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Short Communication: Polymorphism of *myostatin* gene and its association with body weight traits in a hybrid of GAMA chicken (*Gallus gallus domesticus* Linn. 1758)

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Abstract. *Tanjung A, Saragih HTSSG, Trijoko, Soenarwan HP, Widianto S, Mahardhika IWS, Daryono BS. 2019. Polymorphism of* myostatin *gene and its association with body weight traits in a hybrid of GAMA chicken (*Gallus gallus domesticus *Linn. 1758). Biodiversitas 20: 3207-3212.* An experiment was conducted to detect SNP of the *myostatin* gene and its association with the body weight of hybrid chicken crossbreed from F_1 *Kamper* and BC_1 Broiler. Four F_1 *Kamper* hens were crossbred with BC_1 Broiler rooster. Day old chick (DOC) hatched were maintained for 49 days with body weight measurement every seven days. The blood samples from 49 days old chicken were taken for DNA isolation by Chelex 5% method and then amplification of the *myostatin* gene. PCR products were sequenced, and sequence alignment was performed using Clustal Omega to obtain SNP. The SNP obtained was analyzed by the Pearson correlation test with the body weight of forty nine-days-old chickens. The body weight of the hybrid chicken is higher than of *Pelung* chicken but lower than the Broiler. There are 7 SNPs in *myostatin* gene exons included 2 Adenine insertions, 1 Guanine deletion, and four substitutions (C2244G, G2283A, T4842G, G7378T) that yield nine haplotypes. Six haplotypes had different protein sequences with *Myostatin* protein, while three haplotypes were identical to *Myostatin* protein. The correlation analysis showed that there was a strong positive correlation (r = 0.736) between normal *Myostatin* protein and mutant to chicken body weight at 49-days-old. Adenine insertion to nucleotide 2099-2100 of *myostatin* gene had a very strong positive correlation (r = 0.800) to 49-days-old chicken body weight, although T4842G substitution had a strong negative relationship (r = -0.773) to 49-days-old chicken body weight. Adenine insertion to nucleotide 2099-2100 of *myostatin* gene could be a genetic marker of heavier body weight of the hybrid chicken.

Keywords: Adenine insertion, chicken body weight, genetic marker, *myostatin* gene, polymorphism

INTRODUCTION

Indigenous Indonesia chickens have to be maintained optimally to support small scale poultry industry Indigenous Indonesia chickens germplasm can be the solution for fulfilling the increasing demand for domestic food consumption (Daryono et al. 2010). Improvement of productivity and competitive quality of local broiler chicken can be achieved through selective breeding of indigenous Indonesia chicken breeds. Selective breeding is aimed to produce a superior chicken breed with adjusted phenotype quality according to human needs (Cheng 2010).

Pelung Blirik Hitam has several distinguished characters such as higher posture and body weight compared with other indigenous breeds (Daryono et al. 2010). Body weight of male *Pelung* chicken can reach 3.37 kg, and females can reach 2.52 kg (Daryono et al. 2010). Hidayat and Asmarasari (2015) stated that in the period of 20 weeks body weight of *Pelung* can reach 1663 gram/head heavier than other meat producer indigenous chicken such as *Kampung* (1408 gram/head), Black Kedu (1480 gram/head), White Kedu (1320 gram/head) and *Nunukan* (1203 gram/head). Meanwhile, Broiler Cobb 500 has distinguished productivity and a high growth rate in the early phase (7 to 18 weeks). The male and female Broiler Cobb 500 can reach 1,599.17 grams and 1,540.46 grams (Hassan et al. 2016).

