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Genetic variability of IGF1 and IGF2 and correlation to body weight in Kedu chicken of Indonesia

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Abstract. *Rosidi, Suswoyo I, Mugiyono S, Ismoyowati, Tugiyanti E. 2024. Genetic variability of IGF1 and IGF2 and correlation to body weight in Kedu chicken of Indonesia. Biodiversitas 25: 4846-4852.* This study aims to examine differences in genetic variability using Insulin Growth Factors (IGF) to estimate the body weight of selected Kedu chickens. A total sample 37 chickens consist of 10 White Kedu, 10 Cemani Kedu, 10 Red Kedu, 5 Black Tongue Kedu, 1 Blorok Kedu, and 1 Lurik Walik Kedu. Next, their blood is taken from the chicken samples and analyzed in the laboratory. The identification of the IGF-1 and IGF-2 genes polymorphism was done by PCR and sequencing methods. The results showed two Single Nucleotide Polymorphism (SNP) of IGF1: c.166 T>A and c.169 A>G and IGF2: c.248 G>A and c.540 C>T. The frequency of genotypes was informed on GG, GA and AA with values of 0.71, 0.1 and 0.19, respectively. The base pair of 540 showed the existence of genotypes CC, CT and TT with values of 0.28, 0.62, and 0.1 respectively. The value of heterozygosity obtained at each base pair was c. 248 and the heterozygosity value was 0.3648, while the heterozygosity value of c. 540 was 0.4838. The correlation analysis showed that at c. 248 had a value of 0.57, which was relatively greater than that at c. 540, namely 0.15. The c. 248 genotypes of AA had greater body weight than the rest. In conclusion, AA genotypes in the IGF-1 and IGF-2 genes had a relatively high body weight compared to that of other genotypes, but IGF-1 had a relatively low association (correlation), so incompatible as a marker for future selection. An IGF marker can be used to estimate the body weight of the upcoming offspring of Kedu chickens.

Keywords: Body weight, genetic variability, IGF-1, IGF-2, Kedu chicken

Abbreviations: IGF: Insulin Growth Factors

INTRODUCTION

Native Indonesian chickens are distinctive from exotic and commercial breeds, and therefore, should be the core of genetic diversity conservation (Ulfah et al. 2015). The advantage of local chickens, such as Kedu chickens, compared to other chickens, is relatively easy and inexpensive maintenance. However, the development of local chicken can face several constraints that include but not limited to low growth rate, high mortality, and low egg production. To improve native chicken productivity, enhancement can be made in breeding, feeding, and management practice (Hidayat and Asmarasari 2015). Specifically, there remains ample room for improvement and characterization of Kedu chickens. Identification can be done primarily on qualitative phenotypic characteristics (feather color, skin, shank, and comb) and quantitative traits (morphometrics, productivity, and resistance to disease or parasites). Qualitative traits of the phenotype are needed to determine the characteristics of the performance of certain Kedu chickens that can be clearly distinguished (visually) from other local chicken species. The multivariate analysis enables measurement of certain body parts which can be the identifier (differentiator) of a livestock group or family. Chicken genetic diversity can also be identified from their biomolecular area.

It generally takes a long time to identify the body weight of an individual Kedu chicken's offspring, and consequently it is high cost. The average body weight of black Kedu chicken is reportedly 1,955.50±161.88 g (Mustofa et al. 2016) and it can be estimated through the correlation between genes and body weight of the chickens. All organisms have Single Nucleotide Polymorphism (SNP) in the most evenly distributed and scattered genomes. Regarding ducks, the low availability of duck-specific DNA chip platform has been the driving factor to a scarce number of studies on population genetics and genomic selection. The reduction of genome complexity is feasible through restricting the enzymes for digestion in reduced-representation method to apply a sizeable sample into SNP assay. Detecting genome-wide SNP can be effectively done using Genotyping by Sequencing (GBS) for plants, aquatic organisms, and animals like swine, poultry, and cattle (Zhu et al. 2016). Today, many DNA markers are utilized to determine the relationship with Quantitative Traits Locus (QTL) in poultry. SNP or microsatellite markers are very limited in their target enzymes, thus making them specific. The characteristics of egg production and quality are related to candidate gene polymorphisms: IGF-1, GH and GHR.

