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Myostatin (MSTN) Gene Polymorphism Using PCR-RFLP Method in Kerinci Ducks

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ABSTRACT

This study aims to determine body weight, body weight gain, and body measurements of male and female Kerinci ducks, as well as to determine the diversity of MSTN genes and the association of the MSTN gene on body weight, body weight gain and body sizes in Kerinci ducks. The materials used were 96 Kerinci ducks and 96 Kerinci duck blood samples. Data analysis included: T-test, T^2-hotelling, Principal Component Analysis (PCA), genotype frequency, allele, Hardy-Weinberg equilibrium, Heterozygosity, and PIC. The results showed that body weights at 2 and 3 months of age, body weight gain of 2-3 months, and body sizes of 3 months old male Kerinci ducks were significantly different ($P < 0.05$) higher than female Kerinci ducks. Body size markers of male and female Kerinci ducks were the length of the sternum, the length of the shank, and the circumference of the shank, while the shape identifier was the length of the wings. The MSTN|*MboI* gene in Kerinci ducks was polymorphic. The population of Kerinci ducks was in Hardy-Weinberg equilibrium ($P<0.05$). Kerinci duck population heterozygosity showed $H_0<H_e$. The MSTN|MboI gene was associated with BB, PBB, and body measurements of male and female Kerinci ducks, and the best genotype was (-/-).

Keywords: Polymorphism, Kerinci Duck, Gene *Myostatin* (MSTN), Enzyme *MboI*

INTRODUCTION

Indonesia has abundant local livestock diversity, one of which is local ducks. In Jambi Province, one of the local ducks with the potential to be developed is the Kerinci duck. The Kerinci duck is the germplasm of Jambi Province that must be preserved. It is based on the fact that the Kerinci ducks' population has decreased yearly (Dinas Perkebunan dan Peternakan Kabupaten Kerinci, 2021). Efforts can be made to protect Kerinci ducks, one of which is to collect primary data on quantitative phenotype diversity.

Quantitative phenotype diversity can be seen in body weight, weight gain, and measurements. Quantitative characteristics have several weaknesses. Namely, it takes a long time, costs more, and has livestock. With technological advances in the molecular field, factors with economic value, such as body weight, weight gain, and measurements, can be analyzed more deeply on structural genes. The Myostatin gene is one of the structural genes that play an essential role in characteristics that have economic value. The Myostatin gene (MSTN) is a member of the growth sub-gene (Transforming Growth Factor/TGF-β) and functions as a negative regulator of skeletal muscle growth in the body (Bhattacharya et al., 2019). One of the characterization and identification of MSTN genes can be using the molecular identifier of the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

The PCR-RFLP method can be used to study myostatin gene polymorphisms. This method has been developed to determine the different levels of DNA (restriction enzymes) capable of cutting DNA sequences at a certain point. Through the band displayed by electrophoresis, the genetic diversity of the livestock will be known (Hidayati et al., 2016; Mardiah et al., 2022). Until now, the linkage of Myostatin gene diversity with body weight, body weight gain, and body measurements of Kerinci ducks is still rarely done, even though if these two methods, namely molecular and quantitative characteristics, have a relationship, of course, this can be used as the basis for selecting Kerinci ducks in the future.

Based on the description above, research was conducted on "Myostatin Gene Polymorphism (MSTN) Using the PCR-RFLP Method in Kerinci Ducks."

MATERIALS AND METHODS

Materials

This research uses 96 Kerinci ducks consisting of 41 males and 55 females and 96 Kerinci duck blood samples. The materials used are 70% alcohol, cotton, protocol Genomic DNA Purification Kit from Promega, isopropanol, 70% ethanol, agarose powder, TBE Buffer solution, aquades, ethidium bromide (EtBr) staining, loading dye, DNA ladder, forward and reverse primers, Nuclease Free Water, Gotaq Green Mastermix, and the MboI restriction enzyme

Thermoscientific brand. The equipment used is the EDTA K3 vaculab, tube holder disposable syringe size 3 ml, cool box, freezer, oven, autoclave, micropipette, Eppendorf tip pipette, microtube rack, centrifuge, vortec, analytical balance, Erlenmeyer, measuring cup, gel doc, electrophoretic gel system, spin centrifuge, electric heater, PCR machine, and water bath.