Indigenous chicken breed consists of 31 breeds which can be further classified into four functional groups i.e. singing chicken, used in traditional ceremonies, fancy and fighting cock, meat and egg producer (Hidayat and Asmarasari 2015). Selective breeding between *Pelung Blirik Hitam* and Broiler Cobb 500 produced hybrid chicken F_1 Broiler or *Kambro* (*Kampung*-Broiler) (Mahardhika and Daryono 2019). Based on Body Weight (BT) measurement, *Kambro* population (n= 17) with average BT of 1244.14 ± 453.82 grams was significant ($p<.001$) to F_1 *Pelung* (n= 7) with average BT of 602.88 ± 79.93 grams in 8 weeks period with *ad libitum* diet of standard feed (Mahardhika and Daryono 2019). The improvement of *Kambro* performance was significant to F_1

Pelung based on the measurement of linear body weight parameter, vitality parameter, PPa-PBe parameter, and phenotype parameter (Mahardhika and Daryono 2019). *Kambro* has the phenotype combination of parental generation based on phenotype parameter (Hassan et al. 2016). In this study Backcross I of Broiler which derived from F_1 Broiler (♀Broiler x ♂*Pelung*) crossbreed with its *Pelung* male parent was used. This BC_1 Broiler has higher growth than local Indonesian chicken and more uniform character than the F_1 Broiler (Utama et al. 2018). Both chickens can be used to be parental for selective breeding methods. This method required morphological and molecular character that can be used as a marker.

Accompanying selection for rapid growth, meat-type chickens exhibit an increase in physiological disorders such as obesity, multitrait selection to simultaneously improve fitness and increase production is, therefore, difficult to achieve by direct selection. Molecular marker-assisted selection (MAS) may be required, and the integration of traditional genetic selection and modern molecular methods may be preferred for breeding chickens in the future. Advances in molecular biotechnology enable fast and reliable methods for the accurate diagnosis of mutations responsible for different genetic defects.

Myostatin gene, also called Growth and Differentiation Factor 8 (GDF-8) belonging to TGF-β superfamily, has the function of regulating the growth and differentiation of skeletal muscle (Sharma et al. 2015). There is 44 known polymorphism in the *myostatin* gene. Some of them are associated with growth (Bhattacharya and Chatterjee 2013; Dushyanth et al. 2016). Therefore, this study aimed to detect *myostatin* gene polymorphism in hybrid chicken and its association to body weight so that it can be used as a genetic marker to determine the best individual to become a parent stock.

MATERIALS AND METHODS

Chicken breeding

Chicken used in this study were Day Old Chick (DOC) crossbreed of ♀ F_1 *Kamper* and ♂ BC_1 Broiler (Figure 1), DOC of Broiler as positive control and DOC of *Pelung* as the negative control. The DOC was maintained for 49 days with lighting 24 hours using 10 watts light bulb, air temperature ±30ºC and 40-50% humidity, feed by BR I (Japfa Comfeed) *ad libitum* and body weight measurement every seven days. 1 mL of blood was taken from 49-days-old chicken samples from wings vein using 26G syringe and stored in an EDTA tube and at -20ºC until used for DNA isolation.

DNA isolation

The DNA isolation protocol performed was the Chelex method (Butler 2016) with modified concentration of Chelex concentration according to optimization phase. A total of 10 µL of blood samples were mixed into a 1 mL TE 10 mM pH 8 in 1.5 mL Eppendorf tube and then centrifuged at 13,000 rpm for 3 min. The centrifugation supernatant was discarded and 200 µL Chelex 5%, 18 µL DTT 0.01 M, and 2 µL Proteinase K 10 mg/mL were added. The mixture was incubated at 56ºC for 1 hour with vortex every 15 min. Then incubated in 100ºC for 8 min and centrifuged at 13,000 rpm for 3 min. The supernatant containing DNA isolate (±150 µL) was transferred to a new Eppendorf tube for storage at -20ºC until used as a PCR template.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction was done using the master mix MyTaq HS Red Mix of Bioline. Primer was used to amplify the *myostatin* gene fragment are listed in Table 1. The mixtures added into each PCR tube were 25 µL PCR master mix, 2.5 µL forward primer (10 pM/µL) and 2.5 µL reverse primer (10 pM/µL), 10 µL DNA template (10 ng/µL), and 10 µL ddH_2O resulted in the total volume of 50 µL.