At the species level, the SRAP analysis indicates the adequate number of alleles (ne) is 1.5702, Shannon's Information Index (I) is 0.5057, and Nei's gene diversity (h) is 0.336 (Yan et al. 2019). The Insulin Growth Factor (IGF) gene influences chickens' composition, metabolism,

growth, meat characteristics, and adipose tissue and fat growth. Two types of insulin growth factors are IGF-1 and IGF-2, which bear different sequences and product lengths. IGF can be used to analyze SNP using PCR-RFLP or RT-PCR HRM, which, as reported in a previous study, have shown that dangdut chickens and slow-laughing chickens have similar genotype to that of A homozygote (Abinawanto et al. 2019). Therefore, it is crucial to determine which types of IGF are viable markers for chicken selection in the future based on body weight. Different uses of Insulin Growth Factors are investigated in the present research to estimate body weight in the selection of Kedu chickens.

MATERIALS AND METHODS

Materials

The materials used were 37 Kedu chickens consisting of 10 white Kedu chickens, 10 Cemani Kedu chickens, 10 Red Kedu chickens, 5 Black Tongue Kedu chickens, 1 Blorok Kedu chicken, and 1 Lurik Walik Kedu chicken. Blood samples were taken from each of these chickens and analyzed for DNA. The DNA isolation was performed using centrifugation. DNA isolation uses the phenol-chloroform extraction method (Sambrook et al. 1989). Blood extraction was conducted using a QIA-amp DNA Blood Mini Kit (Qiagen).

The procedure for making 1% Agarose Gel employing 0.25 g of agarose mixed with 25 mL of TBE. The mixture is heated in the microwave for 60 seconds, then added 3 µL of flourosafe DNA (dye). The mixture is added dye on the mold, and the agarose gel is ready for use. The materials for manufacturing agarose gel are TBE buffer agarose powder (89 mM, Tris boric acid, and 2 mM EDTA pH 8.0) and DNA dye in the electrophoresis process (Florosafe DNA stain). The reverse and forward preliminary designs in IGF-1 and IGF-2 genes are presented in Table 1.

Procedures

Assessment of DNA polymorphism

Blood sampling. Blood sample (±3 mL) was taken using a syringe on the inner vein of the chicken wing. The collected blood was then put into a test tube pre-filled with EDTA (Ethylene-Diamine-Tetra-Acetic Acid). A label was attached to each sample and the tubes were stored in an ice flask. The sample was well maintained, and then blood serum was taken for gene polymorphism analysis.

DNA isolation. The blood sample of approximately 200 µL was placed into a microcentrifuge tube containing 20 µL of proteinase K and then mixed. To the mixture was added by 200 µL of buffer AL, which was then homogenized by using a vortex for 15 seconds. Next, it spun down and incubated at 56°C for 10 minutes. The dry GD column (top tube) was re-centrifuged for 10 minutes (5000 rpm). The bottom tube was replaced with a new Eppendorf. It was already coded according to the sample to hold DNA. Elution Buffer or TE (Tris EDTA) was prepared by warming the incubator until it felt warm (pre-heated Elution Buffer). Elution Buffer (100 µL) is inserted into the GD Column Matrix (top tube) using a yellow tip right in the middle. GD Column Matrix d left for 3 minutes so that the column matrix absorbs the Elution Buffer. The GD Column Matrix was centrifuged for 5 minutes (5000 rpm), then added 100 µL pre-heated Elution Buffer to rinse so that the DNA contained in the column matrix went all the way down to the Eppendorf tube. DNA, which went down to the Eppendorf tube, was centrifuged for 2 minutes (5000 rpm).

