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Full Length Research Paper

The allele frequencies of growth hormone gene on the parental and progeny of Ongole-crossbreed cattle population in the North Sulawesi of Indonesia using PCR-RFLP

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The objectives of this research were to identify the allelic frequency of growth hormone *MspI* enzyme-restriction, to define the genetic inequilibrium in selected superior and inferior body weight groups of the cows (G0) and to upgrade genetically the genetic equilibrium of their Ongole-crossbred progenies (G1) population. A total of 74 blood samples were collected from G0 and G1 at the artificial insemination (AI) service center in North Sulawesi Province, Indonesia. Blood samples were screened for the presence of GH *Msp1* enzyme-restriction using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method on 1.2% agarose gel. Data were analyzed using statistical program in Excel XP. The results showed that *Msp1*+ allele frequencies in superior and inferior G0 groups were 0.45 and 0.18, respectively. The *Msp1*+ allele frequencies of G1 born by superior and inferior G0 groups were 0.50 and 0.21, respectively. These increasing frequencies were contributed by *Msp1*+ allele inheritance from *Msp1*+/+ bull genotype (Krista). Allele frequencies of *Msp1*+ in both groups of G0 were not under genetic equilibrium. However, allele frequencies of *Msp1*+ in G1 population were under genetic equilibrium. The AI technique should be maintained involving various bull genotypes for breeding purpose of favorable *Msp1*+/- heterozygous genotypes and balancing the genetic equilibrium in progeny population.

Key words: Growth hormone *Msp1* allele frequency, Ongole-crossbred cow population.

INTRODUCTION

In animal industry, growth traits of animals are always of primary concern during breeding for its determinant economical value. With the development of molecular biology and biotechnology, scientists are able to achieve more accurate and efficient selection goal by marker-assisted selection (MAS). In general, validating the genetic markers of growth traits is the initial and crucial step to establish a MAS system (Allan et al., 2007).

Growth hormone (GH) is an anabolic hormone synthesized and secreted by the somatotroph cells of the anterior lobe of the pituitary in a circadian and pulsatile manner, the pattern of which plays an important role in postnatal longitudinal growth and development, tissue growth, lactation, reproduction, as well as protein, lipid and carbohydrate metabolism (Ayuk and Sheppard, 2006). Effects of GH on growth are observed in several

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tissues, including bone, muscle and adipose tissue, so that GH gene with its functional and positional potential, has been widely used for marker in several livestock species, including the cattle such as *Bos taurus* and *Bos indicus* (Beauchemin et al., 2006). It has been reported that the restriction fragment length polymorphisms (RFLP) of GH were associated with body weight in Grati dairy cows (Maylinda, 2011).

The studies of GH gene *MspI* locus have been reported in Ongole crossbred cattle (Sutarno et al., 2005), Brahman cattle (Beauchemin et al., 2006), Indian Zebu cattle (Shodi et al., 2007) and West coastal Sumatera cattle (Jakaria et al., 2007). Their studies indicated that MspI +/+ and MspI +/- genotypes can be used as the candidate genes in cattle selection for breeding program. The objectives of this research were to identify the allelic frequency of growth hormone (GH) *MspI* enzyme-restriction, to define the genetic inequilibrium in selected superior and inferior bodyweight groups of cows (G0) and to upgrade genetically the genetic equilibrium of their Ongole-crossbred progenies (G1) population.

MATERIALS AND METHODS

Animals and sample collection

This study was carried out in North Sulawesi Province in the Sulawesi Island Northern of Indonesia and 74 female animals (parental cows and their progenies) in the total were used and comprised of 37 cows (age ranging 4 to 5 years old), and their 37 female progenies of Ongole crossbred cattle (age ranging 5 to 56 days old). All parental cows (G0) were reared under private areas belonging to farmers with unknown ancestors. Progenies (G1) were born from those G0 mated by artificial insemination using germ plasmas (semen) of the two Ongole bulls called “Kirsta” and “Tunggul” from “the artificial insemination bull germ plasma center” in Singosari, East Java Province, Indonesia. Prior to blood collection, body weights of animals (G0 and G1) were determined by using a digital weighing scale when animals were standing as described in Ozkaya and Bozkurt (2008). The total of 37 G0 consisted of 20 superior body weight animals (cow weights heavier than at least one fifth standard deviation above the mean) and 17 inferior body weight animals (cow weights lighter than one and half standard deviation below the mean) among cow (G0) population (n = 363 heads, with body weight average of 440.20 ± 58.03 kg) were included in this study as described in Paputungan et al. (2000).