The PCR reaction consisted of predenaturation 95ºC for 1 min, followed by 35 cycles of denaturation 95ºC for 15 sec, annealing 55ºC for 15 sec, and elongation 72ºC for 10 sec and the final elongation 72ºC for 5 min. The PCR product was visualized on 2% agarose gel electrophoresis. The generated amplicons were separated by agarose gel and visualized under UV light by AnalytikJena™ gel imaging system and documented with GelDoc™ Documentation System. The mutant allele and wild-type allele are differentiated by checking the amplicon sizes in reference to size markers. Images of electrophoresis gel were analyzed with ImageLab (V. 6.0.1) to identify normal and mutant bands based on base-pair length with BenchTop 100 bp ladder. Agarose gel was added with 3 µL FloroSafe DNA Stain 1st Base for each running with 4 µL PCR sample and 4 µL 100 bp ladder. Electrophoresis was performed with electroporator Mupid-exU™, and TAE Buffer 0,5X was used as a buffer solution.

Sequencing

The PCR product was sequenced by Sanger sequencing method in 1st Base Company, Selangor, Malaysia.

Table 1. *Myostatin* gene primer was used (Perdamaian et al. 2017)

Primer name*	Primer Sequence (5'-3')	Fragment	Size (bp)
MYTmF	ATGCAAAAGCTAGCAGTCTATG	Exon 1	373
MYTE1R	ACTCCGTAGGCATTGTGATAAT		
MYTE2F	CTGATTTTCTTGTACAAATGGAG	Exon 2	374
MYTE2R	CAATCCATCTTCACCCGGTC		
MYTE3F	AACCCATTTTTAGAGGTCAGAG	Exon 3	381
MYTmR	TCATGAGCACCCGCAACGAT		