Polymerase Chain Reaction (PCR). Specific DNA fragments (IGF-1 and IGF-2 genes) were amplified using the PCR method, a technical synthesis of DNA in vitro that can multiply DNA quickly. Thermal cycler machines performed the amplification of specific DNA fragments of the IGF-1 and IGF-2 genes. The PCR process was carried out with the target of the IGF-1 and IGF-2 genes in a total reaction volume of 25 µL consisting of the KAPA2G Fast Ready-mix PCR kit (Kapa Biosystems) of 12.5 µL dH_2O of 9.5 µL forward and reverse primer of 1 µL each, and finally 1 µL of genomic DNA (Purwantini et al. 2013).

The amplification stages include the stages of pre-denaturation, denaturation, annealing, elongation or extension, and finally, the post-elongation stage. The amplification stages include the stages of pre-denaturation, denaturation, annealing, elongation, or extension, and finally, the post-elongation stage. Pre-denature was performed at 95°C for 5 minutes, followed by denaturation at 95°C for 1 minute, and annealing in IGF-1 gene at 63.9°C while in the IGF-2 gene at 64.3°C for 1 minute. Then, the PCR reaction would be stopped, and the thermocycler condition was maintained at 72°C for 1 minute in a stage called the elongation. At last, post-elongation perfected the DNA lengthening, carried out for 7 minutes at 72°C. The PCR reactions were repeated 35 times a cycle, to get the maximum PCR product. The PCR products were electrophoresed on a 1.5% agarose gel and processed for visual with the help of Ultraviolet light (Purwantini et al. 2013).

DNA sequencing. The sequencing method started with sample preparation, then continued with cycle sequencing, purification, and finally, DNA sequencing. The sample preparation stage included amplifying the target DNA sequence with PCR, visualizing and separating the target DNA sequence using electrophoresis, and regenerating the DNA sequence. In the cycle sequencing stage, we amplified the target DNA sequence using dd NTPs labeled fluorescent substances. The next stage was purification to remove excess dd NTPs that could interfere with sequence readings on sequencer machines. The results of the sequencer machine readings show that the electropherogram was shaped as an up-and-down curve with different colors. The blue, red, black, green, purple, or blue colors indicate the C, T, G, A, and N (error) bases, respectively. DNA sequencing was carried out at Apical Scientific Sequencing Singapore.

Data analysis

Allelic and genotypic frequencies were calculated using the following formula:

Allelic frequency: $xi = \frac{2nii+\sum nij}{2N}$

Genotype frequency: $xij = \frac{nii}{N} x100\%$

Where:
xi : Allele frequency ii
nii : Number of individuals with genotype ii
nij : Number of samples with genotype ij
N : Number of individual samples

The estimate Heterozygosity (He) calculated according to Nei (1987) with formula:

$$He = 1 \sum_{i=1}^{n} (pi)^2$$

The relationship or association between IGF-1 and IGF-2 gene genotypes and chicken body weight was analyzed using correlation analysis. Pearson correlation coefficient formula (Obilor and Amadi 2018).

$$rxy = \frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{\sqrt{[n\Sigma x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2]}}$$

Where:
r : Correlation coefficient
x : Genotype
y : Body weight
n : Number of samples

RESULTS AND DISCUSSION

Heterozygosity based on Insulin Growth Factor (IGF) genes

Insulin Growth Factor-1 gene

The samples that were sequenced were 21 out of 37 DNA isolates, because PCR only produced 21 clear PCR products. After the PCR process, sequencing was carried out and analyzed using the Bio-edit Version 7.1 program utilizing alignment between the MH745029.1 Gen Bank and 21 IGF-1 gene sequencing results. The sequencing results showed gene mutations in several base pairs of IGF-1 gene, from Thymine to Adenine in base pair 166 (T>A), as well as a mutation from Adenine to Guanine in base pair 169 (A>G) (Figure 1).

The genotype, gene frequency and heterozygosity values of the IGF-1 gene in Kedu chickens are in Table 2. There are three genotype frequencies, namely AA, TA and TT at a size of 166 base pair (bp), while at a size of 169 bp there are two genotype frequencies. The resulting ones are AA and GA.