Method

The research method is experimental. This research was carried out in two stages: in the field and the laboratory. Research in the field includes data collection on body weights aged 2 and 3 months, body measurements of Kerinci ducks aged three months, and blood collection. The body measurements were 18 parameters according to the instructions (Putri *et al.,* 2020; Depison *et al.,* 2022). In addition, 2-3 ml of blood samples were taken using a syringe in the wing axillary vein and then put into a 3 ml EDTA tube. After that, the blood is stored in the freezer. Research in the laboratory includes extraction, amplification, and restriction.

The DNA extraction method uses the Genomic DNA Purification Kit protocol from Promega. Electrophoresis using 1.5% agarose gel stained with *Ethidium Bromide* at 100 volts for 60 minutes. The DNA extraction results will be visualized through UV light using *Gel Doc.* The primer was 866 bp in length in Exon 2 with no access to GenBank DQ419906.1. Primer Forward 5'CTG TCC TCC TTG GTC TGG AG3' and Reverse 5'CCT CTA GGA TTC GCT TGG TG3'. Amplification PCR was conducted with a thermocycler from BIO-RAD. The composition is as follows 3 µl Forward and Reverse primers, 2 µl genomic DNA, 10 Nucleus free water/DDW (double distillation water), and 15 µl Gotaq Green Mastermix from Promega, which was inserted into a microtube tube PCR 0.2 ml for total mixture is 30 µl, visualization of PCR products was observed using a UV light and documented with Gel Documentation (BiometraGermany). The amplification process was carried out according to the PCR steps in PCR-RFLP by incubating a water bath with the following composition: 10 l of the PCR product and 10 l of the enzyme restriction *MboI* with the ↓GATC at 37°C for 4 hours. PCR-RFLP was visualized using a UV light and documented with Gel Documentation (Biometra Germany). The genotype identification of each sample was determined based on the band cut's size and pattern, namely the band's length compared to a 1000 bp (DNA ladder) marker.

Data analysis

T-test, T^2-*Hotelling*, and Principal component analysis (PCA)

Differences between body weight, weight gain, and body measurements, as well as between genotypes and body weights, were analyzed using the average difference test (t-test) (Mendenhall, 1987). T^2-hotelling was used to compare the vectors of the average body sizes of male and female Kerinci ducks using (Gaspersz (2006). If the T^2-hotelling showed significantly different results ($P<0.05$), then proceed with the principal component analysis (PCA) was used to determine the determinants of the size and shape of Kerinci ducks using (Gaspersz, 2006).

Genotype frequency, allele, *Hardy-Weinberg* equilibrium, heterozygosity, and PIC

Genotype frequency was calculated based on the number of genotype alleles divided by the number of samples. Allele frequency is calculated by summing all alleles divided by 2N. MSTN gene allele frequencies derived from the analysis of PCR-RFLP Mbo1 were calculated using the formula (Nei, 1987). The genetic diversity (genetic variability) is done through observation of the estimated frequency of heterozygosity (Ho), heterozygosity expectations (Hi), and standard error heterozygous expectations (Weir, 1996; Nei, 1987). An allele informative level is calculated using a value approach informative polymorphic content (PIC) (Botstein et al., 1980). Hardy-Weinberg Equilibrium w a s tested with *Chi-square* ($X^{2)}$ (Hartl and Clark, 1997).

RESULTS AND DISCUSSION

Average body weight and body weight gain of Kerinci Ducks

The average body weight of 2 and 3 months and body weight gain at the period of 2-3 months for male and female Kerinci ducks are presented in Table 1.

