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Characterization of Myostatin (MSTN) Gene Using PCR-RFLP Method in KUB Chicken

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ABSTRACT

This study aims to obtain MSTN gene diversity and MSTN gene association. The materials used in this study were 96 KUB chickens and 96 KUB chicken blood samples consisting of 41 males and 55 females. This research method is an experiment. Data collected included body weight, weight gain and body size of male and female KUB chickens, and diversity and association of MSTN genes. Data analysis used mean difference test (T-test), T^2-Hotelling, Principal Component Analysis (PCA), Genotype and allele frequencies, Hardy-Weinberg equilibrium, Heterozygosity and Polymorphic Information Content (PIC). The results showed that body weight at 1 and 2 months of age, body weight gain at 1-2 months and body size of male KUB chickens at two months were significantly ($P<0.05$) higher than female KUB chickens. The body size characteristic of male and female KUB chickens is chest circumference (CC) while the body shape characteristic is wing length (WL). The MSTN|MspI gene in KUB chickens is polymorphic. The KUB chicken population is in Hardy-Weinberg imbalance. Heterozygosity of the KUB chicken population showed a value of Ho (0.31) < He (0.51). The MSTN| MspI gene of KUB chicken was associated with body weight, weight gain and body measurements and the best genotype was (-/-).

Keywords: Diversity, KUB chicken, *Myostatin* gene (MSTN), Msp1 enzyme

INTRODUCTION

Indonesia is known as a tropical country with a wealth of germplasm diversity, including KUB chicken. KUB chicken is a native chicken resulting from research innovations from the Livestock Research Center, Ciawi-Bogor (Ratnawati et al., 2020). The existence of KUB chicken as germplasm needs to be maintained and preserved; for efforts that can be made to preserve it, it is necessary to characterize its quantitative characteristics. Quantitative characteristics of economic value can be seen from the performance of livestock. However, quantitative characterization for improving the genetic quality of livestock is less accurate than molecular selection.

With the advancement of technology in the molecular field, economically valuable characteristics can be carried out with a more in-depth analysis of their structural genes. The myostatin gene is one structural gene affecting livestock productivity *(*Mardiah et al*.,* 2021*)*. The *Myostatin* gene *(MSTN)* is a member of the growth subgene *(Transforming Growth Factor/TGF- β)* and functions as a negative regulator of skeletal muscle growth in the body (Bhattacharya et al., 2019).

The PCR-RFLP method can be used to look at the diversity of the myostatin gene. This method has been developed to determine various levels of DNA (restriction enzymes) that can cut DNA sequences at a certain point. Through the bands displayed on electrophoresis, the genetic diversity of livestock will be known (Hidayati et al., 2016; Mardiah et al., 2022). Based on the description above and the lack of information about the *myostatin* gene in KUB chickens, it is necessary to research "Characterization of the *myostatin gene (MSTN)* using the PCR-RFLP method in KUB chickens".

MATERIALS AND METHODS

The materials used in this study were 96 KUB chickens and 96 KUB chicken blood samples consisting of 41 males and 55 females The materials used in this study were 70% alcohol and cotton for blood preservation, protocol *Genomic* DNA *Purification Kit* from Promega, isopropanol, 70% ethanol, agarose powder, TBE Buffer solution, distilled water, *ethidium bromide* (EtBr) staining, *loading dye*, DNA ladder, *forward* and *reverse* primers*, Nuclease Free Water, Gotaq Green Mastermix,* and *Thermoscientific* brand *MspI* restriction enzyme. *The* equipment used was *hand glove, vaculab EDTA K3*, *tube holder, 3 ml disposable syringe, cool box,* stationery, *freezer*, oven, *autoclave*, *micropipette* 200 µl, 1000 µl, 100 µl, 20 µl, Eppendorf pipette tip *(yellow, blue, white*), *Eppendorf microtube* size 0,2 ml, 1.5 ml, and 2 ml*, microtube rack*, *centrifuge, Vortec,*

analytical balance, erlenmeyer, measuring cup, *gel doc, electrophoresis power supply*, *electrophoretic gel system,* gel printer, *well comb*, *mini spin centrifuge,* electric heater, PCR *Thermocycler* machine, and *water bath.*

The research method is experimental. This research was conducted in two stages: in the field and the laboratory. Field research included data collection of body weight at 1 and 2 months, body measurements of 2-month-old KUB chickens, and blood sampling.

