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Phenotype Diversity and Gene Myostatin (MSTN) of Bangkok Chicken using PCR-RFLP

Triana Mardiah, Depison*, and Helmi Ediyanto

Animal Science Study Program, Faculty of Animal Science, University of Jambi, Jambi, 36361, Indonesia

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* Corresponding author:
Telp. +62 812 7446 999
E-mail: depison.nasution@unja.ac.id

ABSTRACT

This study aims to characterize the phenotype and determine the diversity of the Myostatin (MSTN) gene at Bangkok chickens using the Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) method. This study used 50 Bangkok chicken blood samples taken from the *axillary vein* on the wing. DNA was extracted using the protocol *Genomic* DNA *Purification Kit* from Promega and then amplified by PCR (*polymerase chain reaction*) using a pair of primers F: 5'GGT TTT GAC GAC ATG AGC CT3' R: 5'CAG GTG GAA TGT CAT GCA GA3' with product length 955 bp. Amplification products were cut using restriction enzyme *MboI* with cutting site ↓GATC. MSTN|*MboI* fragments of the Bangkok chicken were electrophoresed using 2% agarose gel and visualized using doc gel. The average difference test (T-test) on body weight and weight gain of Bangkok chickens from DOC to 3 months by gender. Polymorphism analysis includes allele frequency and genotype. Male and female Bangkok chickens have low phenotype diversity. The MSTN|MboI gene fragment is monomorphic with band positions of 492 bp, 244 bp, and 219 bp resulting in a genotype of ++, and there is one type of allele with a + allele frequency of 100%.

Keywords: Bangkok chicken, Enzyme MboI**,** Local chicken, Myostatin gene, Polymorphism

Introduction

Poultry farming in national development is very strategic because poultry in Indonesia spearheads the need for animal protein. Poultry, which is crucial to the availability of animal proteins a local chicken. Local chickens are native chickens or chickens that have long been adopted in Indonesia. Local chicken can be developed because it can be a meat producer, egg producer and can be used as a hobby. The Bangkok chicken is one of the local chickens popular with the community as a hobby and can produce meat and eggs.

Bangkok chicken is a fighting chicken known as King's Chicken which comes from gallus descent in Muangthai, Thailand, has a prominent physique with strong muscles (Junaedi and Nurcholis, 2017). Chicken Bangkok has a well-developed potential small-scale and commercial, in helping fulfilment of animal protein. But genetic data on phenotypic characteristics of chicken Bangkok not much is known about the features of the helpful phenotype information in determining the genetic quality of livestock that will be a consideration in the selection of crosses.

Effort were made to determine the genetic quality of Bangkok chickens, incuding collecting basic data in the form of phenotypic and genetic characteristics and diversity in the population through characterization. Characterization is a method in selection programs for Bangkok chickens through economically valuable traits known as quantitative characteristics, including body weight and weight gain. Elements quantitative are influenced by external environmental factors such as the availability of feed and climate. This makes the selection based on quantitative characteristics takes longer and numbers of livestock more.

The progress in the molecular field can be used as an alternative in the selection program for Bangkok chickens. What can carry out the molecular selection on genes directly related to traits that have economic value. One of the economically valuable genes is the Myostatin gene which acts as a growth controller. One of the members of the growth sub gene (Transforming Growth Factor/TGF) that plays a role in controlling mass muscle growth is the Myostatin Gene (MSTN) (Batubara, 2017). Myostatin gene is a negative regulator of muscle growth in poultry and other animals, by destroying this negative

regulator an important aspect of promoting muscle growth in chickens (Bhattacharya *et al.*, 2017). One of the characterization and identification of the Myostatin gene can use the PCR-RFLP marker.