DNA extraction

The genotyping process was conducted at the Biotechnology Lab. Department of Biological Science, Faculty of Mathematics and Natural Science, Sam Ratulangi University, Manado. Blood samples of those cows (G0), their progenies (G1) and two Ongole bulls as source of germ plasmas (mated by artificial insemination) were collected during July 2011 from Jugular vein of cow or calf in 10 ml EDTA (10%) tubes during the *MspI* selection experiment and stored in the refrigerator (4^{o}C) until ready for DNA isolation. Genomic DNA from whole blood of Ongole crossbred G0 and their calves (G1) were purified by standard protocol using proteinase K digestion as described by DNA extraction kit (AxyPrep Blood Genomic DNA Miniprep kit, AXYGEN Biosciences, Union city, CA, 94587, USA).

Genotyping for GH and allele identification

Following the genomic DNA isolation, the animals were genotyped for GH locus using PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism) and 1.2% agarose gel electrophoresis (Sulandari and Zein, 2003). Amplification of the fragment of 329 bp at intron 3 (Gordon et al., 1983; Dybus, 2002) was done with PCR, using forward primer 5’-CCCACGGGCAAGAATGAGGC-3′; reverse primer 5’-TGAGGAACTGCAGGGGCCCA-3’ (Mitra et al., 1995). The reaction mixture of PCR was performed by using 1x Taq pol 25 µL of master mix (Axygen Biosciences, CA, USA). Composition PCR kit Reaction (Solis Biodyne) (master mix containing $MgCl_2$ 1.5 µM 1x reaction) consist of 5 µL Firepol Master Mix (Ready-to-Load), 1 µL Primer GH5 (10 pmol/µL), 1 µL Primer GH6 (10 pmol/µL), 0.75 µL $MgCl_2$ (50 µM), 14,5 µL H_2O (MiliQ water), and 2 µL Sample of DNA (total volume of 25 µL). The final concentrations of 25 µL PCR for reaction component consisted of Taq Polymerase 1.2 U, Reaction Buffer B 1x, dNTPs 200 µM (each of dATP, dCTP, dCTP, dTTP), Primer (forward) GH5 0.4 µM, Primer (reverse) GH6 0.4 µM, and $MgCl_2$ 3.0 mM. The mixture was placed in thermal cycler PCR machine (Biometra T personal type) with the conditions of the thermal cycler as follows: the initial denaturation temperature step at 94°C for 5 min for 1 cycle followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s and a final extension at 72°C for 1 min. To digest this fragment, a protocol of restricted fragment length polymorphism (RFLP) with restriction enzyme *MspI* was used to recognize the particular site of CC↓GG. The PCR product of GH gene was digested at 37°C for 3 h by *MspI* enzyme. The reaction consisted of 2 µL Buffer V2 10X, 7.5 µL H_2O, 0.5 µL Enzyme Msp I (20 U/µL), and 10 µL PCR product. The digested products were separated by horizontal electrophoresis (100 volts, 30 min) in 1.2% agarose gels in 1x buffer TBE (Tris, Boric acid, EDTA).

Agarose gels were made by weighing 1.2 g agarose powder and placed into 100 ml Buffer TBE (Tris-Boric-EDTA) 1x. Agarose solution was boiled on the hot plate. The warm agarose was poured into comb printing tools to form several wells. A compact agarose gel was moved into electrophoresis tool immersed with Buffer TBE 1x. Loading samples were done by dropping 9 µL PCR product of digested DNA mixed with 1 µL loading dye into each well of agarose gel and into control well of DNA ladder 100 bp (loading dye was included in master mix Firepol). Following the end of PCR and RFLP process, the products were then subsequently electrophorated using 1.2% agarose gel. Each sample of the digested DNA of 10 µL was added by 2 µL of loading dye. The mixture was dropped in artificial hole of agarose gel to run the process of electrophoresis. The products of electrophoresis were immersed in the 10% ethidium bromide for 20 min to identify polymorphism of alleles based on the length of the band. The picture of DNA band products was visually taken on the UV-transiluminator using camera and compared with DNA Ladder (Marker) for allele and genotype identification (Table 1).

