

Polymorphism of 5'UTR myostatin gene indel (g.1256/TTTTA) and its association with body weight in Boerka crossbred goat

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Received April 09, 2020; Accepted June 26, 2020

ABSTRAK

Penelitian ini bertujuan mengidentifikasi keragaman fragmen 5'UTR gen Myostatin (MSTN) dan asosiasinya terhadap bobot badan kambing Boer, Kacang, dan Boerka. Sampel DNA diambil sebanyak 149 buah dari Loka Penelitian Kambing Potong Sungei Putih, Sumatera Utara. Identifikasi polimorfisme menggunakan metode *direct sequencing* dan *Polymerase Chain Reaction - Restriction Fragment Length Polymorphism* (PCR-RFLP). PCR-RFLP menggunakan enzim restriksi DraI terhadap indel g.1256/TTTTA. Analisis ragam bobot badan dilakukan dengan *General Linear Model* (GLM). Hasil penelitian menunjukkan bahwa fragmen 5'UTR gen MSTN monomorfik pada kambing Kacang namun polimorfik pada kambing Boer dan Boerka. Frekuensi genotipe kambing Boer 0,40(AA), 0,43(AB), 0,17(BB); Kacang 1,00(AA); Boerka 0,53(AA), 0,37(AB), 0,10(BB). Frekuensi alel kambing Boer 0,62(A), 0,38(B); Kacang 1,00(A); Boerka 0,72(A), 0,28(B). Frekuensi genotipe AB paling tinggi pada Boer, namun frekuensi genotipe AA paling tinggi pada Kacang dan Boerka. Indel g.1256/TTTTA berpengaruh signifikan ($P < 0,05$) hanya pada bobot lahir (BW) kambing Boer, namun tidak signifikan terhadap parameter bobot badan lainnya baik pada kambing Boer maupun Boerka. Genotipe AA memiliki bobot lahir paling tinggi ($P < 0,05$) dari AB, namun tidak berbeda nyata dengan genotipe BB. Indel g.1256/TTTTA dapat dijadikan sebagai penciri genetik untuk sifat pertumbuhan (bobot lahir) kambing Boer namun tidak pada Boerka.

Kata kunci : 5'UTR, Indel TTTTA, gen MSTN, RFLP, sekuensing

ABSTRACT

This study aimed to identify the variation of 5'UTR Myostatin (MSTN) gene and its association to body weight in Boer, Kacang, and Boerka goats. DNA samples were obtained from 149 heads of the goats from the Indonesian Goat Research Center, Sungei Putih, North Sumatera. Polymorphism identification was conducted by direct sequencing and PCR-RFLP with DraI as the restriction enzyme for indel g.1256/TTTTA. Analysis of variance for body weight was carried out using the General Linear Model (GLM). The 5'UTR MSTN gene/DraI was monomorphic in Kacang but polymorphic in Boer and Boerka. Genotype frequencies for Boer 0.40(AA), 0.43(AB), 0.17(BB); Kacang 1.00(AA); Boerka

0.53(AA), 0.37(AB), 0.10(BB). The allele frequencies for Boer 0.62(A), 0.38(B); Kacang 1.00(A); Boerka 0.72(A), 0.28(B), respectively. AB was the most frequent genotype among Boer, but AA was the most frequent in Kacang and Boerka. Indel g.1256/TTTTA has a significant effect ($P < 0.05$) only on birth weight (BW) of Boer, but no significant effect on other bodyweight parameters both in Boer and Boerka. AA genotype has the highest BW ($P < 0.05$) than AB, but it's not significantly different from BB. Indel g.1256/TTTTA could be used as a genetic marker for the birth weight of Boer but not in Boerka goats.

Keywords: 5'UTR, indel TTTTA, MSTN gene, RFLP, sequencing

INTRODUCTION

Kacang goat is one of Indonesia's local livestock. These goats were set as Indonesian domestic goat breed based on a decision of the Indonesian Minister of Agriculture No: 2840/Kpts/LB.430/8/2012. The superiorities of Kacang goats are known; have a good environment adaptive ability (Santoso *et al.*, 2016; Septian *et al.*, 2015), quickly produce offspring (Wahyuni *et al.*, 2016), and more resistant to parasitic gastrointestinal infections (Batubara, 2006). As a tropical breed, Kacang goat was prolific (Panjono *et al.*, 2012), smaller body size but had many offspring (Mulyono *et al.*, 2018). Low body weight with an adult male weight of 24.05 ± 3.95 kg makes Kacang goat not as ideal as meat-producing livestock (Batubara *et al.*, 2012; Wahyuni *et al.*, 2016).