One of the techniques used to multiply DNA strands is the PCR method which uses a pair of primers with temperature conditions that have been regulated through enzymatic reactions by DNA polymerase (Alfaruqi *et al.*, 2020). The technique of cutting homologous DNA fragments uses restriction enzymes. It produces DNA fragments with different sizes from one allele to another to describe polymorphisms in homologous DNA sequences. RFLP (*Restriction Fragment Length Polymorphism*) has several advantages, one of which is that DNA can be propagated quickly using the *polymerase chain reaction* (PCR) method and its fragment polymorphism through restriction enzymes to identify genotypes because techniques are increasingly intensive to be used as genetic markers (Jakaria *et al.*, 2007). Phenotypic characteristics and diversity of Myostatin gene(MSTN) Bangkok chicken can be used as a reference in the future selection and development of chickens in Bangkok.

Materials and Methods

The material in the field research used was 50 Bangkok chickens consisting of 30 females and 20 males, commercial feed produced by PT. Japfa Comfeed Indonesia Tbk, namely BR 1 for 0-1 months of age with an energy composition of 3020-3120 Kcal/kg, protein 22-23%, fat min 5%, Calcium min 0.9% and phosphor min. 0.6% and BR 2 for 1-3 months of age composition 4100 Kcal/kg, 20-21% protein, 5% fat min, 0.9% min calcium, and 0.6% min phosphorus with digital scales and writing instruments with digital scales and stationery. In laboratory research, the materials used are blood samples taken from Bangkok chickens aged three months, totalling 50 samples. The equipment used is hand glove, EDTA K3 vaculab, tube holder, disposable syringe size 3 ml, cool box, stationery, freezer, oven, autoclave, micropipette 200 µl, 1000 µl, 10 µl, 20 µl, Eppendorf tip pipette (yellow, blue, white), Eppendorf microtube size 0.2 ml, 1.5 ml and 2 ml microtube rack, centrifuges, Vortec, analytical balance, Erlenmeyer, cups, measuring gel doc, power supply electrophoresis, electrophoretic gel system, gel printer, well comb, mini spin centrifuge, electric heater, PCR machine, and water bath. Materials used in this study is the chicken blood Bangkok, alcohol 70% and cotton for preservatives blood, protocol Genomic DNA Purification Kit from Promega, isopropanol, ethanol 70%, powder agarose, a solution of TBE Buffer, distilled water, staining ethidium bromide (EtBr), loading dye, DNA ladders, forward and reverse primers, Nuclease Free Water, Gotaq Green Mastermix, and restriction enzymes from the MboI Thermoscientific brand.

This research went through several stages, namely: data collection on body weight and body weight gain in the field, blood sampling, DNA extraction, and PCR-RFLP. The method used in research in the area is a method of experimentation or direct observation. How to maintain chickens in colony cages by feeding and drinking continuously (ad libitum). The cage used is 4x3x1.8 m and is equipped with feed, drinking places, and lighting. Every month, body weight was measured. The data collected includes the weight of DOC up to 3 months and body weight gain. What carried outweighing body weight and weight gain of bodies at the age of DOC until three months using digital scales. Each chicken is given a nametag on the wing.

Bangkok Chicken blood samples were taken using a syringe in the axillary vein of the wing. Blood was born on the skin that was smeared with alcohol first. 1-2 ml of blood was born and then put into a 3 ml tube and mixed with EDTA powder to not clot. Blood samples were stored in a cool box for a while, after which the blood was held in the freezer before further processing. The DNA extraction method uses the protocol Genomic DNA Purification Kit from Promega. The results of DNA isolation will be tested using gel agarose 1.5% stained with Ethidium Bromide on an electrophoresis device with a voltage of 100 volts for 60 minutes. The DNA extraction results will be visualized through UV light using Gel Doc. Amplification of candidate genes using primary pairs as shown in Table 1.

PCR amplification using a Thermoocycler from BIO-RAD. The composition is as follows: 3 µl primer (forward and reverse), 2 µl genomic DNA sample, 10 µl nuclease-free water (ddH2O) plus 15 µMaster mixes (Taq Polymerase) for a total mixture of 30 µl. The sample is in the spinner first so that it is then put into the PCR machine tube with the optimal temperature, which can be seen from Table 2.