Data analysis

The PCR-RFLP data were analyzed by allele frequency (Nei, 1987). The allele frequency was calculated by counting methods as follows:

Table 1. Band of the fragment after *Msp1* enzyme digestion

Length ofDNA band (bp)	Identified allele	Genotype
224 103	Normal allel (Msp1 +)*	Msp1 +/+
327 224 103	Msp1 + and Msp1 -	Msp1 +/-
323	Mutant allel (Msp1 -)**	Msp1 -/-

*Cut by *Msp1* enzyme; **Uncut by *Msp1* enzyme.

$$x_i = \frac{(2n_{ii} + \sum n_{ij})}{2N}$$

Where, x_i is the *Msp1*+ allele frequency, n_{ii} is the number of cattle with the genotype of *Msp1*+/+, n_{ij} is the number of cattle with the genotype of *Msp1*+/-, **N** is the total number of cattle tested.

The equilibrium test of the observed *Msp1*+ genotype frequency compared with the expected *Msp1* + genotype frequency was calculated using Chi-square test ($\mathcal{X}^2$) (Byrkit, 1987; Walpole, 1993) as follows:

$$\mathcal{X}^2 = \sum \frac{(f_o - f_e)^2}{f_e} = \sum \frac{f_o^2}{f_e} - N$$

Where, $\mathcal{X}^2$ is the Chi-square distribution, f_o is the observed frequency, and f_e is the expected frequency of the i-th cell.

Estimation of heterozygosity of Ongole crossbred cattle in North Sulawesi was calculated using formula (Jakaria et al., 2007) as follows:

$$H_0 = \sum_{i \neq j} \frac{N_{1ij}}{N}$$

Where, H_0 is the frequency of heterozygosity, N_{1ij} is sum of the heterozygote animals at locus 1, N is total of the animals analyzed.

The rate of expected heterozigosity ($\hat{h}$) was calculated on the base of allele frequency at each DNA locus (Nei, 1987) as follows:

$$\hat{h} = 2n\ (1 - \sum x_i^2)\ /\ (2n-1)$$

Where, $\hat{h}$ is the expected heterozigosity value of locus, and x_i is the *Msp1*+ allele frequency.

The standard error (SE) of expected heterozygosity (H_e) was calculated using formula (Jakaria et al., 2007) as follows:

$$V_{s1}(H_e) = \frac{2}{2n(2n-1)} \{2(2n-2)(\sum x_i^3 - (\sum x_i^2)^2) + \sum x_i^2 - (\sum x_i^2)^2\}$$

Where, $V_{s1}(H_e)$ is the variance of heterozygosity, and x_i is the allele frequency of *Msp1*+. Standard error (SE) of heterozygosity = $\sqrt{V_{s1}(H_e)}$. Data were analyzed using software of the statistical program function in Excel XP (2007).

RESULTS AND DISCUSSION

The *Msp1* allele frequencies and its genetic equilibrium in superior and inferior body weight cow groups

The digestion of 327 bp PCR product for growth hormone (GH) gene with restriction endonucleases *Msp1* enzyme differentiated alleles marked *Msp1*+ and *Msp1*-. The *Msp1* digestion of the PCR products produced digestion fragments of 104 and 223 bp for *Msp1*+ allele and of 327 bp for *Msp1*- allele. The size of fragment for *Msp1*- allele was 327 bp after restriction digestion (Figure 1). The population of Ongole crossbred G0s and their G1s were detected and had three genotypes. The *Msp1*+/+ homozygous genotype (224 bp and 104 bp) was detected in 11 animals, while the *Msp1*+/- heterozygous genotype (327 bp, 224 bp, 104 bp) was detected in 29 animals. The *Msp1*-/- homozygous genotype (327 bp) was detected in 34 animals. The frequencies of genotype and alleles determined in the population for each superior and inferior body weights of G0s are presented in Table 2.

The superior body weight population of G0 was determined for the frequencies of *Msp1*+/+ homozygous genotype, *Msp1*+/- heterozygous genotype, and *Msp1*-/- homozygous genotype indicating the values of 0.10, 0.70, and 0.20, respectively. This case suggested a superiority of the *Msp1*- allele frequency of 0.55 compared with the *Msp1*+ allele of 0.45 in the population of superior body weight G0. However, the inferior body weight of G0 population was found only for the frequencies of *Msp1*+/+ homozygous genotype and *Msp1*-/- homozygous genotype of 0.18 and 0.82, respectively.