One effort to improve the genetic quality of Kacang goats was through crossing with Boer buck goat. Boer goat was a beef type with fast body growth, good meat quality, and parasitic tolerance (Chong *et al.*, 2019; Elieser *et al.*, 2012; García-Muñiz *et al.*, 2019). Boer has weaning weights 23.4 ± 9.7 kg at the age of 112 days (Menezes *et al.*, 2016) and adult male 120-150 kg at the age of 2-3 years (Nurgartiningasih *et al.*, 2006). Boer and Kacang goat crosses were called Boerka goat that a new superior goat (Ginting and Mahmilia, 2008). The Boerka goat was reported to have a birth weight, weaning weights, one-year weight, body weight gain, and carcass percentage higher than the Kacang goat (Doloksaribu *et al.*, 2005; Priyanto *et al.*, 2002; Setiadi *et al.*, 2001; Triyantini *et al.*, 2002). The genetic quality of Kacang goats can be improved through molecular-based growth selection. One gene that can be used was the MSTN gene because that affects the growth of muscle mass (Batubara, 2017; El Shafey *et al.*, 2016).

MSTN was a member of the transforming growth factor-beta superfamily, which is also known as the growth differentiation factor (GDF)

8 (Hayashi *et al.*, 2018). MSTN acts negatively towards the regulation of skeletal muscle mass development (Sun *et al.*, 2020; Yue *et al.*, 2020). The MSTN gene inhibits the work of the Myf5 and MyoD gene factors, which are related to the mechanism of differentiation of precursor cells into myoblasts (McPherron and Lee, 1997). The deactivation of the MSTN gene influences adipose tissue mass in addition to bone muscle mass (Dominique and Gérard, 2006) and associated with muscle hypertrophy (Kvedaras *et al.*, 2019). This gene was specifically expressed during the development of the embryonic phase and adult skeletal muscle. MSTN gene polymorphisms cause growth acceleration and muscle mass of yellow catfish (Zhang *et al.*, 2020), double muscular in cattle and goat (Grisolia *et al.*, 2009; He *et al.*, 2018), affect the carcass quality and lamb meat quality (Grochowska *et al.*, 2019). MSTN gene polymorphisms were exciting things that could be used to increase livestock meat production (Aiello *et al.*, 2018).

Some goats breed shown polymorphisms in the MSTN gene. Several studies reported the existence of 5'UTR polymorphisms in local goats in Iran (Abdolmohammadi *et al.*, 2016), China (Li *et al.*, 2008; Zhang *et al.*, 2012), and India (Singh *et al.*, 2014). 5'UTR was the region of the mRNA upstream from the protein-coding region (Manzella *et al.*, 2020). 5'UTR has an important role such as; affect the level of mRNA transcription, mRNA decay, translation rates (Feng *et al.*, 2019), control the process of translational initiation (Arend *et al.*, 2018), translational repression mediator (Theil *et al.*, 2018), and regulation of gene expression (Araujo *et al.*, 2012; Liao *et al.*, 2013; Zhang *et al.*, 2018). The indel of TTTTA in the 5'UTR goat MSTN gene has a significant effect on body weight and size (Li *et al.*, 2008) on birth weight, 90-day weight, 300 days age weight and birth body length of Boer goat (Zhang *et al.*, 2012), whereas the indel study of the TTTTA in the 5'UTR MSTN

gene in Indonesian goats have never been done.

Therefore, the objective of this study was to analyze the genetic diversity of the 5'UTR MSTN gene fragment and associate it with the body weights of Boer, Kacang, and Boerka goats. The results of this study are expected to be used as basic information in the development of goat breeding programs in Indonesia, especially in the Indonesian Goat Research Center, Sungei Putih, Deli Serdang, North Sumatera.