Note: *F: Forward primer; R: Reverse primer

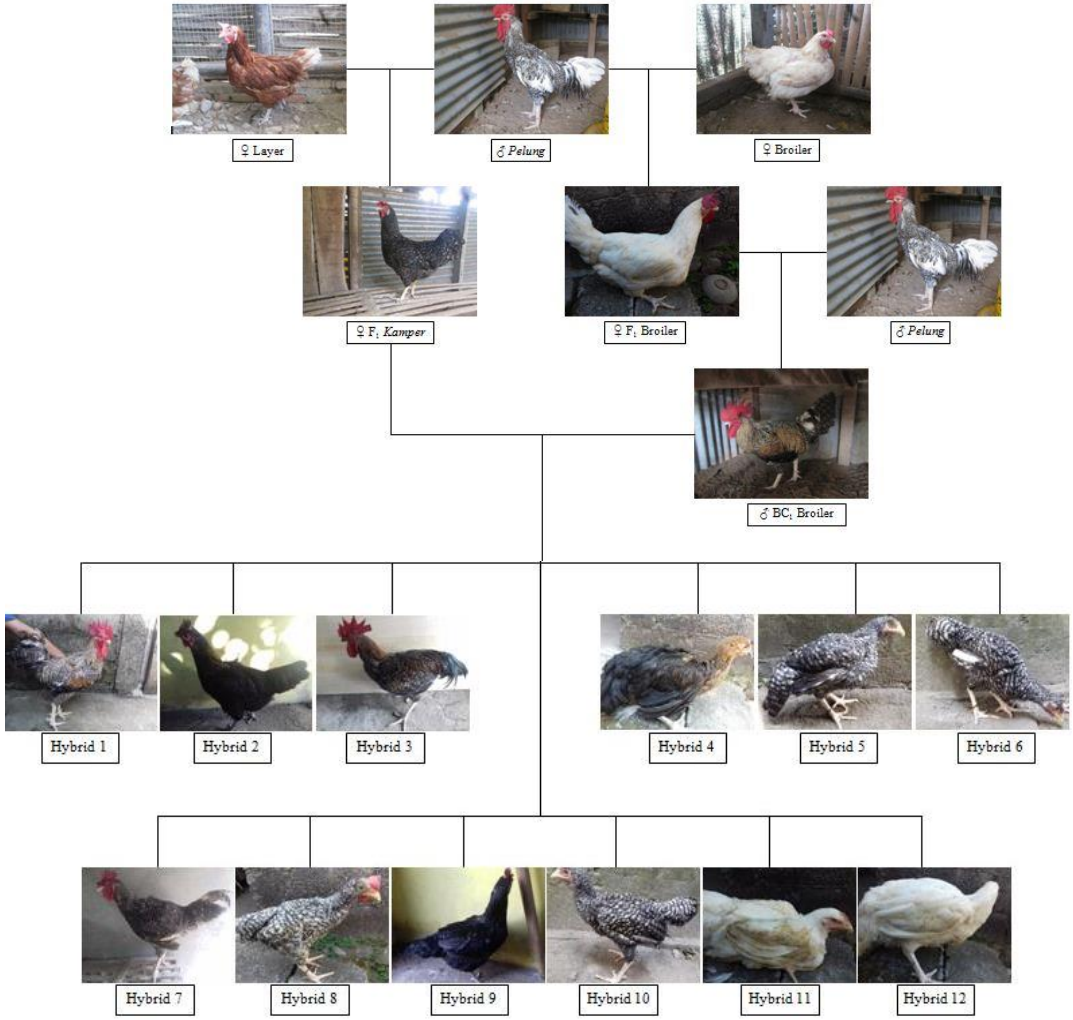

Figure 1. Pedigree of hybrid chicken from crossbreed F_1 *Kamper* and BC_1 Broiler

Data analysis

The data obtained were DOC body weight 0d-49d (Table 2) and was analyzed using ANOVA ($p<0.05$) statistical test and post hoc LSD method (reference ?) to assess the significance between chicken strains. The hybrid chicken was separated by sex (6 males and six females) for weighing by using digital scale ©KrisChef. *Myostatin* gene sequencing result was processed using Gene Studio software, multiple sequences alignment between individuals using Clustal Omega software, and SNP association with chicken weight by using Pearson correlation test (Arnedo et al. 2007) in SPSS software. The polymorphisms obtained in this study lies in the coding region, so it is necessary to predict the amino acid changes using Sequence Translation Sites software online. The results of each haplotype translation then made alignment using Clustal Omega software to observe differences in protein arrangement between haplotype.

RESULTS AND DISCUSSION

Chicken growth

The purpose of the formation of this new chicken strain in addition to having a phenotype character as a local chicken also has faster growth than local chicken so it can be harvested more quickly to increase the production of domestic chicken meat. In this study, we used ♂Backcross I Broiler, which is the offspring of pure line broiler to increase the rate of growth of hybrid chick. In addition, the purpose of F_1 *Kamper* use is to inherit the laying traits obtained from the Layer parent (Figure 1).

The hybrid chicken has higher growth rate compared to *Pelung* chicken, but has lower growth rate when compared to Broiler chicken (Table 2). ANOVA test results indicated a significant weight difference between the three types of chickens. The growth of hybrid chicken that lies between the growth line of *Pelung* and Broiler chicken due to the hybrid chicken has a lineage of both. Hence, it is important to further investigate the cause of differences in the growth of these chickens, by assessing *myostatin* gene polymorphism that has been known to be one of the genetic markers for chicken growth.

Table 2. Hybrid, Pelung, and Broiler chicken body weight 0-49 days

Chicken line	Days-old						
	7	14	21	28	35	42	49
Hybrid [n=12]	66.83±8.419[b]	125.00±15.892[b]	184.17±25.778[b]	268.08±50.581[b]	350.75±81.114[b]	435.58±88.584[b]	529.92±117.586[b]
Pelung [n=5]	33.60±3.507[a]	45.20±8.871[a]	62.80±19.267[a]	95.80±26.205[a]	143.00±37.703[a]	196.60±33.568[a]	289.40±56.871[a]
Broiler [n=6]	194.00[c]	461.00[c]	842.00[c]	1309.00[c]	1817.00[c]	2347.00[c]	2897.00[c]

