growth hormone (IGFBP-1 to 6) with high-affinity, as well as the growth hormone receptor (GHRHR, GHR, IGF-IR and IGF-IIR) (Hjortebjerg and Frystyk 2013). Most of the IGF-I hormones are bound by IGFBP-3, the molecules are source of available hormone IGF-I in the bloodstream, stimulating proliferation, migration, differentiation of cells (Hjortebjerg and Frystyk 2013; Kessler et al. 2013; Nguyen et al. 2013), and apoptosis by modulating gene transcription (Forbes et al. 2012). According to Othman et al. (2014), IGFBP-3 can be used as a marker for the function of growth, metabolism, reproduction, immunity and energy balance in cattle. SNP is a condition where the presence of a different nucleotide constituent of a gene, and studies of SNP on IGFBP-3 gene in sheep, goat, cattle and buffalo have been reported (Rodríguez et al. 2013; Mahrous et al. 2014; Ramesha et al. 2015; Rasouli et al. 2016). SNP in the IGFBP-3 gene was reported in Egypt cattle (Othman et al. 2014), Holstein and Cross between Holstein-Haryana (Choudhary et al. 2007), Hanwoo (Kim et al. 2005) and various Chinese cattle (Gao et al. 2009; Liu et al. 2014). The presence of SNPs in IGFBP-3 gene affected the phenotype appearance, body weight gains (Choudhary et al. 2006; Rasouli et al. 2016), IGFBP-3 serum concentration (Choudhary et al. 2007), body circumference (Gao el at. 2009), and has been used as a candidate gene for meat production (Mahrous et al. 2014).

The study of IGFBP-3 gene has not been reported in Indonesian cattle, or in Zebu cattle and their offspring in Indonesia. This study was conducted on common commercial cattle in Indonesia, which were mainly Brahman and Brahman Cross. The objective of this study was to identify the SNP in Brahman and Brahman Cross cattle so that it can be used as a genetic marker for further research.

MATERIALS AND METHODS

Samples collection

Twenty-six blood samples were collected from 10 of Brahman and 16 of Brahman Cross cattle, which were reared intensively at PT. Widodomakmur Perkasa farm, Klaten, Central Java, Indonesia. The blood samples were taken from the jugular vein using vacuum tubes (Venoject) containing an anticoagulant K_3EDTA. The samples were stored in -20°C until they were processed for DNA extraction.

DNA extraction

Isolation of DNA was performed using Extraction Kit (Geneaid, Taiwan) in the Laboratory of Genetics and Animal Breeding, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Polymerase Chain Reaction (PCR)

Primer arranged based on 14 sequences of cattle IGFBP-3 gene (GenBank accession No: KF899894, AY306011, DQ536398, AY601888, AY338971, DQ536397, JQ711181, NC_0073302.6, AC_000161, U83465, KF899893, DQ536399, AY3355439, AF305712), in order to obtain primer; forward (5'-GCC TGG GTA TCC AGA GAT CA-3') and reverse (5'-GAT GGT GCT CAC CTG CTT TT-3') to amplify 563 bp DNA fragments gene located in the part of intron 2, exon 3 and part of intron 3. The sequences were also used as comparison data to reveal the SNPs. Polymerase Chain Reaction performed in a total reaction of 30 µL, containing 0.75 µL of DNA, 1.5 µL of both forward and reverse primers, 15 µL PCR Kit (KAPA BIOSYSTEMS, USA) and 11.25 µL aquabidest (Otsuka, Indonesia). The reactions were performed using a thermal cycle (PEQLAB Primus 25 advanced, Germany) with a predenaturation temperature at 94°C for 5 minutes, followed by 35 cycles of reaction; denaturation at 94°C for 1 minutes, annealing at a temperature of 56°C for 1 minutes and extension at 72°C for 1 minute, then the last step was a final extension at 72°C for 5 minutes. The quality of the PCR product was determined using gel electrophoresis (2%), the thick and clearly DNA bands was the preferred result (Lee et al. 2012).

Genotyping

The 30 µL/samples of PCR product and 20 µL of primers were sent to the PT. Genetics Science, Indonesia, for sequencing. A total of 34 sequences of IGFBP-3 genes (10 Brahman cattle, 10 Brahman Cross cattle and 14 GenBank sequences (www.ncbi.nlm.nih.gov)) aligned using Bioedit (version 7.2.5) to reveal the SNPs and to determine the restriction enzyme that can recognize the SNPs. Genotyping with PCR-RFLP method were used to confirm the SNP in all samples. Three restriction enzymes (*Pvu*II, *Nla*III and *Hae*III) were used for PCR-RFLP, and the cleavages were visualized by 3% gel electrophoresis.