MATERIALS AND METHODS

Animal and Samples

This research was carried out in the animal molecular genetics laboratory, Animal Science Faculty, IPB University. One hundred and forty-nine blood samples were taken from the Indonesian Goat Research Center, Sungei Putih, Galang, Deli Serdang, North Sumatera. The samples consisted of Boer (30 heads), Kacang (29 heads), and Boerka goats (90 heads). The goats were maintained in one location with the same maintenance management in a cage and given a feeding concentrate and forage feed (*Indigofera zoolingeriana*, *Brachiaria humidicola*, *Brachiaria ruziziensis*, *Pennisetum purpureum cv mott*). Data were collected during the years of 2016-2018 of body weights at birth (BW), 3 months (M3W), 6 months (M6W), 9 months (M9W), 12 months (M12W), and Average daily body weight gain (ADG) between birth to 12 months of age. Blood samples 3 ml were collected from the jugular vein using a venoject needle and kept in the vacutainer tube containing K₂EDTA anticoagulant, then stored in a refrigerator (temperature \pm 4°C).

DNA Extraction and 5'UTR MSTN Gene Amplification

DNA extraction, according to the extraction procedure of Genomic DNA mini kit (Geneaid Biotech Ltd). Amplification using the PCR method with the master cycler gradient machine (ESCO, Singapore). Amplification was performed at a total volume of 25 μ L consisting of 2.5 μ L (1.1-15.7 ng) DNA templates, 12.5 μ L PROMEGA green master mix, 9.4 μ L nuclease-free water, 0.3 μ L (25 pmol) forward primer and 0.3 μ L (25 pmol) reverse primer.

The primer design refers to the National Center for Biotechnology Information (NCBI) with access number EF591039.1. Primer was designed using the Primer 3 program, Multiprimer Analyzer and Primer Stat. The primer design

results were a forward primer 5'-AAGAGCCAATCACAGATCCC-3' and reverse primer 5'-ACTAGAACAGCAGTCAGCAG-3' with a product length of 635 bp. Amplification of MSTN gene DNA through 5 stages. The first stage was the denaturation process at 95°C for 5 minutes. The second, third, and fourth stages were cycles repeated 35 times with steps; denaturation at 95°C for 10 seconds, annealing at 57°C for 20 seconds, and extension at 72°C for 30 seconds. The fifth stage ends with elongation primers at 72°C for 5 minutes. DNA amplification products were extracted on 1.5% agarose gel for 35 minutes and photographed using UV Transilluminator. The use of agarose gel according to the procedure of Lee *et al.* (2012).

DNA Sequencing and PCR-RFLP

Ninety-five selected samples amplicon with a volume of 22 μ L for each sample were sequenced by commercial laboratory service at First BASE Laboratories (Malaysia). Direct sequencing using ABI Prism 96-capillary 3730xl DNA analyzer (Applied Biosystems, USA). Indel g.1256-1260 (TTTTA/-) was identified using the PCR-RFLP method on the remaining samples until all (149) samples were identified. PCR-RFLP uses the DraI restriction enzyme with a cut site (TTT|AAA). The RFLP was carried out at a total volume of 7.2 μ L consisting of 5 μ L of PCR (amplicon) products, 0.9 μ L nuclease-free water, 0.7 μ L buffer enzyme, 0.6 μ L DraI enzyme and incubated at 37°C for 12 hours. RFLP products were electrophoresed on 2% agarose gel for 43 minutes and photographed using UV Transilluminator.

Data Analysis

Sequencing results were analyzed using the software Finch TV 1.4, Bioedit 7.2, and MEGA 7.0 Tamura *et al.* (2013). The allele and genotype frequency were calculated with Popgene32, according to Nei and Kumar (2000). Hardy-Weinberg equilibrium using Popgene32 with chi-square based on the method of Hartl and Clark (1997). The ANOVA of the 5'UTR MSTN gene was analyzed using SAS 9.4 software (SAS Institute, USA) with a mathematical model of the General Linear Model (GLM) at level probability 0.05. Further tests were carried out by Least Square Means to find out the significant differences between genotypes. A mathematical model was formulated as follows; $Y_{ijk} = \mu + \alpha_i + \beta_j$

+ $\gamma_k + z_l + \epsilon_{ijklm}$. Where: μ is the overall mean for each trait; α_i is the effect of i^{th} genotype, i is 1,2,3; β_j is the effect of j^{th} sex, j is 1,2; γ_k is the effect of k^{th} birth type, k is 1,2,3; z_l is the effect of l^{th} birth season, l is 1,2; ϵ_{ijklm} is random error.