Insulin Growth Factor-2 gene

The samples used in the IGF-2 study were as many as the IGF-1 study. Sequencing results showed gene mutations in several sizes (base pairs). There is a mutation at size 248 bp, namely a change from Guanine to Adenine (G>A) and a change at size 540 bp, namely a change from Cytosine to Thymine (C>T) (Figure 2). These results are in accordance with the statements of other researchers who stated that a higher efficiency in selecting chickens with high growth rate can be achieved by combining QTLs, SNPs, sequencing technologies, and candidate gene characteristics related to growth (Thu et al. 2020).

The association between IGF gene and body weight in Kedu chickens is illustrated in Table 4. The lowest correlation was obtained from IGF-1 169 bp (A>G) and the highest from IGF-2 248 bp (G>A). The Insulin Growth Factor (IGF) gene is a gene that plays a role in growth, body composition and metabolism, meat characteristics, and the growth of adipose tissue and fat in chickens. It was reported that the main mediator of Growth Hormone (GH) is the IGF-1 or the insulin-like growth factor 1 was the main enabler of cell growth and differentiation in childhood as well as anabolic effect in adults (Bailes and Soloviev 2021).

The genotype, gene frequency and heterozygosity values of the IGF-2 gene in Kedu chickens are in Table 3. At a size of 248 bp, the GG genotype has a higher relative frequency value compared to the GA or AA genotypes. The GG genotype has a value of 0.71, while at 540 bp the CT genotype has a higher relative frequency value of 0.62 compared to CC or TT genotypes.

Table 1. The primary reverse and the forward design in IGF-1 and IGF-2 genes

		Sequence	Product size
IGF-1	P	TGGTTGCTAAAGGATCGCAG	395 bp
	R	TGGTTAACTTTCGGGTGGCT	
IGF-2	P	AACCATTGGTGGGGGAGGAC	654 bp
	R	GTGGGTTACTGACACTGCCC	

Notes: P: Primary forward, R: Reverse primer

Table 2. Genotype frequency values, genes, and heterozygosity of the IGF-1 gene in Kedu chickens

Base pair	Genotype			Gene frequency			Heterozygosity
	AA	TA	GA	TT	G	A	T	
166 T>A	0.34	0.52		0.14		0.60	0.40	0.48
169 A>G	0.71		0.29		0.145	0.86		0.25

Table 3. Genotype frequency value, gene frequency, and heterozygosity of Kedu chickens IGF-2 gene

Base pair	Genotype						Gene frequency				Heterozygosity
	GG	GA	AA	CC	CT	TT	G	A	T	C	
248	0.71	0.10	0.19				0.76	0.24			0.36
540				0.28	0.62	0.1			0.41	0.59	0.48

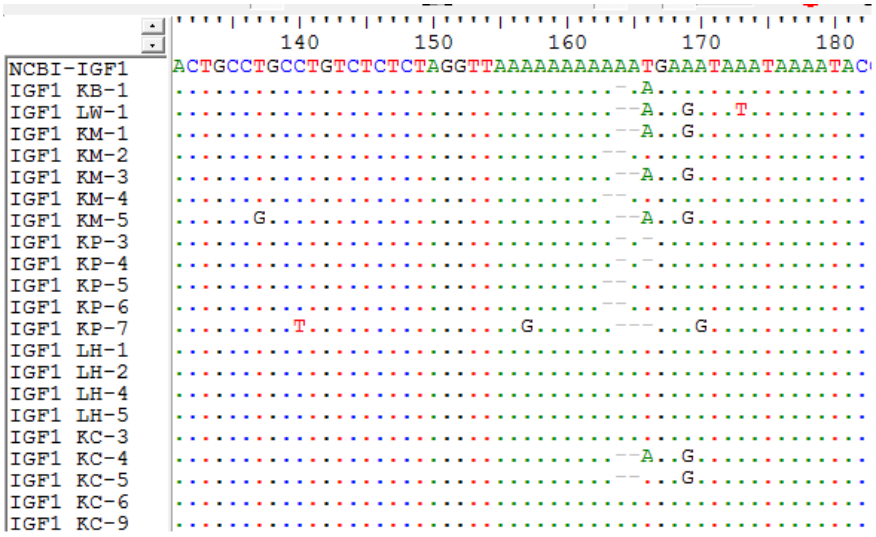

Figure 1. Sequencing result in the IGF-1 gene. A: Adenine; G: Guanine; T: Thymine; C: Cytosine