This also suggested a superiority of *Msp1*- allele of 0.82 compared with *Msp1*+ allele of 0.18 in the population of inferior body weight G0. The total population of superior and inferior body weight G0 indicated also the allele frequency for each *Msp1*+ and *Msp1*- of 0.32 and 0.68, respectively. The level of heterozygosity at GH locus was 0.32 (Table 2) indicating that Ongole crossbred cows were polymorphic as indicated by Dorak, (2006) that the minimum value of polymorphism for general acceptance was 1%.

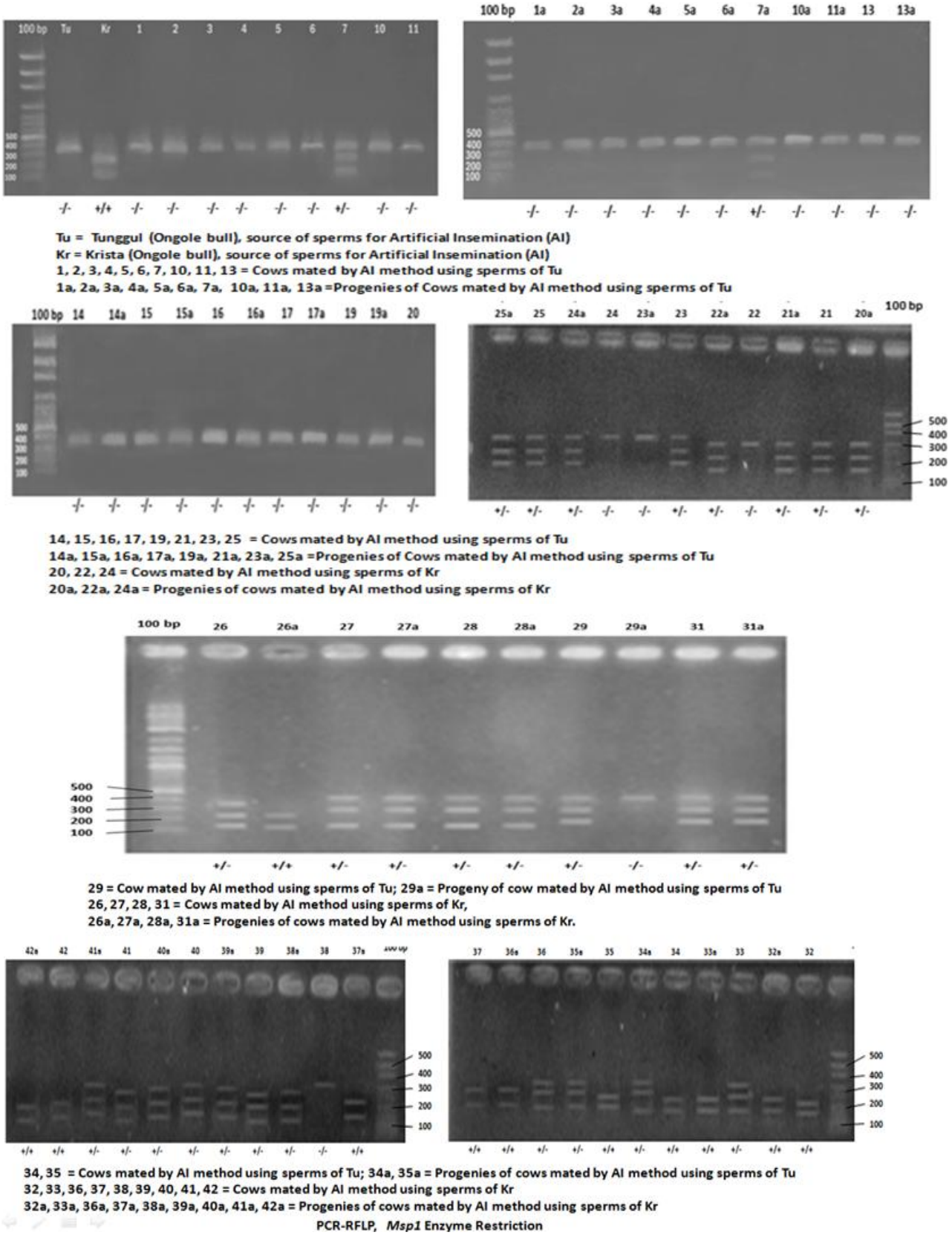

Figure 1. Genotyping results of *Msp1* enzyme restriction in GH locus detected by agarose gel electrophoresis.

Table 2. Genotype and allele frequencies of *Msp1* (+) and *Msp1* (–) at GH locus in Ongole crossbred parental cows (G0).