RESULTS AND DISCUSSIONS

Polymorphisms in 5'UTR MSTN Gene

The 5'UTR MSTN gene was successfully amplified using primer at an annealing temperature of 57°C for 20 seconds, with a length of PCR product was 635 bp (Figure 1). These results indicate that fragments have excellent specifications and can be further processed through direct sequencing analysis. Visualization of direct sequencing results on Finch TV shows that there were three different banding patterns; normal, deletion, and double band until the end of the product sequence (Figure 2). The alignment of sequencing results for the EF591039.1 genebank shows the five base pairs indel (g.1256/TTTTA), but no other SNPs were found (Figure 3). This is in agreement with previous researches which reported the presence of the same indel in eighteen local Chinese goat breeds (Li *et al.*, 2008; Zhang *et al.*, 2012), four Iranian goat breeds (Abdolmohammadi *et al.*, 2016) and seven Indian goat breeds (Singh *et al.*, 2014). But, the Indian goats have shown some new SNP besides the TTTTA deletion. Boer and Boerka goats have shown polymorphisms, but monomorphism in Kacang goat (Figure 3). Amplification RFLP product of the 5'UTR MSTN gene (Figure 4) showed three genotypes; AA (635 bp), BB (430 bp, 205 bp), and AB (635 bp, 430 bp, 205 bp).

Genotype and Allele Frequency of 5'UTR MSTN Genes

PCR-RFLP shows that the 5'UTR MSTN gene in Boer and Boerka goats has three

genotypes; AA (deletion), AB (heterozygous), and BB (normal). But the Kacang goat has only one genotype AA (deletion). The highest AA genotype frequency was in Boerka goats (0.53), the AB genotype was highest in Boer goats (0.43) while the BB genotype had the lowest frequency among the others. Li *et al.*, (2008) and Zhang *et al.*, (2012) reported three genotypes (AA, AB, BB) in local Chinese goats with the frequency of AA and AB dominant than BB. Genotype frequencies in seven local Indian goats have only two genotypes (AA, AB) (Singh *et al.*, 2014). One local Iranian goat breed was shown three genotype polymorphisms (AA, AB, BB), but three other breeds have only two genotypes (AA, AB). The results of this study indicate a pattern of genotype frequency in local goats in 4 countries (Indonesia, India, Iran, and China). Chinese local goats have three genotypes, Iranian have 3 and 2 genotypes, local Indian goats have two genotypes, while local Indonesian goats (Kacang) only have one genotype. This is related to the position of the country to the equator. Where the area around the equator is a tropical region bounded by latitude (Reis *et al.*, 2018). Latitude forms 3 main climates (cold zone, temperate climate zone, and hot lowland). In this zone, certain varieties of plants and animals are formed that can adapt to the environment (Asfaw *et al.*, 2019).

This study found two alleles (Table 1) A and B with the highest frequency in A. A allele in Boer goats was 0.62 and 0.72 in Boerka goats. So, the 5'UTR MSTN gene in Boer and Boerka goats were polymorphic because it had at least two alleles with a relative frequency greater than 0.01 (1%) (Nei and Kumar, 2000).

Hardy-Weinberg Equilibrium of 5'UTR MSTN Gene in Population

The genotype balance of 5'UTR MSTN gene in the population was tested by chi-square (χ^2).

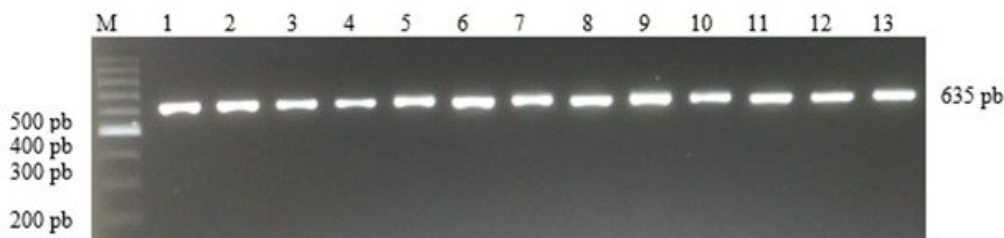


Figure 1. Amplification PCR Product of 5'UTR MSTN Gene Fragment using 1,5% Agarosa Gel. M = Marker 100 bp DNA; 1,2,3,.....13 = Sample Code