Table 1. shows that the average body weight of male Kerinci ducks aged 2 and 3 months was 1264.84 ± 35.51g and 1682.56 ± 52.42g. The average body weight of female Kerinci ducks aged 2 and 3 months was 1035.47 ± 44.01g and 1422.85 ± 43.35g, respectively. The results of this study are not much different from those of Khanza et al. (2021), who stated that the body weights of male and female Kerinci ducks aged two months were 1283.60 ± 70.61g and 1137.72 ± 41.09g.

Table 1. Average of body weight of male and female Kerinci ducks aged 2 and 3 months and body weight gain of male and female Kerinci ducks aged 2-3 months.

Age (g)	Male (41)	Female (55)
Body weight 2 months	1264.84 ± 35.51[a]	1035.47 ± 44.01[b]
Body weight 3 months	1682.56 ± 52.42[a]	1422.85 ± 43.35[b]
Body weight Gain 2-3 months	417.72 ± 62.83[a]	387.38 ± 40.98[b]

Description: Superscript letters with slight differences in the same row were significantly different (P<0.05)

The results of this study are higher than several other studies, namely the body weight of male Alabio ducks aged three months of 1347.92 ± 97.53g (Setiyono and Bekti, 2019), female Alabio ducks aged three months of 1237.00 ± 40.94g (Son et al., 2015. This condition shows that the body weight of male and female Kerinci ducks aged 2 and 3 months are still quite good compared to several other studies.

The average body weight gain of male and female Kerinci ducks aged 2-3 months was 417.72 ± 62.83g and 387.38 ± 40.98g. The results of this study are higher than those of Putra et al. (2015), who stated that the average body weight gain of male and female Alabio ducks aged 2-3 months was 374.12g and 261.33g, respectively. Asiamah et al. (2020) stated that the average body weight gain of male and female Leizhou Black ducks aged 2-3 months in the first generation was 284.42g and 314.44g, respectively. It means that the body weight gain of male and female Kerinci ducks aged 2-3 months, the results of this study were still quite good compared to several other studies.

The results of the average difference test showed that the body weight of male Kerinci ducks aged 2 and 3 months and the body weight gain of male Kerinci ducks aged 2-3 months were significantly different (P <0.05) higher than the average body weight and body weight gain of female Kerinci ducks. This difference is due to a male duck's influence on hormones. It is the opinion of Sari et al. (2021), who states that male poultry has testosterone hormone produced by the testes so that the body weight of male chickens is higher than female chickens. Furthermore, Prawira et al. (2021) stated that male poultry has the hormone testosterone, which functions as a steroid, and androgen is a growth regulator.

Average body sizes of Kerinci Ducks

The average body sizes of male and female Kerinci ducks aged three months include beak length (BL), beak width (BW), head length (HL), head height (HH), neck length (NL), back length (BL), sternum length (StL), wing length (WL), femur length (FL), tibia length (TL), shank length (SL), shank circumference (SC), third finger length (TFL), chest circumference (CC), body length (BL), head circumference (HC), neck circumference (NC), and tibia circumference (TC).

The results of this study are not much different from those of Tarigan et al. (2015), which state that the body sizes of male Bali ducks are more significant than female Bali ducks. The average body sizes of male Kerinci ducks aged three months were significantly different (P<0.05) than that of female Kerinci ducks. This condition indicates that the body skeleton of male Kerinci ducks is higher than that of female Kerinci ducks. It is the opinion of Prawira et al. (2021), who states that the greater the size of the body frame, the greater the body weight of the livestock. Furthermore, Sitanggang et al. (2016) stated that the larger the size of the body's skeleton, the larger the body size.

T^2-*hotteling* analysis and principal components analysis of body size

The results of the T^2-*hotteling* analysis in this study obtained a statistical value of 6264.76, an F-value of 292.50, and a P-value of 0.01. The results of this study showed that the body sizes of male Kerinci ducks were significantly different (P<0.01) than that of female Kerinci ducks. This difference is thought to be because the body skeleton of a male duck is more significant than a female duck's. Sari et al. (2021) state that male chickens have larger body sizes than female chickens, so male chickens have higher performance than female chickens. Sadick *et al.* (2020) stated that the size of the body skeleton determines the size of an animal's body.