Phenotype measurement

The phenotypes measured include birth weight, weaning weight and Average Daily Gain (ADG). Birth weight was measured within 24 hours after birth, and weaning weight was measured in the age range of 6 months then adjusted within 205 days. Birth weight and weaning weight also adjusted to sex of calves and age of dam. ADG was calculated by dividing the gain difference between birth and weaning weight with age (days). To minimize confounding factors may occur, similar management of cows and calves and similar calf birth-time (seasons) were used.

Statistical analysis

Data phenotype (birth weight, weaning weight and ADG) compared to genotype data using One-Way ANOVA statistical design. Genotype frequencies in populations were analyzed using statistical examination Chi-square to determine the status of the law of Hardy-Weinberg equilibrium.

RESULTS AND DISCUSSION

Recent studies have reported an association between SNPs in IGFBP-3 gene with the economic trait on ruminant livestock, including significantly correlated to the birth weight, the body weight of 12, 18 and 24 months (Choudhary et al. 2007), weaning weight and ADG from birth to weaning (Rasouli et al. 2016), the chest width and chest circumference (Gao et al. 2009), and the differences in serum concentrations of IGFBP-3 as a result of the SNP (Choudhary et al. 2006). This is due to the significance of IGFBP-3 in the IGF-I regulation; they regulate the amount of the IGF-I to their receptors. IGFBP-3 have their own receptors or can penetrate the cell membrane and influence intra-cell mechanism (Clemmons 2016). IGF-I is proliferation agent and provides cell survivability. IGFBP-3 as the major carrier protein of IGF-I is also active in intracellular and be in action as anti-proliferative agent (Baxter 2014). The presence of SNP in IGF-1 gene also proved a significant effect on postnatal growth, mainly the growth of muscles and bones (Yazdanpanah et al. 2013), the milk fat content, and long days open (Abdolmohammadi and Zamani 2014). The concentration of circulating IGF-I have a significant effect on body weight and Body Condition Score (BCS) (Mirzae and Rezaei 2014). In the present study, the IGFBP-3 gene in 10 of Brahman cattle and 16 of BX cattle were isolated, amplified (Figure 1) and 20 animals (10 Brahman and 10 BX) were sequenced then aligned with the comparator reference sequence (GenBank) to reveal the SNP. All animals were digested with three restriction enzyme to

confirm the SNP. Brahman and BX cattle were selected in this study due to their superiority, resistance to heat stress and ticks, and have good growth than the common local cattle (Porto-Neto et al. 2014), so they are potential for further development in Indonesia. Brahman were composite cattle which developed from crosses between *Bos indicus* (Gir, Krishna Valley, Guzaret, Nellore) with *Bos taurus* (Shorthorn) cattle. BX cattle were developed from crosses between Brahman, Hereford, and Shorthorn cattle with the blood proportion respectively, 50, 25 and 25% (Hardjosubroto 1994). The example of visualization result of IGFBP-3 genes amplification (PCR product) can be seen in Figure 1; the thick and clearly DNA band indicates the good quality of amplification.

From the 34 sequences were identified 3 SNPs that spread in the most of the sequences (Figure 2). The SNPs located in intron 2 at nucleotide base number 3,930, 3,996 and 4,177 based on the NCBI database (www.ncbi.nlm.nih.gov) with the referenced sequence no. AC_000161.1, that mentioned cattle IGFBP-3 gene has 8,406 bp of full-length sequence. Based on the similarity of the SNPs, the 34 sequences can be divided into 7 genotypes (Table 2). Brahman and BX cattle were split into 3 genotypes.