Table 3. SNPs of *myostatin* gene

Sample	Position in the *myostatin* gene							Haplotype
	Exon 1					Exon 2	Exon 3	
	2099-2100 A insertion	2108-2109 A insertion	2109 G deletion	C2244G substitution	G2283A substitution	T4842G substitution	G7378T substitution	
AF346599	-	-	g	c	g	t	g	Reference
Hybrid 1	a	-	g	c	a	k	g	1
Hybrid 2	-	a	g	s	a	k	g	2
Hybrid 3	a	-	g	c	a	k	g	1
Hybrid 4	-	a	g	s	a	g	g	3
Hybrid 5	a	-	g	c	r	k	g	4
Hybrid 6	-	a	g	s	r	k	g	5
Hybrid 7	a	-	g	c	a	k	g	1
Hybrid 8	-	-	g	c	r	g	g	6
Hybrid 9	-	a	g	s	a	k	g	2
Hybrid 10	a	-	g	c	a	k	g	1
Pelung 1	-	a	g	s	a	k	k	7
Pelung 2	-	a	-	g	a	k	k	8
Pelung 3	-	a	-	g	a	g	g	9
Pelung 4	-	a	g	s	a	g	g	3
Pelung 5	-	a	-	g	a	g	g	9

Note: *a: adenine; g: guanine; c: cytosine; t: thymine; s: guanine or cytosine; r: adenine or guanine; k: guanine or thymine

Myostatin gene polymorphism

Myostatin gene polymorphism has been known to associate with the growth of chicken weight (Zhang et al. 2011). The SNPs obtained in this study (Table 3) were based on previous research by Bhattacharya and Chatterjee (2013). There are 7 SNPs, i.e. 2 Adenine insertions (2099-2100 and 2108-2109), 1 Guanine deletion (2109), and four substitutions (C2244G, G2283A, T4842G, G7378T) on the three exons of the *myostatin* gene. Two of the SNP obtained were the substitution of G2283A also obtained in *Bian* chickens which is the indigenous chicken from China and substitution of T4842G also obtained in *Kampung*, *Merawang*, and *Sentul* chicken which are the indigenous chicken of Indonesia (Zhang et al. 2011; Zhang et al. 2012; Khaerunnisa et al. 2016). Several SNPs in the *myostatin* gene such as C7552T, C7638T, and T7661A has existed in noncoding exon 3 (Zhang et al. 2011). Some SNPs have found in the 5' regulatory region, and the exon 1 *myostatin* gene is A326G, C334G, G673A, G985C, G1085A, A1278T, C1346T, G1375A, A1473G, G1491A, and G2283A (Zhang et al. 2012; Zhang et al. 2012). In addition, some of the polymorphisms that have been obtained in *myostatin* genes in chickens include G167A, T177C, G304A, A322G, and C334T in regulator region 5' and A6935G and A7263T in the 3' regulatory region (Gu et al. 2002). Give discussion based on your findings, not just describing the SNP polymorphism.

These seven SNPs from nine haplotypes. The result of protein alignment (Figure 2) suggested there were differences in the protein arrangement between haplotype resulting from polymorphism. There are two protein patterns of nine haplotypes based on Genbank reference. The first type that is is identical to the GenBank reference were haplotype 6, 8, and 9 predominantly found in *Pelung* chicken. The second type that was different from GenBank reference where haplotype 1, 2, 3, 4, 5, and 7 possessed by hybrid chicken. The difference of protein arrangement in hybrid chickens is caused by insertion mutation between nucleotides to 2099-2100 and nucleotides to 2108-2109, so there is a frameshift mutation of codon that resulted in changing of protein arrangement after mutation point. Frameshift mutation often leads to changes in protein structures resulting in protein malfunctions or protein degradation that should be formed. This is consistent with the theory that *myostatin* gene mutations cause an increase in weight due to *Myostatin* protein degradation, so that myoblast proliferation occurs continuously (Ye et al. 2006). The mutations that have been obtained in the coding region are the mutations in the coding region of exon 1 G2283A acquired but do not cause changes in amino acids (silent mutation) (Zhang et al. 2011). Changes in the composition of these amino acids cannot be known whether the phenotypic changes that occur, so it needs to be further investigated.

