Morphometric Diversity and Polymorphism of Melanocortin-4 Receptor (MC4R) Gene in Red Kedu and Kampung Chickens

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ABSTRAK

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Penelitian bertujuan untuk membandingkan perbedaan morfometrik dan mengetahui adanya polimorfisme gen MC4R pada ayam Kedu Merah dan Kampung. Materi penelitian adalah ayam Kedu Merah dan Kampung sebanyak 98 ekor. Metode yang digunakan yaitu eksperimental dengan pengukuran morfometrik pada ayam berumur 37 minggu. PCR menggunakan sepasang primer MC4R berdasarkan kode akses GenBank AB01221 untuk mengamplifikasi target PCR sepanjang 221 base pair. Analisis data menggunakan uji-t untuk membandingkan morfometrik antara ayam Kedu Merah dan Kampung, kemudian perhitungan frekuensi genotipe, frekuensi gen, heterozigositas, dan jarak genetik untuk mengetahui adanya polimorfisme. Analisis variansi untuk mengetahui pengaruh genotipe terhadap bobot badan dan Panjang shank. Hasil penelitian menunjukan bahwa terdapat perbedaan nyata (P<0.05) antara ayam Kedu Merah dan Kampung baik jantan dan betina pada beberapa parameter morfometrik. Sekuensing produk PCR ditemukan SNP pada base pair 54G>C. Nilai frekuensi genotipe GC dan GG pada ayam Kedu Merah sebesar 0.51 dan 0.49, sedangkan pada ayam Kampung sebesar 0.32, 0.50, dan genotipe CC 0.18. Nilai frekuensi alel G dan C pada ayam Kedu Merah dan Kampung, masing-masing sebesar 0.74, 0.26, dan 0.66, 0.34. Nilai heterozigositas sebesar 38% dan 45%. Jarak genetik pada ayam Kedu Merah dan Kampung memiliki hubungan kekerabatan dekat yakni 0.42. Gen MC4R berpengaruh tidak nyata (P>0.05) pada bobot badan dan panjang shank sehingga gen MC4R tidak dapat digunakan sebagai kandidat marker assisted selection.

Kata Kunci: Ayam Kampung, Gen MC4R, Morfometrik, Polimorfisme, Ayam Kedu Merah

ABSTRACT

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The aim of this research was to compare morphometric differences and to determine the presence of MC4R gene polymorphisms in Red Kedu and Kampung chickens. This research used a total of 98 Red Kedu and Kampung chickens. The 37-week-old chickens were subjected to experimental study with morphometric measurements. PCR used a pair of MC4R primers based on GenBank access number AB01221 to amplify the PCR targets 221 base pairs long. Data analysis used the t-test to compare the morphometrics between Red Kedu and Kampung chickens. The genotype frequency, gene frequency, heterozygosity, and genetic distances determine the presence of polymorphisms. Analysis of variance to determine the effect of genotype on body weight and shank length. The results showed significant differences (P<0.05) between male and female Red Kedu and Kampung chickens in terms of body morphometric parameters. Sequencing of the PCR product found SNP in base pair 54G>C. GC and GG genotype frequencies of Red Kedu chicken were 0.51 and 0.49, while those of Kampung chicken were 0.32, 0.50, and the CC genotype was 0.18. Allele frequency for G and C of Red Kedu and Kampung chickens were 0.74 vs. 0.26 and 0.66 vs. 0.34, respectively, and the heterozygosity was 38% and 45%, respectively. The genetic distance between Red Kedu and Kampung chickens showed a close kinship of 0.42. Conclusively, the association of the MC4R gene had no significant effect (P>0.05) on body weight and shank length, and therefore, the MC4R gene could not be used as a marker assisted selection.

Key Words: Kampung Chickens, MC4R Gene, Morphometric, Polymorphism, Red Kedu Chickens

INTRODUCTION

Local chickens that spread across Indonesia have positively influenced the diversity of local poultry genetic resources. Efforts to preserve local chickens are carried out by zoning the original habitat (the maintenance system) to maintain the population in a genetic program. Local chickens are common source of animal protein derived from poultry, and local chicken meat is very popular in Indonesia. Local chicken meat production has continued to increase in recent years. The local chicken population in 2021 showed a 0.31% increase, amounting to 306.4 million (DJPKH 2022). In other words, local chicken production increases with

local chicken population and people's purchasing power in Indonesia (Zandos et al. 2021). As a genetic source, local chickens produce meat and eggs and contribute to the ecology and the socio-economic and culture of village communities (Partasasmita et al. 2017).