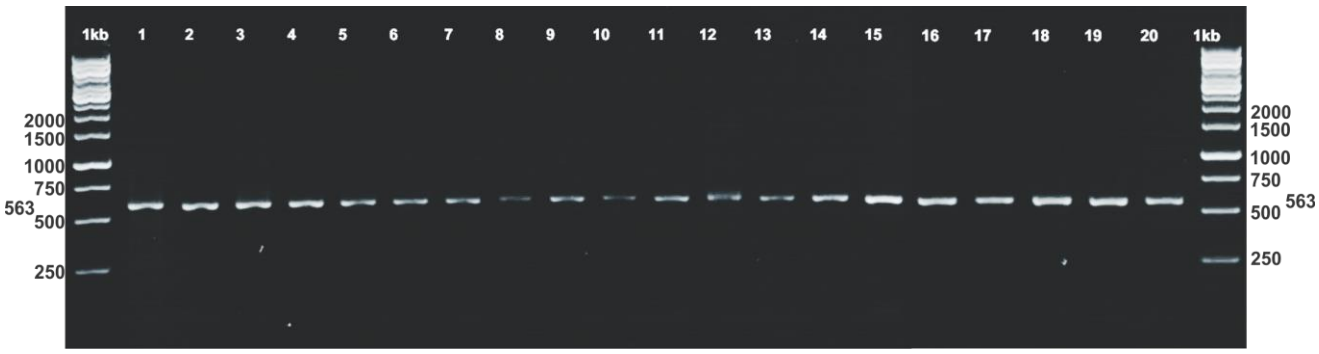

Figure 1. Visualization of PCR product by 2% electrophoresis gel; BX (lane 1-10); Brahman (lane 11-20)

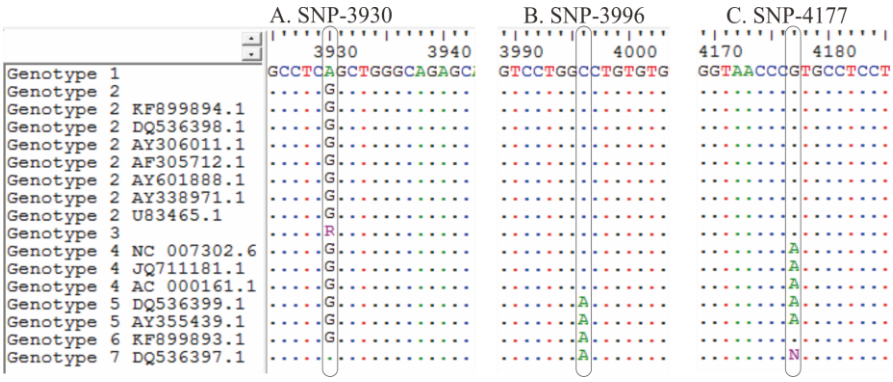

Figure 2. Alignment of 34 sequences IGFBP-3 gene revealed 3 SNP; A. SNP-3,930 (G>A), recognized by *Pvu*II, B. SNP-3,996 (A>C), recognized by *Hae*III, C. SNP-4,177 (G>A), recognized by *Nla*III

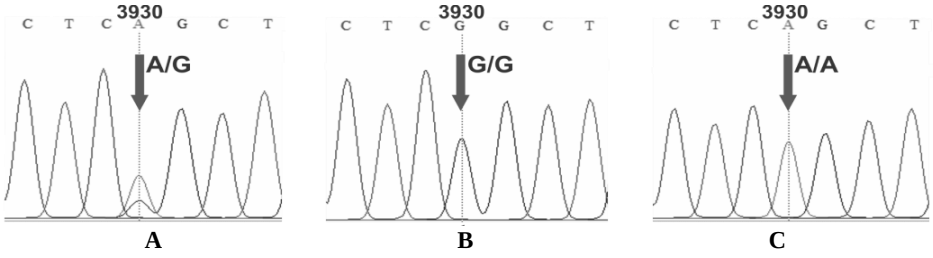

Figure 3. Sequencing chromatography of genotype 3, double peaks indicating heterozygous gene and a single peak indicating homozygous gene. A. SNP-3,930 heterozygous A/G, B. SNP 3,930-homozygous G/G, C. SNP-3,930 homozygous A/A

The sequencing chromatogram showed double peaks which indicate the presence of heterozygous genes (Figure 3). Double peaks are shown on genotype 3 in the nucleotide number 3,930. Heterozygosity was also the basis for determining the genotypes distribution. The digestion results with specific restriction enzymes were used as evidence of heterozygous genes which clearly produced visible variations cleavages visualized by gel electrophoresis.

The SNPs located in Brahman and BX IGFBP-3 gene sequence were identified using restriction enzymes *Pvu*II, *Hae*III and *Nla*III, respectively (Figure 5). Although no SNP-3,996 and 4,177 were found in Brahman and BX cattle that were alignment (Figure 2), but the analysis using three enzymes was still performed because the SNPs were possible to appear in 6 unsequenced BX samples. As shown in Table 2 and supported by previous research (Choudhary et al. 2006; 2007, Cheong et al. 2008, Othman et al. 2014), that SNP-3,996 and 4,177 have a tendency to appear in *Bos taurus* cattle. Brahman and BX cattle were Taurine influenced cattle so there is a possibility of the SNPs on them.