Cow phenotype (G0)	n	Enzyme/Locus		*Msp1* genotype and its frequency			X^2	*Msp1* allele frequency		X^2
				+/+	+/-	-/-		+	-	
Superior body weight (BW)[1)]	20	*Msp*1/GH	Ob	2 (0.10)	14 (0.70)	4 (0.20)		0.45 (18)	0.55 (22)	
			Ex	3 (0.15)	7 (0.35)	10 (0.50)		0.30 (12)	0.70 (28)	
							20.2*			8.93*
Inferior body weight (BW)[2)]	17	*Msp*1/GH	Ob	3 (0.18)	0	14 (0.82)		0.18 (6)	0.82 (28)	
			Ex	2 (0.12)	6 (0.35)	9 (0.53)		0.35 (12)	0.65 (22)	
		Heterozigosity (h) ± Standard Error (SE)						0.32 ± 0.090		
		Expected Heterozigosity ($\hat{h}$)						0.91		

Ob, Observed; Ex, Expected. [1)] Superior BW was the cow with high body weight more than 450 kg per head. [2)] Inferior BW was the cow with low body weight less than 350 kg per head. [*)] $X^2 = 20.2 > X^2_{.05}\{2\} = 5.991$; and $X^2 = 8.93 > X^2_{.05}\{1\} = 3.84$; the values denote that genotypic and allelic cow population were not in genetic equilibrium (p>0.05).

This polymorphism value suggested that in Ongole crossbred cows, there was high variability in the GH locus offering opportunity to use GH genotype criteria for selection in breeding program.

Based on the Chi Square test (Table 2), it was found that genotype and allele frequencies of GH *Msp1* were not under genetic equilibrium (P>0.05). This inequilibrium of genotypic and allelic frequencies of GH *Msp1* in this animal population caused the instability of genotypic and allelic frequencies of GH gene from G0 generation to the next generation due to the selection of superior and inferior parental cows without random mating system in animal population at this location of study.

Van Vleck et al. (1987) stated that factor affecting genetic equilibrium was selection program using selected male and female animal parents for the next generation without random natural mating system, such as the artificial insemination mating system.

The *Msp1* allele frequency and its genetic equilibrium in G1 born by G0 groups

Out of 17 genotyped progenies (G1) born by inferior G0 group, three G1 animals were found to be heterozygous genotype for the *Msp1*+/-. The *Msp1*+/- heterozygous genotype and *Msp1*- mutant allele frequencies were 0.18 and 0.79, respectively (Table 3). In their inferior G0 group, this heterozygous genotype frequency was not found, indicating zero genotype frequency as shown in Table 2. Based on the Chi Square test (Table 3), it was found that allele frequency of GH *Msp1* in G1 population was under genetic equilibrium (P<0.05). The genetic equilibrium of GH *Msp1* alleles in this animal G1 population caused the stability of genotypic and allelic frequencies of GH gene from G1 generation to the next generation due to the combination use of bull genotypes (Krista with *Msp1*+/+ and Tunggul *Msp1*-/- genotypes) as described in Figure 1. The application of artificial insemination technique involving the various genotypes of GH *Msp1* alleles in bulls as the animal sires of parental cows (G0) supported the genetic equilibrium in G1 population at this location of study.

In this case, the heterozygous genotype frequency of GH *Msp1*+/- was not found in group of inferior body weight cows causing the instability of genotypic and allelic frequencies of GH gene in animal population (Table 2). This *Msp1*+/- heterozygous genotype frequency was found only in group of the superior body weight G0 indicating a trend of heterosis effect inherited by alleles of both *Msp1*+ and *Msp1*-.

The genetic inequilibrium in G0 population can be genetically upgraded by mating of the various *Msp1* genotypes of sires (Krista = Kr_$Msp1^{+/+}$ and Tunggul = Tu_$Msp1^{-/-}$) as described in Figure 1, using either AI technique or natural mating system for the next progeny G1 population.The distribution of those improved progenies to the farmers was the main improvement using normal allele (*Msp1*+) of bull, such as

Table 3. Genotype and allele frequencies of *Msp1* (+) and *Msp1* (–) at GH locus in Ongole crossbred progenies (G1).