The equations of body size and shape, total diversity, and eigenvectors of male and female Kerinci ducks aged three months are presented in Table 2.

Table 2. Equations of body size and body shape with total diversity and eigenvectors of male and female Kerinci ducks aged three months.

Kerinci duck			Equation	KT (%)	Λ
Male	Body Size	=	0.133BL + 0.264BW + 0.255HL + 0.239HH + 0.265NL + 0.269BL+ 0.297StL + 0.101WL + 0.232FL + 0.277TL + 0.289SL + 0.288 SC + 0.87TFL + 0.227CC+ 0.212BL + 0.247HC + 0.263 NC + 0.134TC.	56.6	10.18
	Body Shape	=	0.081 BL+ -0.018 BW+ -0.047 HL+ -0.186 HH+ -0.062 NL + -0.117 BL+ 0.02 StL+0.555 WL+ 0.134 FL+ 0.042 TL+ -0.046 SL+ 0.057 SC+ 0.541 TFL+ -0.231 CC+ -0.271 BL+ 0.127 HC+ -0.117 NC+ 0.395 TC.	1.40	7.8
Female	Body Size	=	0.237 BL + 0.221 BW + 0.241 HL + 0.228 HH + 0.243 NL + 0.242 BL + 0.265 StL + 0.199 WL + 0.252 FL + 0.242 TL + 0.258 SL + 0.262 SC + 0.224 TFL + 0.217 CC+ 0.213 BL + 0.221 HC + 0.228 NC + 0.238 TC.	69.4	12.49
	Body Shape	=	0.315 BL+ -0.009 BW+ 0.222 HL+ 0.157 HH+ 0.117 NL+ 0.375 BL+ 0.189 StL+0.412 WL+ 0.056 FL+ -0.133 TL+ -0.271 SL+ -0.281 SC+ -0.182 TFL+ -0.408 CC+ -0.096BL+ -0.255 HC+ -0.123 NC+ -0.073 TC.	6.7	1.21

Information: BL = Beak Length, BW = Beak Width, HL = Head Length, HH = Head Height, NL = Neck Length, BL = Back Length, StL = Sternum Length, WL = Wing Length, FL = Femur Length, TL = Tibia Length, SL = Shank Length, SC = Shank Circumference, TFL = Third Finger Length, BC = Bust Circumference, BL = Body Length, HC = Head Circumference, NC = Neck Circumference, TC = Tibia Circumference.

Table 2. shows that the body size score equation for male and female Kerinci ducks has a total diversity of 56.6% and 69.4%, respectively of total diversity. The highest eigenvectors in the body size equation in male and female Kerinci ducks were the sternum length (StL), shank length (SaL), and shank circumference (SaC). It means that the length of the sternum (StL), shank length (SL), and shank circumference (SC) are characteristics of body size because they have the most significant contribution to the body size equation.

The equation for the body shape score of male and female Kerinci ducks has eigenvectors of 7.8% and 1.21%, respectively. This percentage is the most significant proportion of diversity among the main components that determine body shape. The highest eigenvector obtained in the body shape equation of male and female Kerinci ducks was wing length (WL). It means that wing length (WL) is a marker of body shape because it has the most significant contribution to the equation of body shape. It is different from the research results of Suryana *et al.* (2014), which stated that the highest eigenvector in the body shape of male and female Albino ducks was beak length. The cause of the differences in body size and shape in each line is thought to be due to genetic and environmental differences (Putri *et al.,* 2020).

DNA extraction and gene amplification *Myostatin* in Kerinci Ducks

DNA extraction is a series of processes to separate DNA from other cell components (proteins, fats, and carbohydrates) (Hidayati et al., 2016; Nova et al., 2016). DNA extraction from 96 Kerinci duck blood samples using the DNA Purification Kit protocol from Promega was electrophoresed using 1.5% Agarose, which was visualized using UV light via Gel Doc. More details are presented in Figure 1.