Free-range chicken, despite its low production, makes a major contribution to the community's economy, increases food security, and potentially provides financial profits and market outreach (Zandos et al. 2021). The advantages of free-range chickens are high adaptability to the new environments, good feed modifiers, and resistance to disease, parasites, and tropical climate stress (Sumantri et al. 2020). The distinctive characteristics of free-range chickens are their non-specific feather colors (yellow, red, black, white, and a mixture of black), medium and small body size, but strong and tough muscle structure which that so that native chickens are also called fighting cocks (Partasasmita et al. 2017).

Identification of Kedu chickens, is very important for sustaining genetic quality improvement program in Indonesia. Descriptively, the qualitative characteristics of Kedu chickens include feather color, shank color, skin color, comb color, and comb shape. These qualitative characteristics can influence the production of kedu chickens (Untari et al., 2013). Kedu chicken can produce up to 124 eggs per year (Telnoni et al. 2021).

One of the efforts to increase the productivity of local chickens is through selections. The first step before selecting local chickens is characterization which, according to Saputra et al. (2021), is the basis for breeding livestock by identifying the morphometrics. The quantitative characteristics of chickens can be measured from its various body parts in order to find the correlations or associations in estimating the body weight. Estimating the value of this correlation is the first important step in the selection (Djegho & Kihe, 2020).

Another way to carry out selections on the measurement of local chickens is using genetic markers by identifying candidate genes coding for economic traits. The economic characteristics of livestock are polygenic traits which are mostly controlled by the number of genes. The diversity of traits is influenced by two factors: genetic and environmental factors. The value of genetic parameters in a population can be used as a guide for improving genetic quality. One of the heritability values or inheritance rates that is often used in local chickens is body weight (Saputra et al. 2021).

The Melanocortin-4 Receptor (MC4R) gene is one of the most influential genes on the growth traits of livestock that have economic value. Melanocortin-4 Receptor gene is responsible for regulating feed intake, energy balance, body weight and bone development of chickens. Melanocortin-4 receptor genes play an important role in regulating food intake, energy expenditure, body weight, metabolism, obesity and energy balance (Zhang et al. 2017). The association between the MC4R gene and body weight has a significant effect on body weight of chickens at 2, 4, and 10 weeks of age. There is a significant relationship between MC4R and livestock body growth because the MC4R gene can affect the activity or function of a protein related to body weight of chickens (Kubota et al. 2019). This study aims to determine differences in morphometric characteristics, identify MC4R gene polymorphisms and their association with body weight and shank length in Kampung and Kedu chickens.

MATERIALS AND METHODS

Animals and experimental design

All procedures in this study were approved by the Research Ethics Commission, Faculty of Veterinary Medicine, Gadjah Mada University (Record no. 047/EC-FKH/Eks./2022). This experimental study used 98 Red Kedu and Kampung chickens, comprising 9 male and 40 female of each strain. Phase 1 research was carried out when the chickens were 20-37 weeks old, kept in battery cages, and fed continuously with rations composed of 40% corn, 30% bran, and 30% laying hen concentrate (18.23% crude protein, 5.83% crude fat, 5.68 % crude fiber, 2825 kcal/kg metabolic energy (ME), 1.09% lysine, 0.35% methionine, 3.60% calcium (Ca), and 0.92% phosphorus (P)). Drinking water was provided ad libitum.

In Phase 2, the materials used were chicken blood samples, EDTA as an anticoagulant, 70% alcohol, DNA isolation kit materials, proteinase K, PCR core kit materials, 2 pairs of primers, TBE 10x buffer, ethanol absolute, fluoroVue gel stain, DNA stain Leadder, 16% acrylamide, 3% glycerol, 10% APS, TEMED, formamide, and aquabidest. Blood collection and measurement were undertaken using caliper, metline, stationery, camera, digital scale, disposable syringe, ice flask, and vacutainer tube. Equipment for DNA isolation consisted of a set of DNA isolation kits, micropipette, centrifuge, waterbath, thermocycler machine, duoplate, collection tubes (white top, yellow top, and blue top). Tools for PCR were PCR machines, 0.2 ml PCR tubes, and a set of PCR kits, while the tools for electrophoresis were measuring cups, gloves, Erlenmeyer flasks, submarine electrophoresis, gel pen glass, comb, and UV visualization.