The enzymes produced large size and specific among other (Figure 4), so the determination of the genotype by analyzing of the enzyme digestion results can easily be done by identifying the DNA cleavages produced. The information will be very useful for similar advanced research that using the PCR-RFLP genotyping method.

Figure 4 illustrates the cutting position by the restriction enzyme *Pvu*II, *Hae*III and *Nla*III on IGFBP-3 gene in the sample used which produce two types of digestion (x and y) that establish three genotype combinations (Table 1). Digestion cleavages with sizes above 100 bp will be easily visualized by Agarose gel electrophoresis, so the three genotypes can be identified by RFLP method which has the advantage of being low cost, fast, simple, accurate and reliable (Tabit, 2016). As shown in Figure 5, SNP-3,930 was revealed as evidenced by the variation of digestion results using the restriction enzyme PvuII. While the SNP-3,996 and 4,177 were monomorphic, as evidenced by identical digestion results.

Three SNPs were in the intron 2 on the fragment gene targeted and in sufficient detail were located at nucleotide number 3,930 (SNP-3,930), produce AA, AG, GG gene with the frequency were 23.08%, 42.31%, and 34.61%, respectively. The allele frequencies at SNP-3,930 was A (44.23%) and G (55.77%). Distribution of genotypes based on the SNP in the sample population was consistent with Hardy-Weinberg equilibrium (Table 3).

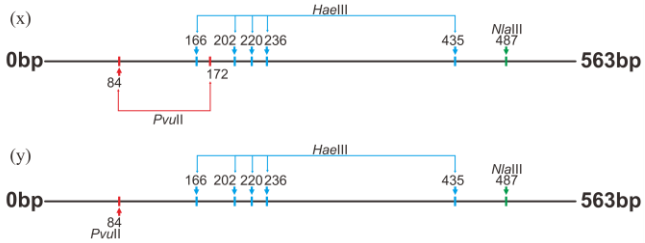

Figure 4. Restriction enzymes cutting site illustration that can be divided into two types (x and y)

Table 1. Restriction enzyme mapping on 3 genotypes of IGFBP-3 gene that include Brahman and Brahman Cross cattle

Genotype	*Pvu*II (CAG'CTG)		*Hae*III (GG'CC)		*Nla*III (CATG')	
	Site	*Fragments size*	*Site*	*Fragments size*	*Site*	*Fragments size*
1 (x, x)	2	84, 88, 391	5	166, 36, 18, 16, 199, 128	1	489,74
2 (y, y)	1	84, 479	5	166, 36, 18, 16, 199, 128	1	489,74
3 (x, y)	2 1	84, 88, 391 84, 479	5	166, 36, 18, 16, 199, 128	1	489,74

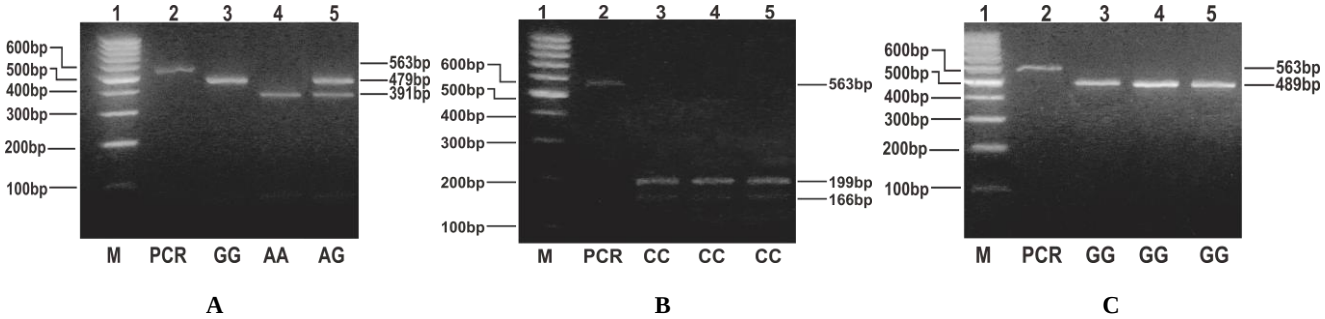

Figure 5. PCR-RFLP visualization with gel electrophoresis (3%). Targeted sequence IGFBP-3 (563 bp) digested with three restriction enzymes: A. *Pvu*II; showed three type of cleavage on SNP-3,930, B. *Hae*III; monomorphic on SNP-3,996, C. *Nla*III, monomorphic on SNP-4,177. M= marker ladder 100 bp, PCR = PCR product