The DNA extraction method used the *Genomic* DNA *Purification Kit* protocol from Promega. Electrophoresis used a 1.5% *agarose* gel stained with *Ethidium Bromide* at 100 volts for 60 minutes. The extracted DNA was then amplified using one pair of primers with an estimated product of 955 bp, which was expected to amplify the *myostatin* gene. These primers were designed using the Primer3 program based on GenBank with accession number AF346599. Primer Forward: $^{5'}$GGT TTT GAC GAC ATG AGC CT$^{3'}$ and Revers: $^{5'}$CAG GTG GAA TGT CAT GCA GA$^{3'}$. PCR amplification using a BIO-RAD brand PCR machine. The PCR amplification products obtained were then cut with the restriction enzyme *Msp1* (*Arthrobacter luteus I*) with the CC↓GG cutting site according to the gene locus. Myostatin|Msp1 gene restriction results can be seen after electrophoresis using 2% agarose stained with Ethidium Bromide (EtBr) at 200 for 120 minutes.

T-test, T^2 -Hotelling and Principal Component Analysis

According to the instructions, the T-test was used to determine differences between body weight, weight gain, body measurements, and between male and female KUB chicken genotypes (Mendenhall, 1987). If the T test^2 -Hotelling showed significant results ($P < 0.05$), then data processing in each group of livestock continued with Principal Component Analysis (PCA). Principal Component Analysis to determine the determinants of body size and shape of KUB chickens (Gaspersz, 2006).

Genotype Frequency, Alleles, Hardy-Weinberg Equilibrium, Heterozygosity, and PICs

Genotype frequency is the proportion or percentage of a particular genotype in a population, calculated based on the number of genotypes divided by the total sample. Hardy-Weinberg (H-W) equilibrium with chi-square X^2 test aims to compare observed data with hypothesized or *expected* values. Genetic *variability* is done through the estimation of the frequency of observed heterozygosity (Ho) and expected heterozygosity (He) is calculated using the formula Nei (1987). *Polymorphic Information Content* (PIC) was calculated using the Botstein et al. formula (1980).

RESULTS AND DISCUSSION

Average Body Weight and Weight Gain of KUB Chickens

The average 1 and 2 months body weight and 1-2 months body weight gain of male and female KUB chickens are presented in Table 1. Based on Table 1. the average body weight of male KUB chickens 1 and 2 months old is 382.30 ± 11.62 g and 771.99 ± 51.30 g, while female KUB chickens 1 and 2 months old are 370.93 ± 11.63 g and 715.32 ± 45.37 g. The results of this study are higher than the results of research by Utama et al. This study's results are higher than those of Utama et al. (2021), which states that the body weight of 1-month-old KUB chickens is 374.79 ± 24.47 g and two months 755.39 ± 30.11 g. The results of this study are higher than the results of research by Utama et al. Furthermore, according to Depison et al. (2022), the body weight of KUB chickens aged 1 and 2 months is 321.14 g and 699.52 g. The results of this study are higher than those of several other studies.

Table 1. Average body weight of male and female KUB chickens at 1 and 2 months of age and body weight gain of male and female KUB chickens.

Parameters	Male	Females
1 Month BW (g)	382.0±11.62^a	370.93±11.63^b
2 Months BW (g)	771.99±51.30^a	715.32±45.37^b
PBB 1-2 Months	389.69 ± 41.85^a	344.39±37.22^b

Notes: Superscript letters with slight differences in the same row significantly differ ($P<0.05$).

The results of this study are higher than some other local chicken studies. Average body weight of male Merawang chickens aged 1 and 2 months 320.28 ± 32.97 g and 700.96 ± 47.13 g, females aged 1 and 2 months 267.21 ± 9.96 g and 602.37 ± 32.86 g (Sari et al., 2021), male Kampung chickens aged 1 and 2 months 330.91 ± 28.89 g and 692.16 ± 36.98 g, female chickens aged 1 and 2 months 318.41 ± 19.95 g and 670.76 ± 21.30 g (Prawira et al., 2020). The description above shows that the body weight of male and female KUB chickens aged two months is quite good compared to several other studies.