Body weight and morphometric measurement

Morphometric measurements were carried out on Red Kedu and Kampung chickens aged 37 weeks. Body measurements included body weight using a digital scale, while beak length and wattle length were measured using a caliper. Measurements of chest circumference,

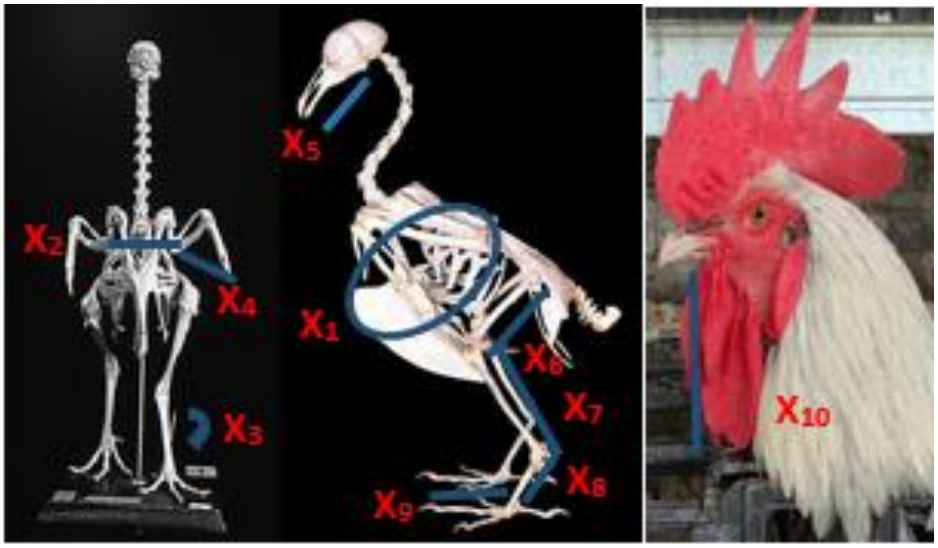

Figure 1. Local chicken body size. Description: X_1 : chest circumference, X_2 : chest width, X_3 : shank circumference, X_4 : wing length, X_5 : beak length, X_6 : femur length, X_7 : tibia length , X_8 : shank length, X_9 : 3rd digiti femur length, and X_{10} : wattle length

wing length, chest width, femur length, tibia length, shank length, shank circumference, and 3rd digit femur were measured using metline. Measurement: X1. The measurement of the chest circumference, namely the circumference from the sternum front to the back (mm); X2. The width of the chest was measured between the anterior and posterior limits on the sternum (mm) (Ismoyowati et al. 2018); X3. Shank circumference is measured by circling the center of the shank (mm) (Sophian et al. 2021); X4. Wing length is measured from the base of the humerus to the tip of the wing bone (mm); X5. Beak length was measured from the base to the tip of the beak (mm); X6. The measurement of the femur was measured from the base to the tip of the femur (mm); X7. Tibia length measurement from the patella to the tip of the tibia (mm); X8. The length of the shank was measured following the length of the tarsometatarsus (mm); X9. The length of the third digit was measured from the base to the tip of the third finger (mm); X10.The length of the wattle from the length of the base of the wattle to the tip of the wattle (mm) (Ismoyowati et al. 2018). Qualitative observations included feather color, feather pattern color, plumage, shank color, comb type, comb color, earlobe color, and eye color

Blood sample and DNA isolation

Three ml of blood were drawn from the subcutaneous area of each chicken (n=98 samples), then put into a vacutainer tube containing EDTA, then transferred 50 µl of it into a 1.5 ml Eppendorf tube and stored in a refrigerator. DNA isolation was carried out following the procedure of FavorPrepTM Plant Genomic DNA Extraction Mini Kit Protocol by Favorgen. DNA isolation was carried out in several stages including cell isolation, cell wall and membrane lysis, DNA extraction, DNA purification, and precipitation.