Table 2. Distribution of genotypes based on the SNPs

Geno-type	Cattle breed		n total
1	Brahman	n=4	6
	BX	n=2	
2	Brahman	n=1	16
	BX	n=8	
	Bos taurus: GenBank KF899894.1, DQ536398.1, AY306011.1, AF305712.1, U83465.1	n=5	
	Bos indicus: GenBank AY601888.1, AY338971.1	n=2	
3	Brahman	n=5	11
	BX	n=6	
4	*Bos taurus*: GenBank NC007302.6, JQ711181.1, AC000161.1		3
5	*Bos taurus*: GenBank DQ536399.1, AY355439.1		2
6	*Bos taurus*: GenBank KF899893.1		1
7	*Bos taurus*: GenBank DQ536397.1		1
Total			40

Table 3. Genetic equilibrium based on SNP-3,930 on Brahman and BX cattle

Breed		Genotype			Allele frequency		X^2
		AA	AG	GG	A	G	
Brahman	Observed	4	5	1	65.00	35.00	0.97
	Expected	4.23	4.55	1.23			
BX	Observed	2	6	8	31.25	68.75	0.26
	Expected	1.56	6.88	7.56			

Note: X^2 value < $X^2_{0.05;1}$ (3.841)= consistent with Hardy-Weinberg equilibrium

Table 4. Correlation between genotype and birth weight (BW), weaning weight (WW) and average daily gain (ADG) of Brahman and BX cattle

Genotype	n	Mean		
		BW	WW	ADG
Brahman				
*Pvu*II +/+ (AA)	4	36.45±5.08	113.32±17.43	0.36±0.07
*Pvu*II +/- (AG)	5	34.46±6.93	119.93±7.29	0.41±0.04
Total	9	35.35±5.90	116.99±12.35	0.38±0.06
BX				
*Pvu*II +/+ (AA)	2	27.50±1.55	137.70±42.83	0.41±0.10
*Pvu*II +/- (AG)	6	30.83±6.15	132.44±37.51	0.56±0.23
*Pvu*II -/- (GG)	8	35.81±9.69	101.80±17.48	0.57±0.23
Total	16	32.90±8.17	131.24±38.52	0.55±0.23
Grand total	25	33.32±6.69	126.65±30.91	0.49±0.18

The SNP on nucleotide number 3,996 (A>C) has been widely reported (Choudhary et al. 2006; Cheong et al. 2008; Othman et al. 2014), and has been confirmed to exert a significant effect on the economic traits of cattle (Choudhary et al. 2006). SNP-3,930 and SNP-4,177 have not been reported and the phenotypic correlations are still unknown. The SNP was located in the intron, so they did not cause amino acid changes (silent polymorphism). But intron also has important function such us, it is known that intron has direct role in regulating gene expression, rate of transcription, gene translation, level of post-transcriptional, also determine the fate of RNA molecule, the stability of RNA, efficiency of translation, and subcellular localization (Barrett et al. 2013). Intron has also an indirect role as shown by the correlation of intron length with the efficiency of natural selection and is known to provide a source of new genes, and several kinds noncoding functional RNA. Mutation in the intron can produce disease-associated allele or trait-associated SNP (Jo and Choi 2015). It can be inferred that SNP in the intron region is also important.

The presence SNP in the IGFBP-3 gene was no correlated to the phenotypes (Table 4). This SNP has not been reported in previous research. IGFBP-3 gene has been investigated on small ruminants such as goats and sheep much more than in cattle. Eight SNPs have been detected in exon 2 of IGFBP-3 gene, 6 of them produce non-synonymous protein coding or non-synonymous mutation (Sharma et al. 2014) which can lead to a significant metabolic abnormality that is desirable or not desirable (Veneroni-Gouveia et al. 2015; de Camargo et al. 2015). IGFBP-3 gene expression was higher in the liver and *longisimus dorsi* muscles, thus contributing to the development of these organs (Zhang et al. 2015), increased ADG from birth to weaning and weaning weight (Rasouli et al. 2016), but there is not enough evidence to suggest that SNPs are associated with milk production (Zhou et al. 2016).

In conclusion, this study revealed one SNP in the IGFBP-3 gene of Brahman and Brahman Cross that were located in the intron 2 at nucleotide number 3,930. The SNP can be as a marker candidate for growth for further research on Brahman and BX cattle. SNP can be identified by restriction enzyme *Pvu*II. This information will be useful for further similar research.

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