The amplification results can be seen by electrophoresis using 1.5% agarose, stained with Ethidium Bromide (EtBr) with a voltage of 100 volts for 60 minutes. Furthermore, the gel will show the bands formed in each good groove containing the DNA sample of the PCR product. Determination of the size of each Myostatin gene fragment formed on the agarose gel was carried out by comparing the position of the band started with the part of the DNA band ladder. The results of the amplification of the PCR product obtained were then cut with the restriction enzyme MboI (Moraxella bovis I) with the ↓GATC cutting site according to the gene locus with the following composition: 10 µl of the PCR amplification results and 10 µl of the restriction enzyme for a MboI total of 20 µl which would then be incubated using Waterbath. To get an idea of the gene loci and their cutting sites, see Table 3.

Gene restriction MSTN|MboI can be seen after electrophoresis using Agarose 2% stained with Ethidium Bromide (EtBr) at a voltage of 200 for 120 minutes. The genotype identification of

each sample was determined based on the band cut's size and pattern, namely the band's length compared to the 1000 bp (DNA ladder) marker.

Data analysis

T-test. The mean difference test (t-test) was used to see differences in the weight of DOC ages, up to ages three months, and body weight gain, which were grouped according to the gender of Bangkok chickens and according to the genotypes obtained and analyzed with the formula following (Mendenhall, 1987):

$$t = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\frac{\sum (X_{J1} - \overline{X}_1)^2}{n_1(n_1-1)} + \frac{\sum (X_{J2} - \overline{X}_2)^2}{n_2(n_2-1)}}}$$

where:
t = t arithmetic value
X1 = sample mean in the first group,
X2 = sample point in the second group,
XJ1 = the value of the j-th observation in the first group
XJ2 = the value of the j-th statement in the second group
n1 = The number of samples in the first group, and
n2 = number of examples in the second group.

Genotype frequency and allele. Genotype frequency is the proportion or percentage of a particular genotype in a population, calculated based on the number of alleles of a genotype divided by the number of samples:

$$F = \frac{\Sigma X_i}{N}$$

where:
xi = observed genotype
N = Total Population

The allele frequency of the gene myostatin (MSTN) is the proportion of an allele in a population compared to all the alleles occupying the same loci, obtained from the PCR-RFLP marker analysis were analyzed using the formula of Nei and Kumar (2000):

$$X_i = \frac{(2n_{ii} + \sum_{j \neq i} n_i t)}{2N}$$

where:
xi = frequency of the i-th allele,
nii = number of individuals with genotype ii,
nij = number of individuals with genotype ij,
N = total number of samples.

Results and Discussion

Body weight of Bangkok chicken

Average body weight in male and female chickens Bangkok DOC up to 3 months are in Table 4.

Table 4 average DOC weight of male and female Bangkok chickens is 40.15 g and 37.95 g. The results of this study are higher than some research results. Pagala *et al.* (2019) stated that the average DOC weight of male and female Bangkok chicken was 36,76 g and 37 g. KUB chickens was 31.50 g and 30.12 g (Suryana, 2017). The DOC weights of Kampung, Sentul and KUB chickens were 26.38 g, 33.85 g, and 31.06 g (Irmaya *et al.*, 2021).

The average body weight of Bangkok chickens aged one month male and female were 420,33 g and 361.22 g, respectively. The results of this study are higher than the results of research. Pagala *et al.* (2019) which states that the average body weight of male and female Bangkok chickens is 265,36 g and 256,19 g. Urfa *et al.* (2017) said that one-month-old KUB chickens had an average body weight of 221 g. Putri *et al.* (2020) stated that the body weights of Super, KUB, and Kampung chickens were 349.47 g, 235.64 g, and 224.68 g.

From this study, the average body weight of two-month-old Bangkok chickens, male and female, was higher than several other studies on local chickens. Pagala *et al.* (2019) stated that the average body weight of male and female Bangkok chickens was 648,62 g and 624,47 g. The average body weights of KUB, Sentul, and Arab chickens were 713.15 g, 632.17 g, and 5 91.20 g (Puteri *et al.*, 2020).