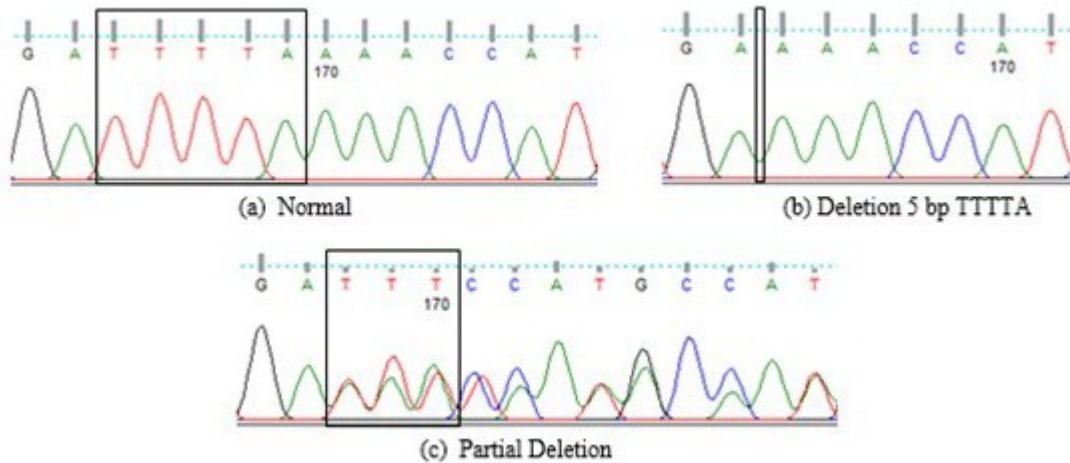


Figure 2. Visualization Indel (g.1256/TTTTA) 5'UTR MSTN Gene in Boer, Kacang, and Boerka Goat

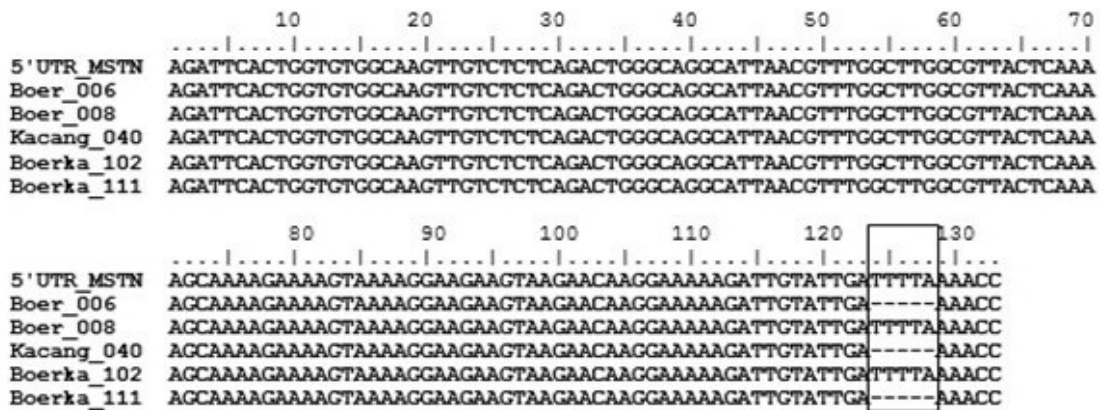


Figure 3. Alignment Partial Sequence 5'UTR MSTN Gene in Boer, Kacang, dan Boerka Goats with GenBank EF591039.1

The χ^2 test aims to determine whether the population was still in Hardy-Weinberg Equilibrium (HWE) or not. The population was said to be balanced if the calculated χ^2 value was smaller than χ^2 tables ($P < 0.05$) (Allendorf *et al.*, 2013). Boer, Kacang, and Boerka goats were still in HWE (Table 1). The frequency of alleles and genotypes does not change from generation to generation indicates that the population was in balance (Allendorf *et al.*, 2013). HWE only applies to populations that are ideal where there are no disturbances that affect genotype and allele frequencies (Waples and Allendorf, 2015). Therefore the overall principle of HWE makes

several assumptions such as; random mating, unlimited population sizes, no mutations, no selection, single population, no migration, non-overlapping generations, and diploid inheritance (Meirmans, 2018).