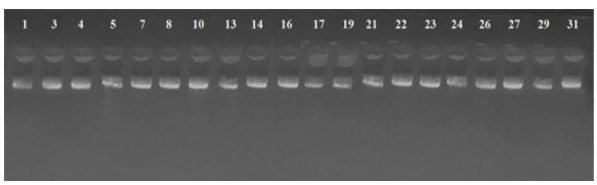

Figure 1. Electrophoresis of DNA extraction from dilution

Description: numbers 1, 3, 4, ... 31 = individual sample

The amplification of the exon two myostatin gene fragment used a primer with a product length of 866 base pairs (bp). The PCR product amplification results were visualized

through electrophoresis using 1.5% Agarose. The genotype identification of each sample was determined based on the band cut's size and pattern, namely the band's length compared to a 1000 bp (DNA ladder) marker, which can be seen in Figure 2.

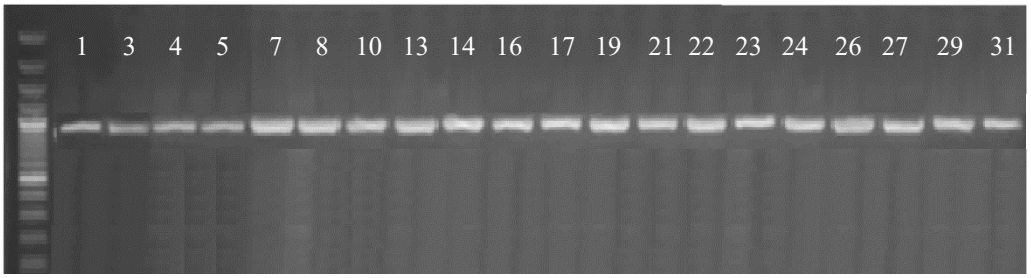

Figure 2. The electrophoresis results of the myostatin gene PCR product using 1000 bp DNA Ladder.

Figure 2. shows that the amplified Myostatin gene is 866 bp long. The Myostatin gene PCR product amplification at an annealing temperature of 60˚C for 45 seconds obtained the band's position according to the length of the primer used and visible. DNA bands obtained are clear and not too thick, meaning that the concentration of DNA obtained is relatively high. Some DNA samples whose concentration level is still low can be seen from the thin bands, but some other DNA samples have bands that are not too thick so that DNA can be used for the next step. The annealing temperature is an essential factor in the success of amplification. The annealing temperature must be optimal, if the annealing temperature is too high, it will cause the primer to not stick to the DNA, and the amplification will fail. At the same time, if the annealing temperature is too low, the primer will stick to the other side of the DNA, resulting in poor-quality DNA (Ramadhan et al., 2019).

Genotype and allele frequency

The diversity of the Myostatin gene in Kerinci ducks was identified using the MboI cleavage enzyme with GATC cutting sites at positions 866 bp, 422 bp, 230 bp, and 214 bp, resulting in three genotypes, namely +/+, +/-, and -/-, and the two alleles + and - are presented in Figure 3.

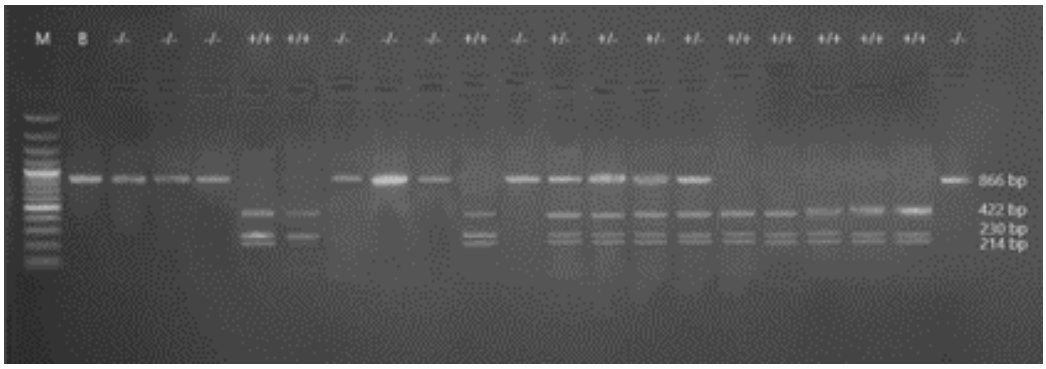

Figure3. Visualization of RFLP MSTN|*MboI*
Information : M = Marker, B=Blank Fragment amplification gene MSTN

The result of gene MSTN|*MboI* genotyping analysis is presented in Table 3.