Male KUB chickens' average body weight gain from 1-2 months of age was 389.69 ± 41.85 g; in female KUB chickens, it was 344.39 ± 37.22 g. The results of this study are higher than the research of Depison et al. (2022), which states that the body weight gain of 1–2-month-old KUB chickens is 378.49 ± 110.83 g. The results of this study are higher than those of Depison et al. The results of this research are higher than those of several other native chicken studies. The average body weight gain of Merawang chickens aged 1-2 months was 380.67 ± 23.24 g for males and 335.16 ± 25.80 g for females (Sari et al., 2021), 382.11 ± 32.85 g for males and 345.84 ± 46.83 g for females (Abdu et al., 2021). This condition shows that the body weight gain of KUB chickens aged 1-2 months is quite good from other studies.

The results of the mean difference test showed that the body weight of KUB chickens aged 1 and 2 months and body weight gain of KUB chickens aged 1-2 months were significantly different ($P < 0.05$) higher than the average body weight and body weight gain of female KUB chickens. This difference is thought to be due to the influence of hormones possessed by male livestock. It is the opinion of Sari et al. (2021), who state that male livestock have testosterone hormone produced by the testes so that the body weight of male livestock is higher than that of female livestock. According to Hapsari (2015), a testosterone hormone functions as an androgen steroid, a growth regulator. High steroid secretion in males results from the high secretion of the hormone testosterone produced by the testes, so the growth rate in male chickens is higher than in female chickens.

Average Body Measures of KUB Chickens

The average body measurements of male and female KUB chickens aged two months include BL= Beak Length, HL= Head Length, **BW**= Beak Width, HH= Head Height, HC= Head Circumference, NL= Neck Length, NC= Neck Circumference, WL= Wing Length, UBL= Upper Body Length, LBL= Lower Body Length, BH= Body Height, CL= Chest Length, CW= Chest Width, CC= Chest Circumference, SL= Shank Length, SC= Shank Circumference, TL= Tibia Length, TC= Tibia Circumference, TFL= Third Finger Length and PBD= Pubic Bone Distance.

This study showed that the average body measurements of male KUB chickens aged two months were significantly different ($P<0.05$) than those of female KUB chickens aged two months. The results of this study are not much different from several other studies. Prawira et al. (2021) stated that the average body size of male Kampung chickens is higher than that of female Kampung chickens. Supported by Sari et al. (2021) research, the average body size of male Merawang chickens was higher than that of female Merawang chickens. The difference in average body measurements indicates that the skeletal growth of male KUB chickens is greater than that of female KUB chickens. It is the opinion of Sitanggang et al. (2016), who stated that the larger the size of the animal's body skeleton, the larger the body size.

T^2 -Hotteling Analysis and Principal Components of Body Measures

T2-Hotelling analysis is conducted on body measurements between males and females simultaneously in KUB chickens. This analysis explains the differences between male and female KUB chicken groups. This study showed that the body measurements of male KUB chickens were significantly different ($P < 0.01$) than female KUB chickens. The difference in body size of male and female KUB chickens is thought to be due to genetic factors. It is the opinion of Hikmawaty et al. (2014), which states that the body size frame of livestock can differ from one another due to genetic factors. Principal component analysis (PCA) is one way to determine the discriminant between the size and shape of the chicken body. The size and shape equation, total diversity (TD), and *eigenvalue* (λ) of male and female KUB chickens are presented in Table 2.

The highest eigenvector in the body size equation in male and female KUB chickens is the chest circumference (CC) Breast circumference (BC) is a body size characteristic because it contributes the largest to the size equation. The results of this study are the opinion of Utama et al. (2021), who stated that the characteristic of Kampung chicken body size is the chest circumference (CC) so that it can be used as a

selection parameter in increasing body score and as a consideration for purification policies. Putri et al. (2020) body measurements can be used to estimate the description of body shape as a characteristic of a particular nation. Wing length is the highest eigenvector in the male and female KUB chicken body shape equation. It means that wing length is a body shape characteristic because it contributes most to the shape equation. The results of this study are the opinion of Rahayu et al. (2021), who stated that the characteristic of a Super chicken body shape is wing length. Thus, it can be stated that WL can be used as a selection parameter to improve the body shape score of chickens.