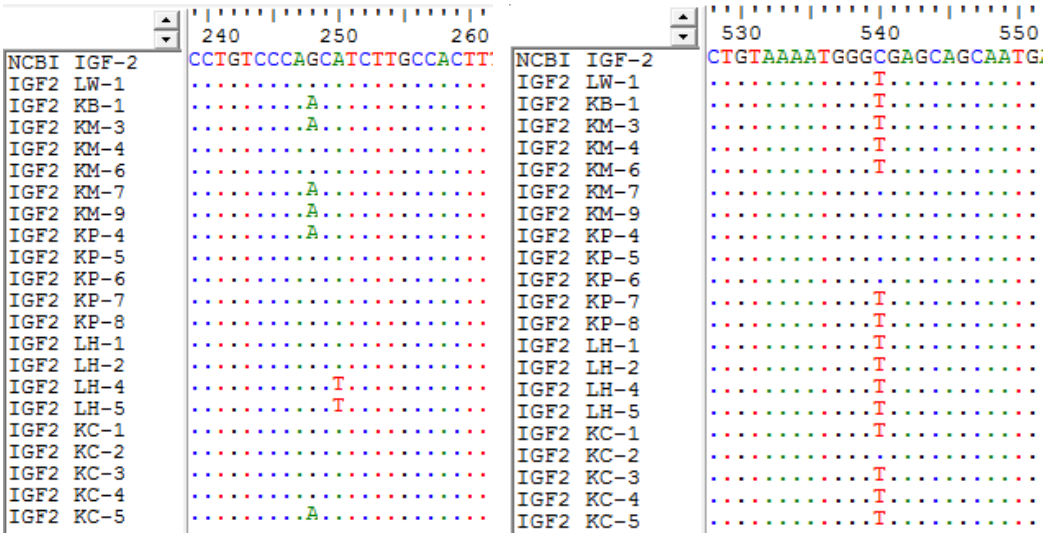

Figure 2. Sequencing results in the IGF-2 gene

Table 4. Associations between Insulin Growth Factor gene and body weight in Kedu chickens

	Base pare	Genotype	Sum	Average body weight (g)	Correlation
IGF-1	166 (T>A)	TT	3	1,550.98	0.31
		TA	11	1,663.01	
		AA	7	1,724.29	
	169 (A>G)	AA	15	1,669.73	
		GA	6	1,661.67	0.02
IGF-2	248 (G>A)	GG	15	1,644.23	0.57
		GA	2	1,843.50	
		AA	4	1,912.50	
		CT	13	1,682.31	0.15
	540 (C>T)	CC	6	1,763.39	
		TT	2	1,775.00	

The genotype frequency produced at 166 bp has the highest relative frequency value among other genotype frequencies. The TA genotype has a frequency value of 0.52, while in the size of 169 bp, the most considerable genotype frequency value was found in the AA genotype (0.71). Allele A has a higher frequency than other alleles at 166 bp or 169 bp, namely 0.60 and 0.855, respectively. Furthermore, the selected allele was fixed in Asian chickens. Meanwhile, the TSHR indicated that before the gene flow started, the frequency approached fixation before the onset of gene flow due to selection. As a result, determining allele frequency trajectory by was only insignificantly influenced the precise frequency of the derived allele in Asia. Observing the BCDO2, we found that a slightly higher level of selection may be resulted from the lower frequency of the derived allele in Asia (Loog et al. 2017). It shows that allele A in the IGF-1 gene

is an allele that commonly appears in local chickens both domestic and abroad.