Phenotype of progeny (G1)	n	Enzyme/ Locus		*Msp1* genotype and its frequency			X^2	*Msp1* allele frequency		X^2
				+/+	+/-	-/-		+	-	
Born by superior cows	20	*Msp*1/ GH	Ob	4 (0.20)	12 (0.60)	4 (0.20)		0.50 (20)	0.50 (20)	
			Ex	3 (0.15)	8 (0.40)	9 (0.45)		0.40 (16)	0.60 (24)	
							11.3*			3.73[eq]
Born by Inferior cows	17	*Msp*1/GH	Ob	2 (0.12)	3 (0.18)	12 (0.70)		0.21 (8)	0.79 (26)	
			Ex	3 (0.18)	7 (0.41)	7 (0.41)		0.35 (12)	0.65 (22)	
		Heterozigosity (h) ± Standard Error (SE)						0.40 ± 0.116		
		Expected Heterozigosity ($\hat{h}$)						0.88		

Ob, Observed; Ex, expected. *) $X^2 = 11.30 > X^2_{.05}\{2\} = 5.991$; the values denote that genotypic progeny population were not in genetic equilibrium (p>0.05). Eq) $X^2 = 3.73 < X^2_{.05}\{1\} = 3.841$; the values denote that allelic progeny population were in genetic equilibrium (p<0.05).

Krista with genotype of *Msp1*+/+. Jawasreh et al. (2012) reported that breeding program must be continued as the first step to increase the frequency of the favorable allele in breeding station. Genotypic selection of bulls was the main strategy for distribution of improved animals to the farmers. The increasing frequency of *Msp1*+/- heterozygous genotype of the progenies (G1) to be 0.18 from that of their parental cows (G0) of zero in inferior bodyweight group was the main consequence of using genotypic combination of selected bulls mated by the artificial insemination service program in location of this study. In North Sulawesi Province, the artificial insemination service center at Tumaratas village are using straw containing spermatozoa germ plasma of Ongole bull called “Krista” and “Tunggul” from germ plasma center for artificial insemination (Balai Benih Inseminasi Buatan) Singosari, East Java Province.

The GH gene for *Msp1* enzyme restriction in both bulls of “Krista” and “Tunggul” were *Msp1*+/+ (Kr_*Msp1*$^{+/+}$) and *Msp1-/-* (Tu_Msp1$^{-/-}$), respectively (Figure 1). These different genotypes of bulls may indicate potential polymorphism of GH gene in each generation of Ongole crossbred cattle breeding program in North Sulawesi, as indicated by the increasing heterozygosity value of 0.40 in the progeny (G1) population (Table 3) compared with that of 0.32 in their parental cow (G0) population (Table 2). Do et al. (2012) reported the analysis of gene interaction and found that it might be two or more genes can interact to express a particular phenotype. Multiple gene products can also contribute to the expression of a single phenotype along the biochemical pathways in the cells (Klug et al., 2007).

The heterozygosity estimation value of 0.40 for the progeny generation (G1) indicated that GH-*Msp1* was more informative (polymorphic) in Ongole crossbred cattle in North Sulawesi. The mean of observed heterozygosity was lower than the expected heterozygosity. Heterozygosity value in this study was slightly higher than that of Grati cattle population reported by Meylinda, (2011). Allele frequency of GH *Msp1* in this G1 population was under genetic equilibrium (P<0.05), indicating that breeding program for generating the heterozygous genotype of GH locus of *Msp1*+/- enzyme restriction by artificial insemination system should be maintained for the purpose of increasing the rate of dispersion of these favorable genotypes as indicated by superior body weights of parental cows (G0). This was supported by the fact that a population property of gene pool for GH-*Msp1* under the Hardy-Weinberg equilibrium pattern was a function of both allele frequencies and biological interactions among genes (Carter et al., 2005).

Conclusion

This research indicates that the *Msp1*+ allele frequencies in superior and inferior bodyweights of cow (G0) groups were 0.45 and 0.18, respectively. The *Msp1*+ allele frequencies of progenies (G1) born by superior and inferior bodyweights of G0 groups were 0.50 and 0.21, respectively. These increasing frequencies were contributed by *Msp1*+ allele inheritance from *Msp1*+/+ bull genotype (Krista). The frequencies of *Msp1* alleles in groups of G0 were not under genetic equilibrium (genetic inequilibrium). However, those of *Msp1* alleles in G1 population were under genetic equilibrium. Therefore, the breeding program using various genotypes of bulls and cows (G0) for generating the heterozygous genotype of GH *Msp1*+/- enzyme restriction by the artificial insemination technique should be maintained for the

purpose of increasing these various allele dispersion rates for balancing genetic equilibrium and breeding of those favorable heterozygous genotypes in the Ongole crossbred cattle population.

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