Table 3. Genotype frequency, allele, Hardy-Weinberg (HW) balance test, heterozygosity, and PIC (Polymorphic Information Content)

Galur-Lokus	N	Genotype	Frequency Genotype	Frequency Allele	X^2count	H_0	H_e	Value of PIC
Itik Kerinci MSTN|*MboI*	96	+/+	0.48	66%	3.32^{ns}	0.36	0.45	0.40
		+/-	0.36					
		-/-	0.16	34%				

Table 3. shows that the results of the genotype frequency analysis in the Myostatin|MboI gene in Kerinci ducks are +/+ (0.48), +/- (0.36), and -/- (0.16) with allele frequencies (+) of 66% and (-) of 34%. The results of this study indicated that the *Myostatin* gene in Kerinci ducks was polymorphic. A population can be polymorphic if there is more than one allele at a locus and if one of the alleles is less than 99% (Allendorf *et al*., 2013; Nei and Kumar, 2000).

Equilibrium *Hardy-Weinberg* (HW)

Based on Table 3. X^2count (3.32) < X^2table 0.05 (3.84), this condition indicates that the Kerinci duck population is not significantly different at the 0.05 level; thus, the Kerinci duck population can be expressed in conditions of equilibrium with the Hardy-Weinberg law. This condition indicates that the Myostatin (MSTN) gene in Kerinci ducks is in balance, so it can be stated that mating occurs randomly. Population equilibrium is also caused by the uncontrolled mating system so that there is an opportunity for males to mate with the same female. Allendorf et al. (2013) stated that the population is said to be in balance if the calculated X^2 count is smaller than the X^2 table.

Heterozygosity

Genetic diversity of the gene MSTN|*MboI* of Kerinci ducks were obtained based on the heterozygosity value in Table 3. which shows that the value of H_0<H_e. The observed heterozygosity value was H0= 0.36, and the expected heterozygosity was He= 0.45; this value indicates that the Kerinci duck population has moderate (moderate) conditions with relatively distant genetic relationships. It is presumably because the Kerinci duck population has endogamous marriages. It is the opinion of Machado *et al.* (2003), who states that if the value of H_0 is lower than that of H_e, it indicates the degree of endogamy (marriage in groups). Karabag *et al.* (2016) stated that high genetic diversity has an observed heterozygosity value of more than 0,5. Heterozygosity is a parameter used to measure the level of genetic diversity in a population based on allele frequencies at each locus (Wang *et al.,* 2015).

Polymorphic Information Content (PIC)

Table 3 shows the value of PIC (Polymorphic Information Content) in the MSTN|*MboI*gene for Kerinci ducks is 0,40. PIC value in MSTN|*MboI* gene belongs to the moderate category, which means that the value is quite informative as a marker for the MSTN|*MboI* gene fragment. Tamzil *et al*. (2013) stated that the PIC value could be used to determine genetic information and the presence of polymorphic alleles, meaning that it has the same function as the heterozygosity value. Furthermore, Carsono *et al.* (2014) stated that the PIC value was used as a standard for evaluating genetic markers based on DNA bands resulting from PCR amplification; therefore, the PIC value was divided into three classes, namely PIC> 0.5 = very informative, then 0.25> PIC> 0.5 = medium, and PIC 0.25 = low.

Gene association *Myostatin* with body weight of 3 months age, body weight gain in 2-3 months old, and body measurements of 3 Months Kerinci Ducks

The average body weight of Kerinci ducks aged three months, weight gain in Kerinci ducks aged 2-3 months, and body measurements of male and female Kerinci ducks aged three months, various genotypes identifying the *Myostatin*|*MboI* using PCR-RFLP are presented in Table 4.