Based on the frequency of genotypes and genes obtained, heterozygosity tests were carried out to determine the genetic variations found in Kedu chickens. The PAV-based GWAS results in promoter regions were made evident from the significant association between the *IGF2BP1* genotype and body size. CW1 ($P = 2.32E-14$) and CR ($P = 3.70E-12$) showed the most significant associations, respectively at 4.01% and 3.85% of the phenotypic variation. L1 had a bigger effect on females than the males where phenotypic variations of female was 11.5% and the male was 7.3% for CW1 trait. In addition, L1 indicated that DPW and SL12 had a female phenotype variation of 8.2% and 6.2% (Wang et al. 2021). Previous studies on SNP-based GWAS for chickens and ducks have reported the association between the body weight, head weight, gizzard weight, chest width, leg weight, and wing weight with the SNPs near the *IGF2BPI* (Zhou et al. 2018; Ma et al. 2019; Wang et al. 2020a; Wang et al. 2020b; Zhang et al. 2022). The results showed that the size of 166 bp had a relatively higher heterozygosity value (0.48) compared to that of 169 bp (0.25). The contributing factors to high heterozygosity include crossing. Indonesian native chickens are in the same clade as broiler, except for Arab chickens which have have high egg production (69.1%/hen/period) compared to that of other Indonesian native chickens (Syafwan and Noferdiman 2020). Based on the study results, the heterozygosity value at a size of 166 was relatively high. The probable cause of this was a cross between several Kedu chickens produced. Research findings submitted by Kedu chicken farmers show that it is often possible to cross between Kedu chickens, which results in different types of Kedu chickens.

The IGF-2 gene mutations studied in Kedu chickens are shown in Figure 2. There is a mutation in the size of 248 bp, namely the change from Gu and to Adenine (G>A), and a change in the size of 540 bp, namely the change from Cytosin to Thymine (C>T). Living beings have Single Nucleotide Polymorphism (SNP) in most evenly distributed and scattered genomes. Regarding ducks, the low availability of duck-specific DNA chip platform has been the driving factor to a scarce number of studies on population genetics and genomic selection (Zhu et al. 2016). The values of genotype frequency, gene frequency, and heterozygosity of the chicken Kedu on IGF-2 gene are presented in Table 3, that at a size of 248 bp, the GG genotype has a higher relative frequency value than the GA or AA genotype. Genotype GG has a value of 0.71, while at a size of 540 bp, the CT genotype has a higher relative frequency value of 0.62. A total of 97 DNA samples of broiler lines Hubbard F15 and Cobb E chickens were subjected to the IGF1/HinfI PCR-RFLP analysis and the results showed only two of three genotypes: AA (378 + 244 + 191 bp), and AC (622 + 378 + 244 + 191 bp), and there were higher AA homozygotes (73.20%) than the heterozygotes (26.80%) (Hosnedlova et al. 2020). Gene frequency values obtained in each size are different; at 248 bp are the G allele (0.76) and A allele (0.24). The frequency of genes obtained at 540 bp was the T and C alleles, with frequency values of 0.41 and 0.59, respectively. One contributing factor to gene frequency is selection. The value of heterozygosity in a population can be influenced by the resulting frequency. There was a gap in GH mRNA levels between genotypes after six weeks (breast muscle) and two weeks (liver tissue). The highest BW at the initial stage of life observed in AA genotype of IGF-I gene (Sinpru et al. 2021). Kedu Lurik chickens have an amylase-I locus controlled by two alleles, namely Amy-I ^{B} and *Amy-I*^{C} (Abubakar et al. 2014). The heterozygosity value obtained in 248 bp was 0.36, while in 540 bp was 0.48. The value of heterozygosity was calculated to determine individual genetic diversity in the population. The null allele frequency was detected above 0.2 in only four loci (LEI0196, MCW0020, MCW0287, and MCW0330). On LEI0196 locus, the null allele frequency was identified in RIRI (0.244), RIRII (0.329), BARI (0.276), and BARII (0.224) populations. On MCW0020 locus, allele frequency was observed on RIRII (0.319), BARI (0.212), and BARII (0.272) lines. The MCW0287 and MCW0330 loci were observed in RIRI (0.267 and 0.255), RIRII (0.417 and 0.257), and COL (0.295 and 0.242) (Karsli and Balcıoğlu 2018). The importance of heterozygosity obtained from this study was in contrast with that by Karsli and Balcıoğlu.