Table 1. Length, location, and sequence of primary pairs of Myostatin gene (MSTN) in Bangkok chicken

Locus name	Length (bp)	Location	Sequence Primary	Gene Bank
MSTN|MboI	955	Exon 1	F: ^{5}GGT TTT GAC GAC ATG AGC CT^{3} R: $^{5'}$CAG GTG GAA TGT CAT GCA GA$^{3'}$	AF346599

F: Forward primer R: Reverse primer used for PCR analysis.

Table 2. Optimization temperature of PCR-RFLP analysis

Stage	MSTN		
	Temperature (°C)	Length (hours:minute:second)	Cycle
Predenaturation	95	00:05:00	1x
Denaturation	95	00:00:45	
Annealing	60	00:00:45	35x
Extention	72	00:01:00	
Final extension	72	00:05:00	1x
Retriction enzym MboI	37	04:00:00	

Table 3. Myostatin Gene Locus in Bangkok chicken

Locus name	Cutting sites	Cuts	Cuts Position	Band size
MSTN|MboI	5' ↓GATC 3.'	3	233,10,219	492,234,219,10

Three-month-old male and female Bangkok chickens had an average body weight of 1378.78 g and 1170.20 g. The results of this study are higher than the results of research Fahrudin *et al.* (2016), which states that the bodyweight of three-month-old native chickens has an average body weight of 1044 g, according to Mariandayani *et al.* (2013) that the average body weight of male and female Sentul chickens is 1291 g and 1087 g, Puteri *et al.* (2020) stated that Sentul and KUB chickens aged three months had a bodyweight of 1108 g and 1021 g, meaning that the difference in body weight was thought to be due to the influence of different strains, genetics and the environment (Kusuma and Prijono, 2007; Noor, 2008; Pagala *et al.*, 2015).

The opinion of North and Bell (1990); Wijayanti (2011), Stating that the type and strain of chickens influence the growth rate of chickens. The results of the mean difference test (t-test) showed that from DOC until the age of 3 months, Bangkok male chickens were significantly ($P<0.05$) higher than female Bangkok chickens. The average body weight of male Bangkok chickens is higher than that of female Bangkok chickens. This difference is thought to be due to the influence of gender. This statement is supported by Soeparno (1998) opinion, which states that gender affects growth. At the same age, male cows experience faster growth than female cows. According to Djego *et al.* (2019), differences in body weight in livestock groups fed the same feed are caused by genetic factors. The genetic influence is related to the effect of hormones in male and female chickens, which causes differences in growth rates. According to Hapsari (2015), the hormone testosterone, which functions as a steroid androgen, is a growth regulator. The high steroid secretion in male chickens is caused by four increases in testosterone secretion produced by the testes, so that the growth rate is higher than in female chickens.

Body weight gain of Bangkok chicken

The average weight gain of male and female Bangkok chickens from DOC to 3 months of age is presented in Table 5.

Table 5 shows that the average weight gain of male Bangkok chickens from DOC aged one month, 1-2 months, and 2-3 months respectively were 380.11 g, 446.78 g, and 511.71 g, while the female Bangkok chicken was 323.27 g, 390.86 g, and 418.14 g. The results of this study are higher than those of Rahmat *et al.* (2020), which states that the average weight gain of Merawang chickens aged DOC-1 month and 1-2 months is 109.87 g and 140.73 g. Pagala *et al.* (2019) noted an increase in weight of male Bangkok chickens from one month and 1-2 month are 226,62 g and 383,24, while for female Bangkok chickens are 219,19 and 364,97. While for female Bangkok chickens are 227.05 g, 409.74 g, and 375.06 g. The difference in weight gain is due to genetic differences and environmental conditions, including maintenance management (Subekti and Arlina 2011; Risnajati, 2014).