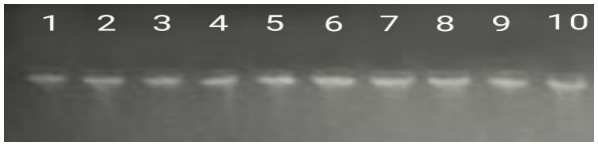
Table 2. Similarities in body size and shape of KUB chickens

Type			Equation	TD (%)	Λ
Male	Body Size	=	0.173BL + 0.18HL - 0.222BW - 0.244HH - 0.245HC + 0.224NL + 0.23NC - 0.183WL+ 0.21UBL + 0.229LBL - 0.245BH + 0.208CL + 0.191CW + 0.245CC - 0.245 SL - 0.245SC - 0.245TL + 0.228TC + 0.225 TFL + 0.23 PBD	82,6	16,5
	Body Shape	=	0.417 BL - 0.456HL + 0.096BW - 0.033HH - 0.032HC + 0.063NL + 0.122NC + 0.456WL + 0.114UBL - 0.092 LBL - 0.032BH +0.139CL - 0.553CW + 0.032CC - 0.032SL - 0.032SC - 0.032TL + 0.001TC + 0.052TFL + 0.134PBD	0,4	7,98
Females	Body Size	=	0.245BL + 0.234HL - 0.041BW + 0.223HH + 0.201HC - 0.256NL + 0.25NC - 0.007WL + 0.247UBL + 0.24 LBL + 0.252BH + 0.25CL + 0.113CW + 0.256CC + 0.23SL + 0.237SC + 0.232TL + 0.245TC + 0.236TFL - 0.254PBD	73,9	14,77
	Body Shape	=	- 0.154BL - 0.08HL + 0.316BW + 0.215HH + -0.412 HC - 0.063NL - 0.15NC - 0.349WL - 0.17UBL - 0.212 LBL - 0.043BH - 0.112CL + 0.382CW - 0.042CC + 0.265SL + 0.27SC + 0.253TL + 0.156TC + 0.258TFL + 0.096PBD	9,3	1,86

Description: TT = Total Diversity, BL= Beak Length, HL= Head Length, **BW**= Beak Width, HH= Head Height, HC= Head Circumference, NL= Neck Length, NC = Neck Circumference, WL= Wing Length, UBL= Upper Body Length, LBL= Lower Body Length, BH= Body Height, CL = Chest Length, CW= Chest Width, CC= Chest Circumference, SL= Shank Length, SC= Shank Circumference, TL= Tibia Length, TC= Tibia Circumference, TFL= Third Finger Length and PBD= Pubic Bone Distance.

DNA Extraction and Amplification of Myostatin Gene (MSTN)

DNA extraction consisted of 96 blood samples of 2-month-old KUB chickens. Using the *DNA Purification Kit* protocol from omega in electrophoresis using 1.5% *agarose,* visualized using UV light through *Gel Doc*. DNA extraction is a series of processes to separate DNA from other cell components (proteins, fats, and carbohydrates) (Hernandez et al., 2006; Hidayati et al., 2016). More details can be seen in Figure 1.



Description: numbers 1,2,3,.....20 = individual samples

Figure 1. Electrophoresis of DNA extraction results

The results of DNA extraction electrophoresis in Figure 1. 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. It indicates that the DNA concentration is equivalent, and the DNA can proceed to the following process (Mardiah et al., 2021), supported by the opinion of Nova et al. (2016), which states that good DNA quality can be seen with clean and clear DNA bands. The quality of DNA produced at the isolation stage determines the success of research based on molecular DNA.

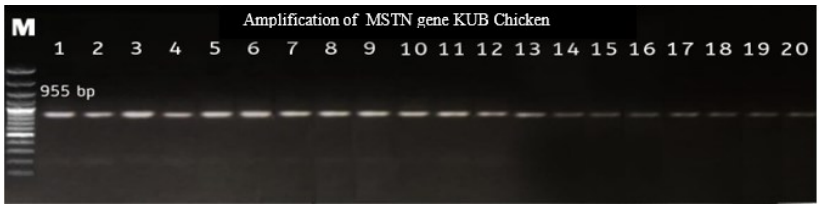
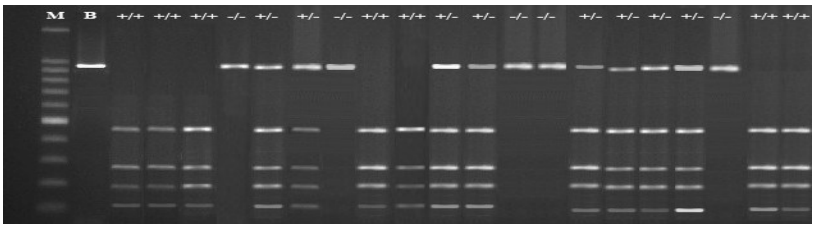