Associations between Insulin Growth Factor gene (IGF-1 and 2) with body weight

Based on Table 4, the genotype obtained at 166 bp has three genotypes, namely TT, TA, and AA, while at 169 bp are two genotypes, namely AA and GA. At 166 bp, the AA genotype is assumed to be the dominant genotype because it has the highest body weight. The TA genotype is heterozygous because it has medium body weight, and the TT genotype is presumably a recessive genotype because it has the lowest body weight. Table 4 shows that the IGF-1 gene has a relatively low correlation of 0.31 at 166 bp and 0.02 at 169 bp. The AA genotype at 166 bp has a relatively high average body weight compared to other genotypes. Meanwhile, the IGF-1 gene has a relatively low correlation to the body weight of Kedu chickens. Since the highest BW of all ages is found in the homozygous type (A1A1), the selection programs for growth can utilize GH as a marker gene (Sinpru et al. 2021). Two clusters appeared after the principal component analysis. In the first cluster was Arab chickens, while the second were Merawang, Pelung, Sentul, Cemani, KUB, Black Kedu, White Kedu, and Broiler chickens. In Italian chickens, 580,961 SNP markers separated clusters of 6 breeds (Strillacci et al. 2017). The microsatellite markers have been reported in a previous study to figure out three clusters into which seven South African native chickens were categorized (Nxumalo et al. 2020). On the other hand, 24 microsatellite markers managed to put five Swedish chicken breeds into two clusters (Abebe et al. 2015). The three main advantages of significant SNPs over microsatellite were more accurate estimates of diversity at the population level, a greater ability to identify groups in clustering methods, and the ability to consider local adaptation (Zimmerman et al. 2020). It suggests that the IGF-1 gene influences body weight by selecting the AA genotype. However, in this study, Kedu chickens with an AA or other genotype cannot be used as

selection characteristics because they have a relatively low correlation value. Therefore, it is better to commit further crossbreeding or selection to get chicken seeds with prospective brood stock criteria.

Table 4 shows two locations where polymorphism occurs, including 248 bp and 540 bp. Six genotypes were produced including three which were obtained from the point of 248 bp with correlation value of 0.57. The genotypes included GG, GA, and AA. The GG genotype is considered recessive because it has the lowest body weight compared to other genotypes. GA is a heterozygous genotype, while AA is the dominant genotype because it has a relatively high body weight. The body weight obtained in the AA genotype was 1912.5 g. At 540 bp, there are three genotypes: CC, CT, and TT. The TT genotype has a relatively high body weight but at a size of 540 bp, it has a relatively low correlation of 0.15. Therefore, at a size of 540 bp, the IGF-2 gene in Kedu chickens cannot be used as a selection character because it has a low correlation.

In contrast to the results of IGF-2 at a size of 248 bp with the AA genotype, it can be used as a selection criterion because it has a relatively high body weight and correlation value. Another study found differences in male legacy and commercial broilers' expression levels of selected somatotropic genes. While no difference was identified in circulating IGF, the higher level of IGF1 and IGF2 in post-hatch Ross muscle was indicative of paracrine IGF signaling that results in bigger pectoral muscle in modern commercial broilers (Vaccaro et al. 2022). Meanwhile, IGFBP2/Eco72I polymorphism found in all three genotypes (AA, AB, and BB), but a very low frequency (4.12%) was detected in BB (265 + 102 bp) homozygotes showed very low frequency (4.12%). The selection program for chickens in the Hubbard F15 and Cobb E broiler line showed that AB (367 + 265 + 102 bp) was the most represented genotypes with a frequency of 56.70% (Hosnedlova et al. 2020). The contributing factors of genes may include breeds, number, and gene mutations. The IGF-2 gene, which has an AA genotype, has a marked effect on body weight and a relatively more excellent correlation and body weight than the other genotypes. Accordingly, the AA genotype can be used as a marker for future selected characteristics related to body weight.

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