Heterozygosity was used to measure the level of genetic diversity in a population-based on allele frequencies. If the objective heterozygosity (H_o) value is greater than the expected heterozygosity (H_e), the population is diverse (Sharma *et al.*, 2016). Based on Table 1, the five base pairs indel (g.1256/TTTTA) MSTN gene in Boer and Boerka goats has low diversity due to the value of H_o less than H_e . H_o and H_e values

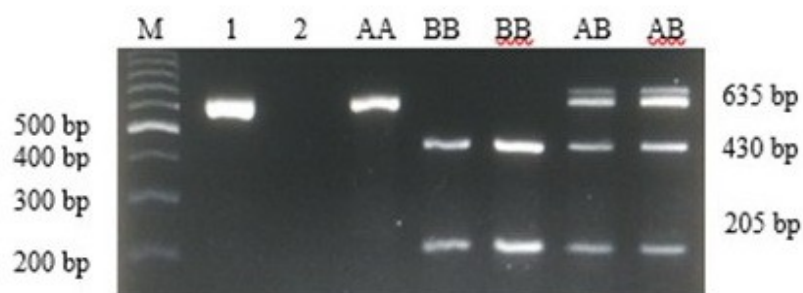


Figure 4. Amplification RFLP product of 5'UTR MSTN gene fragment using 2% agarosa gel. M = marker 100 bp DNA, 1 = DNA template, 2 = mix, AA = deletion, BB= normal, AB = heterozygous

Table 1. Result of Statistical Analysis in the Indel 5'UTR MSTN Gene in Boer, Kacang and Boerka Goat

Marker	Goat Population	(n)	Genotype Frequency			Allele Frequency		Ho	He	χ^2
			AA (n)	AB (n)	BB (n)	A	B			
Indel	Boer	(30)	0.40 (12)	0.43 (13)	0.17 (5)	0.62	0.38	0.43	0.47	0.30 (ns)
g.1256 / TTTTA	Kacang	(29)	1.00 (29)	-	-	1.00	-	-	-	-
	Boerka	(90)	0.53 (48)	0.37 (33)	0.10 (9)	0.72	0.28	0.37	0.41	0.95 (ns)

df = 1; χ^2 table = 3.84; ns = non significant

that were not significantly different indicated that there had been selection activities and there was no random matting (Allendorf *et al.*, 2013; Kolenda *et al.*, 2019)

Association of 5'UTR MSTN Gene with Body Weight

The results of the study (Table 2) five base pairs indel g.1256/TTTTA in the 5'UTR fragment of the MSTN gene were only significant ($P < 0.05$) to the growth in birth weight of the Boer goat, but not significantly in Boerka goats. However, the indel did not show any significant effect on other parameters such as M3W, M6W, M9W, M12W and ADG, both on Boer and Boerka 04 ± 0.82 (Zhang *et al.*, 2008), 3.22 ± 0.13 (Browning *et al.*, 2011) and 3.41 ± 0.80 (Menezes *et al.*, 2016).

The AA genotype in Boer goat birth weight (BW) was significantly different ($P < 0.05$) from the AB genotype but not substantially different from the BB genotype. The AA genotype tends to

have a higher body weight than the BB genotype in birth weight (BW) and 3 month age weight (M3W). Although it has no real effect, the AB genotype has a bodyweight that tends to be higher in; 6 months (M6W), 9 months (M9W), 12 months (M12W) of age and daily weight gain (ADG). In this case, the BB genotype has a bodyweight that is almost the same as the AB genotype compared to the AA genotype. BB genotype in Boerka goats tends to have the highest body weight in BW, M3W, M9W, M12W, and ADG. Although the three genotypes in Boerka goats did not show statistically significant differences, the highest tendency for body weight was found in BB and AA genotypes, compared to AB genotypes respectively.