Figure 2. Electrophoresis results of *myostatin* gene PCR products using 1000 bp *DNA Ladder.*

Figure 2. shows successful PCR product amplification of the *myostatin* gene (MSTN) using an *annealing* temperature of 60ºC for 45 seconds. The amplified *myostatin* gene indicates if the *appropriate annealing temperature is not too high or* too low. The *annealing* temperature used must be optimal; if the *annealing* temperature used is too high, it causes the primer not to stick to the DNA, and the amplification fails, while if the *annealing* temperature used is too low, it causes the primer to stick to the other side of the DNA which results in poor quality DNA (Hidayati et al., 2016; Ramadhan et al., 2019). The *annealing* temperature used is 50-60º C.

Genotype and Allele Frequency

The diversity of the KUB chicken *myostatin* gene was identified using the cutting enzyme *Msp1* CC↓GG at 199 bp, 228 bp, 487 bp, and 41 bp. Getting the results of 3 genotypes namely +/+, +/-, and -/- and 2 alleles + and -. Suhartati et al. (2020) stated that the diversity of the *myostatin* gene in *exon* one cut with the *SatI* enzyme showed that the myostatin gene had three genotypes, namely A/A, A/G and G/G.



Description: L = Ladder, B = Myostatin gene amplification fragment.
Figure 3. PCR-RFLP electrophoresis results in *MSTN|MspI*

Table 3. Genotype frequencies, alleles, Hardy-Weinberg (HW) equilibrium test, and PIC (*Polymorphic Information Content*) values.

Locus Germplasm	N	Genotyping	Genotype Frequency	Allele Frequency	X^2 count	H_0	H_e	PIC Value
Chicken *KUB|MSTN| Msp1*	96	+/+	0.3	58%	13.2	0.31	0.51	0.42
		+/-	0.31					
		-/-	0.26	42%				

Based on Table 3. that the results of genotype frequency analysis on the *Myostatin Msp1* gene in KUB chickens obtained three genotypes, namely +/+ (0.43), +/- (0.31), -/- (0.26) with allele frequency (+) of 0.58% and allele (-) of 0.42%. The results of this study indicate that the *Myostatin* gene in KUB chickens is polymorphic. Nei and Kumar (2000) stated that an allele is declared polymorphic if the frequency of one allele of a gene is less than 99%. The results of this study are not much different from those of other polymorphic studies (Fastawa et al*., 2019*; Al-Sobri et al., 2022; Salsabila et al*.,* 2022).

Hardy-Weinberg equilibrium

The table of Chi-Square test analysis results shows that the X^2 count (13.2) is higher than the X^2 table 0.05 (3.84). This condition illustrates the *Hardy-Weinberg* imbalance ($P<0.05$) in the KUB chicken population. Allendrof et al. (2013) stated that the population is said to be in balance if the calculated value of X^2 is smaller than the X^2 table. Khaerunnisa et al. (2016) stated that the *Hardy-Weinberg* imbalance in the Kampung chicken population shows that the frequency of genotypes and alleles is not constant from generation to generation. The imbalance occurs allegedly due to selection and mating

within the group. Nova et al. (2016) state that Hardy-Weinberg imbalance in a population is caused by selection, absence of random mating and migration.

Heterozygosity

Table 3. shows the value of $H_0 < H_e$. The observed heterozygosity value (H0) was 0.31, and the expected heterozygosity (He) was 0.51. This condition indicates that the diversity of KUB chickens is classified as moderate, with relatively distant genetic relationships. 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).

According to Allendorf and Luikart (2013), the value of heterozygosity is important to know to get an overview of the genetic diversity of a population. The value of heterozygosity is a parameter used to measure a population's genetic diversity level (Ghassani et al., 2022; Putri et al., 2020).