The increase in weight of male Bangkok chickens from DOC until the age of 3 months of male Bangkok chickens was significantly ($P<0.05$) higher than the increase in weight of female Bangkok chickens. The difference in weight gain is thought to be due to the influence of gender. This is by Qurniawan (2016) which states that the

Table 4. Average weight chicken DOC up to 3 months male and female Bangkok chicken

Age	Bangkok Chicken	
	Male	Female
DOC Weight (g)	40,15±2,75[a]	37,95±1,91[b]
Body Weight 1 Month (g)	420,33±40,04[a]	361,22±32,68[b]
Body Weight 2 Month (g)	867,11±72,64[a]	752,07±25,90[b]
Body Weight 3 Month (g)	1378,78±91,07[a]	1170,21±56,68[b]

*Different superscripts in the same line are significantly different ($P<0.05$)

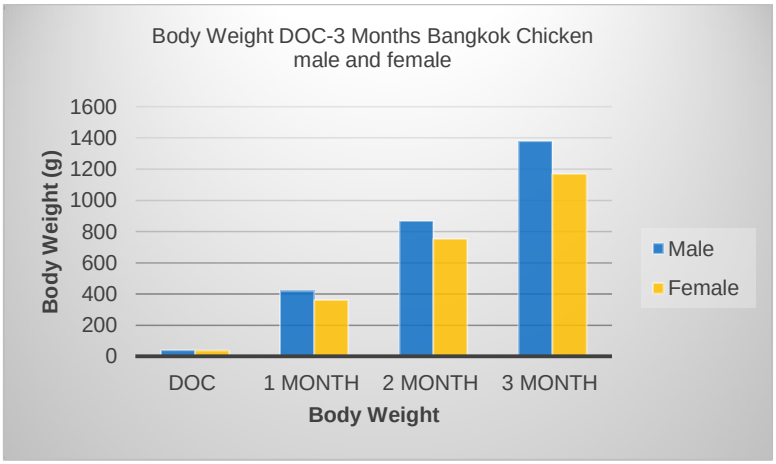

Figure 1. Body weight DOC-3 months Bangkok chicken male and female.

factors that influence weight gain are gender differences. According to Rahayu *et al.* (2014), roosters have testosterone, which stimulates the increased growth hormone secretion. Growth Hormone (GH) can stimulate faster growth (Ma'ruf, 2004).

DNA extraction

DNA extraction aims to obtain DNA with excellent quality, which has high purity. DNA purification is a process to separate DNA from cell lysates (proteins, carbohydrates, lipids) and other contaminants (Hernandez *et al.*, 2006; Nova *et al.*, 2016). Bangkok chicken DNA extraction results from 50 blood samples were electrophoresed using 1.5% Agarose, which were visualized using UV light through Gel Doc presented in Figure 3.

Figure 3 shows that the electrophoresis results of Bangkok chicken DNA extracted as a whole are apparent, quite clear, not thick or thin; this indicates that the DNA concentration is appropriate. DNA can be used for the next step. The opinion is supported by Hidayati *et al.* (2016) statement, which states that two thick and bright bands are qualitatively thought to have a high concentration of DNA produced while three thin bands are thought to have a small DNA concentration. The high and low concentration of DNA is influenced by the process of lysis of the cell nucleus. According to (Yurnalis *et al.*, 2013), an adequately lysed cell nucleus will produce a relatively high concentration of DNA, and the quality of the DNA obtained will be good.

Myostatin gene fragment amplification in Bangkok Chicken

Amplification Myostatin exon one gene used a primer with a product length of 955 bp. The amplification process was carried out for 35 cycles which went through several stages, starting with predenaturation using a temperature of 95^{o}C for 5 minutes followed by denaturation at a temperature of 95^{o}C for 45 seconds, then the annealing stage at a temperature of 60^{o}C for 45 seconds then extension at a temperature of 72^{o}C for 1 minute and the final extension using a temperature of 60 72^{o}C for 5 minutes. The results of the amplification of the PCR product were visualized by electrophoresis using 1.5% Agarose, as shown in Figure 5.