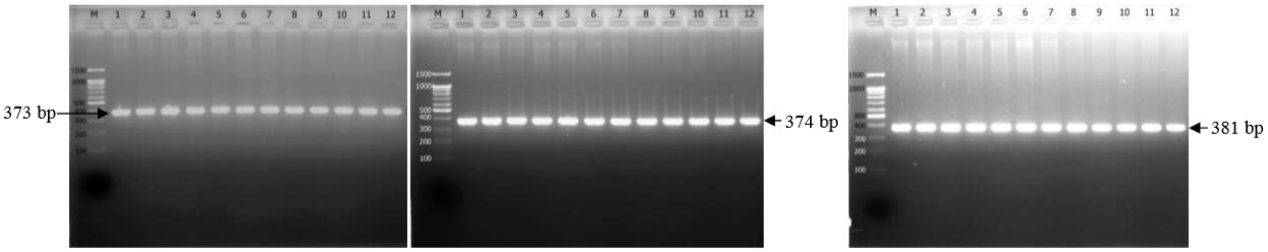

Figure 2. *Myostatin* gene visualization results (A) exon 1 (373 bp); (B) exon 2 (374 bp); (C) exon 3 (381 bp); M: DNA ladder 100 bp; 1-12: sample code of hybrid chickens (arrows indicate size of amplified fragments)

Table 4. Correlation test *myostatin* polymorphism to chicken weight at 49 days-old

Polymorphism	Insertion-A 2099-2100		Insertion-A 2108-2109		Subtitution-C2244G			Subtitution-C2283A			Subtitution-T4842G		
Genotype	-	AA	-	AA	CC	CG	GG	GG	GA	AA	TT	TG	GG
Frequency of genotype	0.5	0.5	0.6	0.4	0.6	0.4	0	0	0.3	0.7	0	0.8	0.2
Average of chicken body weight on 49th-day (g)	473.4	640.2	595.7	498.5	595.7	498.5	-	-	503.3	579.7	-	579.1	395.5
Correlation coefficient	0.800		-0.457		-0.457			0.336			-0.773		
Significance	0.005** (p<0.01)		0.185 (p>0.05)		0.185 (p>0.05)			0.343 (p>0.05)			0.009** (p<0.01)		
Finding	Significant; very strong positive correlation		Not significant; negatively correlated		Not significant; negatively correlated			Not significant; positively correlated			Significant; strong negative correlation		

Mutations in the *myostatin* gene were first observed in mice treated with knockout *myostatin* gene expression of hyperplasia and skeletal muscle fibers hypertrophy, resulting in a doubling of the mice's weight compared to normal. The double-muscling events are also found in Belgian Blue and Piedmontese cattle that have mutations in the coding region of *myostatin* gene (Kambadur et al. 1997; McPherron and Lee 1997; Wiener et al. 2009). Mutation of the *myostatin* gene was also found in sheep and goat. Mutation in sheep *myostatin* associated with double-muscling and birth weight (Gan et al. 2008; Farhadian et al. 2012). While in *goat*, *myostatin* gene polymorphism associated with growth traits (Alakilli et al. 2012). Furthermore, the *myostatin* gene not only regulates skeletal muscle development but also plays a role in metabolism and fat deposition in chicken (Gu et al. 2003).

Association of polymorphism with growth traits

Changes in protein structure due to expressed polymorphism can be observed by analyzing the difference of chicken body weight with Pearson correlation test (Arnedo et al. 2007). Haplotypes having identical protein arrangements to GenBank references are considered normal phenotypes whereas haplotypes having different protein arrangements with GenBank references are considered mutant phenotypes. Both of these phenotypes performed a correlation test on the weight of chickens on 49-day-old using Pearson correlation test. The results from Pearson correlation test suggested that there is a strong positive correlation (r = 0.736) and significant between the normal phenotype and the mutant against the 49-days-old chicken weight (Table 4). This suggests that there is an increase in chicken weight due to the *myostatin* gene polymorphism.

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