Polymorphic Information Content (PIC)

PIC value is divided into three classes: PIC > 0.5 = very informative, 0.25 > PIC > 0.5 = medium, and PIC < 0.25 = low (Al-Sobri et al*.,* 2022). Based on Table 6. above, the PIC value on the *MSTN*|*MspI* gene of KUB chicken is 0.42. The PIC value of the *MSTN*|*MspI* gene is in the moderate category, which means that the primer is quite informative as a marker for the *MSTN*|*MspI* gene. The PIC value is the value of a marker that shows polymorphism in a population (Widhianata, 2019).

Association of *Myostatin* Gene (MSTN) with 2-month-old KUB Chicken Body Weight, 1-2-month-old Body Weight Gain and 2-month-old Body Measures.

Based on Table 7. shows that the average BW of male and female KUB chickens aged 2 months, PBB of male and female KUB chickens aged 1-2 months and body measurements of male and female KUB chickens aged two months genotype (-/-) is higher than genotypes (+/+) 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 genotype (-/-) are higher than genotypes (+/+) and (+/-).

The results of the mean difference test analysis showed that the average body weight, body weight gain, CC and WL of the MSTN gene of KUB chickens using PCR-RFLP genotype (-/-) was significantly different (P < 0.05) higher than the genotype (+/-) as well as the genotype (+/-) was significantly different (P < 0.05) higher than the genotype (+/+). The results of this study differ from the research of Suhartati et al. (2020), which states that the *MSTN*|*MspI* gene has an association with one and 2-month-old BW, 1-2-month-old PBB and body measurements of 2-month-old KUB chickens, the *myostatin SaltI* gene has an association with 12 week old BW and 12-week old body measurements in Kampung chickens with genotypes (+/-) higher than genotypes (-/-) and genotypes (+/+).

Table 4. Average body weight of KUB chicken aged two months, body weight gain of aged 1-2 months and body measurements of KUB chicken aged two months various genotypes

	Genotype		
	+/+	+/-	-/-
2-month Body Weight			
Male	730.26± 22.25 ^{c}	765.77 ± 11.87 ^{b}	847.07 ± 12.31 ^{a}
Females	673.0 ± 8.53 ^{c}	732.5 ± 29.91 ^{b}	774.3 ± 15.92 ^{a}
UN 1-2 months			
Male	357.59 ± 22.65 ^{c}	383.375 ± 11.92 ^{b}	449.1 ± 13.93 ^{a}
Females	312.8± 11.25 ^{c}	349.5 ± 29.43 ^{b}	389.8 ± 19.77 ^{a}
Body Size			
Chest Circumference	254.55±7.93^{b}	255.98 ± 7.58^{a}	258.77± 7.75^{a}
Wing Length	148.98 ±4.98^{a}	149.92± 5.33^{a}	151.39 ±5.69^{a}

Notes: Small letters on the same line differ significantly (P < 0.05).

The results of this study are by the research of Fastawa et al. (2019), which states that the *myostatin* gene is associated with the body weight of 20-week-old Sentul chickens with the (-/-) genotype higher than the (+/-) and (+/+) genotypes. It is thought to be due to a mutation of the *myostatin* gene that results in gene expression that can increase livestock's muscle mass.

Myostatin can increase pectoral muscle weights and weights due to extreme muscle hypertrophy achieved by manipulating TGF-b signalling (Lynch et al., 2019; Tanjung et al., 2019; Zhao et al., 2019). MSTN gene deficiency can exhibit increased muscle mass in cattle, commonly known as double muscling (DBM) (Aiello et al., 2018). Thus, it can be stated that the *MSTN|MspI gene* with the (-/-) genotype is higher than the (+/-) and (+/+) genotypes.

CONCLUSIONS

Based on the results and discussion, it can be concluded that: 1) Body weight, weight gain and body measurements of male KUB chickens aged two months are higher than female KUB chickens. 2) The body size characterizer of male and female KUB chickens is the chest circumference, while the shape determinant characterizer in male and female KUB chickens is the wing length. 3) The *myostatin|Msp1* gene in KUB chickens is polymorphic. 4) The *myostatin|Msp1* gene in KUB chickens is associated with body weight, weight gain and body measurements, and the best genotype is (-/-).

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