Figure 5 shows that the myostatin PCR gene amplification product (MSTN) successfully used an annealing temperature of 60^{o}C for 45 seconds. The myostatin gene was amplified to indicate whether the appropriate annealing temperature was used. This is in the opinion of Hidayati *et al.* (2016), which states that if the annealing temperature is too high, the primer to the template will be separated so that no PCR product is formed, on the other hand, if the

Table 5. Average body weight gain of DOC up to 3 months of male and female Bangkok chickens

Gender	DOC-1 Month	1-2 Month	2-3 Month
MALE	380,11±38,0^{cA}	446,78±36,25^{bA}	511,71±53,89^{aA}
FEMALE	323,27±31,16^{cB}	390,86±22,64^{bB}	418,14±46,07^{aB}

*Different superscripts in the same line are significantly different (P<0.05)

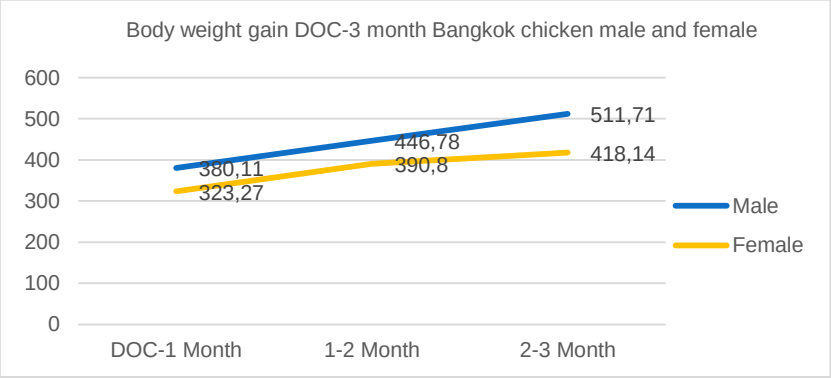

Figure 1. Body Weight gain DOC-3 months Bangkok chicken male and female.

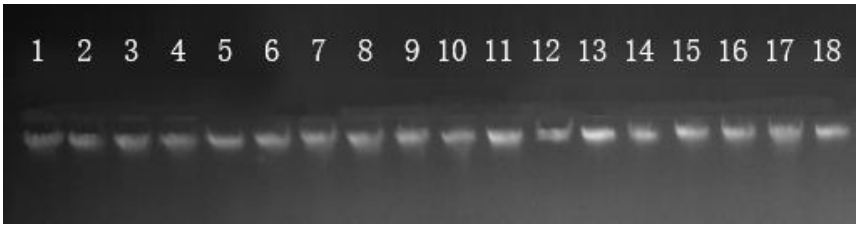

Figure 2. Electrophoresed total of DNA using the DNA Purification Kit protocol from Promega
Description: numbers 1, 2, 3, ... 18 = sample of individuals.

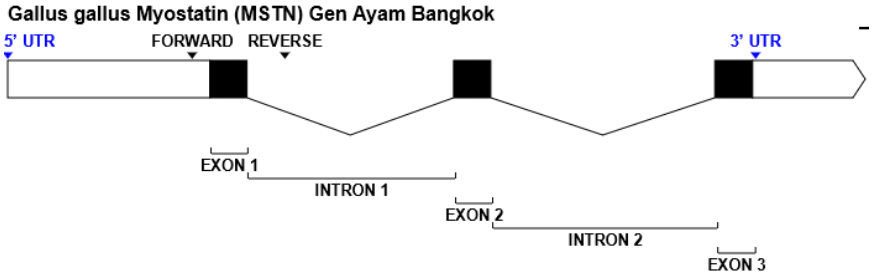

Figure 3. Myostatin Gene Structure of Bangkok Chicken Genbank: AF346599
(Exon 1 = 2050 – 2422 = 373 bp Intron 1 = 2423 – 4516 = 2.094 bp; Exon 2 = 4517 – 4890 = 374 bp Intron 2 = 4891 – 7159 = 2.263 bp; Exon 3 = 7160 – 7540 = 381 bp)

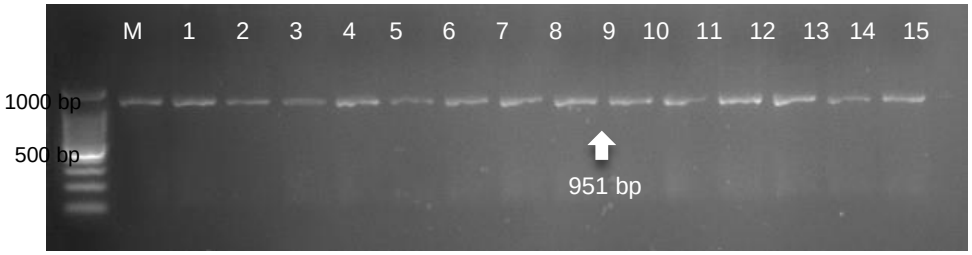

Figure 4. Electrophoresis results of the myostatin gene PCR product using DNA Ladder 1000 bp.