Table 4. Average body weight of Kerinci ducks aged 3 months, body weight gain of Kerinci ducks aged 2-3 months, and body measurements of Kerinci ducks aged three months of various genotypes

Description (g)		Genotypes (96)		
		+/+	+/-	-/-
Body Weight of 3 Months	Male	1450.57±17.19[a]	1500.69±16.27[b]	1573.37±12.49[c]
	Female	1388.71±27.00[a]	1440.52±12.46[b]	1493.86±14.76[c]
	Combined	1414.27±38.57[a]	1466.31±33.29[b]	1530.97±43.15[c]
Body Weight Gain of 2-3 Months	Male	187.30±19.38[a]	216.20±7.35[b]	261.00±4.46[c]
	Female	165.64±11.90[a]	178.47±8.12[b]	193.29±12.85[c]
	Combined	176.72±21.38[a]	194.64±14.39[b]	224.89±35.64[c]
Body Measurements	StL	132.89±2.20[a]	138.80±0.89[b]	141.94±0.89[c]
	SL	46.55±1.46[a]	50.54±1.33[b]	52.49±0.27[c]
	SC	35.87±2.01[a]	38.95±0.45[b]	40.22±0.36[c]
	WL	232.41±1.87[a]	238.08±1.00[b]	239.73±0.12[c]

Information: Superscripts of different letters on the same line are significantly different (P<0,05).

Table 4. shows the average body weight of male and female Kerinci ducks aged three months, the body weight gain of male and female Kerinci ducks aged 2-3 months, and the body measurements of male and female Kerinci ducks aged three months genotype -/- was higher than the genotype +/+ and +/-. The results of this study are not much different from the research of Batubara (2017), which states that the body weight and body measurements of livestock with the (-/-) genotype are higher than the (+/+) and (+/-) genotypes.

The results of the analysis of the mean difference test (t-test) showed that body weight aged three months, body weight gain at 2-3 months old, and body measurements at three months old Kerinci ducks male and female Myostatin (MSTN) gene used the PCR-RFLP MboI marker in all genotypes (-/-) were significantly different ($P<0.05$) with genotypes (+/-) as well as genotypes (+/-) were significantly different ($P<0.05$) with genotypes (+/+). This condition showed that the body weight at three months of age, body weight gain at the period of 2-3 months, and body measurements at the age of 3 months in the Kerinci duck, the Myostatin (MSTN) gene using the PCR-RFLP marker MboI genotype (-/-) was higher than the genotype (+/ -), also genotype (+/-) is higher than genotype (+/+). The Myostatin (MSTN) MboI gene was associated with body weight at three months, weight gain at 2-3 months, and body measurements at three months of age in male and female Kerinci ducks. Zhang et al. (2019) stated that the Myostatin (MSTN) gene has a positive relationship with growth. Dushyanth et al. (2016) noted that the Myostatin (MSTN) gene expression in livestock showed development in various organs such as muscles, brain, and intestines. Furthermore, Dou et al. (2018) stated that the Myostatin(MSTN) gene associated with the growth of chest and thigh muscles in the early stages of livestock growth was not optimal. Thus it can be stated that the genotype (-/-) of the Myostatin (MSTN) MboI gene was higher than that of the (+/-) and (+/+) genotypes.

CONCLUSION

Based on the results and discussion, it can be concluded that: 1) Body weight, weight gain, and body measurements of male Kerinci ducks were higher than female Kerinci ducks. 2) Body measurements markers for male and female Kerinci ducks are sternum length (StL), shank length (SL), and shank circumference (SC), while body shape markers for male and female Kerinci ducks are wing length (WL). 3) *Myostatin* (MSTN) gene in Kerinci ducks is polymorphic. 4) *Myostatin* (MSTN) *MboI* has an association with body weight at three months old, body weight gain at 2-3 months, and body measurements at three months in male and female Kerinci ducks with the best genotype, namely (-/-).

The results of this study are expected to be used as the basis for future selection of Kerinci ducks for livestock breeding and policy makers and business actors.

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