Genotype AA was significant ($P < 0.05$) for the highest birth weight in Boer goat. This shows that 5'UTR MSTN|DraI can be used to select for birth weight traits in Boer goat in Indonesia. Previous studies found a significant association

Table 2 Association of Five Base Pairs Indel g.1256/TTTTA with Birth Weight of Boer, Kacang and Boerka Goat

Goat Population	Body Weight	Genotype			Significantly
		AA (n)	AB (n)	BB (n)	
Boer	BW (kg)	3.29± 0.19a (11)	2.88± 0.48b (12)	2.96± 0.17ab (5)	*
	M3W (kg)	10.13± 1.73 (11)	9.49± 2.91 (11)	9.72± 0.95 (5)	ns
	M6W (kg)	13.43± 3.82 (12)	14.78± 4.59 (13)	12.64± 2.54 (5)	ns
	M9W (kg)	14.84± 3.88 (12)	19.05± 5.51 (13)	16.52± 5.33 (5)	ns
	M12W (kg)	18.74± 4.49 (12)	24.48± 7.71 (13)	20.94± 6.59 (5)	ns
	ADG (g/d)	42.68±12.24 (12)	59.40±21.33 (13)	49.30±17.92 (5)	ns
Boerka	BW (kg)	2.58± 0.50 (48)	2.58± 0.44 (33)	2.71± 0.65 (9)	ns
	M3W (kg)	9.70± 3.24 (48)	8.61± 2.59 (33)	9.72± 3.08 (9)	ns
	M6W (kg)	13.49± 4.64 (48)	11.35± 3.56 (33)	12.76± 5.38 (9)	ns
	M9W (kg)	20.17± 6.59 (48)	17.88± 7.29 (33)	20.24± 8.61 (9)	ns
	M12W (kg)	25.48± 7.10 (48)	23.33± 8.04 (33)	26.76± 9.82 (9)	ns
	ADG (g/d)	62.73±19.15 (48)	56.88±21.85 (33)	65.85±26.45 (9)	ns

BW = birth weight; M3W = body weight at 3 months age; M6W = body weight at 6 months age; M9W = body weight at 9 months age; M12W = body weight at 12 months age; ADG = Average daily body weight gain between birth to 12 months of age; significantly test level (5%); n = number of samples; * significant (P<0.05); ns = non significant

between the indel g.1256/TTTTA markers on the growth traits in several goat breeds in China. Li *et al.*, (2008) report the results of the same study in which the AA genotype significantly affected birth weight up to 3 months of age in eighteen local Chinese goat breeds. The AB genotype in Boer goats was known to have a significant effect (P<0.05) on birth weight, 90-day weight, and 300-day weight (Zhang *et al.*, 2012). However, Bi *et al.*, (2020) reported different results in which the AA genotype in Shaanbei White Cashmere goat had no significant effect on body weight, but had a significant effect (P<0.05) on body size such as body height and height at the hip cross. All of these studies confirm that the g.1256/TTTTA 5'UTR MSTN gene indel can be used as a marker gene for the growth traits of goats. But differences in livestock environments between countries have led to variations in genotypic expression that control the nature of the growth.

CONCLUSION

The five base pairs indel g.1256/TTTTA of 5'UTR MSTN gene fragment was polymorphic in

Boer and Boerka, but monomorphic in Kacang goat. Boer, Kacang, and Boerka goats were still in Hardy-Weinberg equilibrium (HWE). Association of the polymorphism of the MSTN | 5'UTR gene DraI shown a significant difference in birth weight (BW). Genotype AA has only affected the growth of the highest birth weight in Boer goats, but it does not affect the other bodyweight parameters, both in Boer and Boerka goats. Therefore the molecular selection of the MSTN | 5'UTR gene DraI could be used to find the best Boer birth weight with AA genotype, but it is not suitable for Kacang and Boerka goats.

ACKNOWLEDGMENTS

This research was supported and funded by the Indonesian Agency for Agricultural Research and Development (IAARD) Indonesian Ministerial of Agricultural as a part of the 2019 scholarship package. The author would also like to give thank you to Heads of Indonesian Goat Research Center, Sungei Putih, Galang, Deli Serdang, North Sumatera for providing blood samples of goat and data that were used in this

study.

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