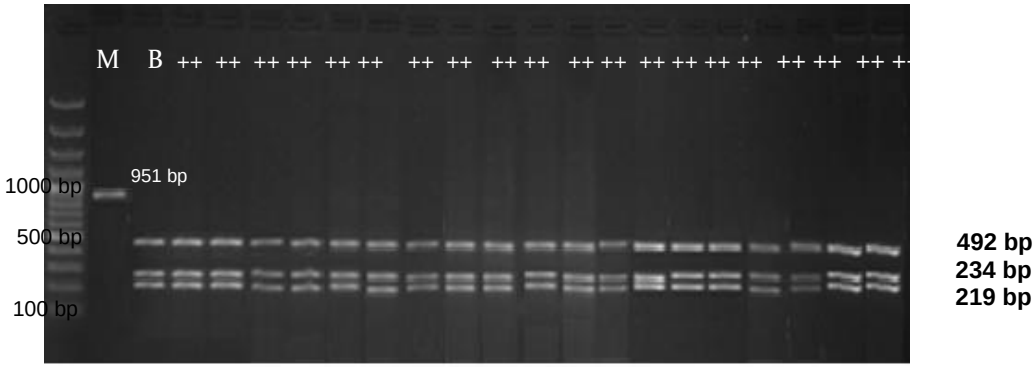

Figure 5. Visualization of result RFLP MSTN|MboI
Description: M = Marker, B=Blank Fragment of MSTN gene amplification.

annealing temperature is too low, primer attachment will occur on a non-specific or inappropriate template.

Genotype frequency and allele

Diversity of Bangkok chicken myostatin gene identified using restriction enzyme cleavage with MboI|GATC cutting site resulted in one genotype (++) and one allele (+) with three strips of bands at 492 bp positions, 244 bp, and 5 219 bp (Figure 6.). The research results conducted by Zhang *et al.* (2012) related to myostatin gene diversity using two primer designs chicken and broilers produced one genotype, namely MM and OO, with a genotype frequency of 1.00. According to Fastawa *et al.* (2019), the frequency of genotypes in a population can experience selection, mutation, population mixing, inbreeding and outbreeding, and genetic drift. The result from analysis genotyping of MSTN gene|MboI is shown in Table 3.

Table 6 shows that the results of the analysis of genotype frequency and allele frequency in the myostatin gene fragment MboI Bangkok chicken are ++ (50), +- (0), and -- (0) with one allele, namely +. The genotype frequency obtained reached a value of 1.00, and the Bangkok chicken population studied was based on the allele frequency of the MSTN|MboI gene, which was considered monomorphic. This is by

Table 6. Genotype and allele frequency

Galur-Lokus	N	Genotipe	Frekuensi Genotipe	Frekuensi Alel
Ayam Bangkok MSTN|MboI	50	(++)	1	100%
		(+-)	0	
		(--)	0	0

the opinion of Nei and Kumar (2000), which state that a gene is polymorphic if one of the allele frequencies is less than 0.99. The study results have similarities with Zhang *et al.* (2012), which states that the myostatin gene in breeder and broiler chickens is monomorphic. Genetic uniformity in a livestock group is caused by the selection process and the lack of new male introductions to the population (Malewa, 2019).

Conclusions

Male and female Bangkok chickens have low phenotype diversity. Fragment cuts gene MSTN|Mboi are monomorphic with ribbon position 492 bp, 244 bp, and 219 bp produces a genotype that is ++, and there is one kind of allele with an allele frequency + 8 at 100%.

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