Primer design and amplification of DNA fragments with PCR

The procedure for preparing the MC4R primer solution in which the PCR primers (MC4R1 F 5'-GAA TTT CAC CCA GCA TCG-3', MC4R1 R 5'-GAG GTT CTT GTT TTG GCT AT-3') match the MC4R DNA sequence (accession number AB012211) (Li & Li 2006) 0.5 µl each, PCR mix 2x powerpoll 6.25 µl, ddH_2O 3.25 µl, and DNA sample 2 µl. The PCR cycle conditions were pre-denaturation at 94˚C for 7 minutes, denaturation at 94˚C for 30 seconds, and annealing at 55˚C for 30 seconds. Elongation occurred when PCR reaction stopped at 72˚C for 45 seconds, then post elongation completed DNA elongation for 10 minutes at 72˚C. The results of the PCR reaction were repeated 35 times to get maximum results. The DNA fragments produced from PCR products were then subjected to electrophoretic tests using acrylamide gel at the SSCP stage.

PCR SSCP and DNA sequencing

At the Single Strand Conformation Polymorphism (SSCP) stage, 12.5 µl PCR product was mixed with 16 µl loading buffer (95% formamide and 5% glycerol) then denatured at 98ºC for 10 minutes. After that, the sample was placed on ice for 5 minutes, then electrophoresed for 17 hours at 10 V/cm in 16% acrylamide gel 3.99 ml, 3% glycerol 0.26 ml, 10x TBE 0.38 ml, aquabidest 2.41 ml, 10% APS 0.075 ml, and TEMED 0.01 ml. The silver stain method was developed to show the bands. The

individual band patterns of the PCR-SSCP were determined under UV visualization (Li & Li 2006).

PCR sequencing was carried out by the Integrated Research and Testing Laboratory at Gadjah Mada University, resulting in nucleotide sequences. The electropherogram graph is marked by different colors of nitrogenous bases, namely green for A nucleotides (Adenine), black for G nucleotides (Guanine), blue for C nucleotides (Cytosine), and red for T nucleotides (Timine) (Ismoyowati et al. 2018). The product sequenced in the MC4R gene sample was read using MEGA 11 Software and the BioEdit program to see the Single Nucleotide Polymorphism (SNP) genotyping by aligning the product sequence according to GenBank access number AB012211.

Statistical analysis

The t-test was used to determine differences in phenotypic characteristics and to compare morphometrics between Red Kedu and Kampung chickens. Data analysis used the t-test (Chernick & Friis, 2003), with the following formula:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{\frac{(N_1 - 1)Sd_1^2 + (N_2 - 1)Sd_2^2}{N_1 + N_2 - 2}} \sqrt{\frac{N_1 + N_2}{N_1 \, x \, N_2}}}$$

where, $\bar{Y}_1$= Mean phenotypic characteristics of Kampung chickens; $\bar{Y}_2$= Mean phenotypic characteristics of Red Kedu chickens; Sd_1= Kampung chickens' variance; Sd_2= Red Kedu chickens' variance; N_1= number of samples of Kampung chickens; N_2= number of samples of Red Kedu chickens.

Calculation of allele frequency, genotype Frequency, and heterozygosity value determined using (Hamillton, 2021). Allele frequencies are calculated using formula (1):

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

where xi= allele frequency ii, nii= Number of individuals with genotype ii, nij= Number of samples with genotype ij, N= Number of individual samples.

Genotype frequencies are calculated using formula (2):

$$xij = \frac{nii}{N} x100\%$$

where xii= frequency of homozygous genotypes (ii), xij= frequency of heterozygous genotypes (ij), nii= number of individuals of genotype ii, nij= number of individuals of genotype ij, N= number of individual samples.

Heterozygosity values in this study were calculated using formula (3):

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

where He = heterozygosity, n = number of alleles, i = alleles, Pi = allele frequency i.

Genetic distance was calculated using formula (4):

$$D = -ln[Gxy/\sqrt{GxGy}]$$

$$Gx = \sum (pi)^2$$

$$Gy = \sum (qi)^2$$

$$Gxy = \sum piqi$$

Kinship relationship determined the kinship between Red Kedu and Kampung chickens and analyzed using MEGA11 software based on their genetic distance. The correlation analysis was carried out to determine the kinship between genotypes and body weight and shank length of local chickens. The correlation value is calculated using the mathematical model of (Chernick & Friis, 2003):

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

where, r = correlation coefficient; X = genotype; Y = body weight and shank length of local chickens; n = the number of repetitions.

Analysis of variance was carried out to determine the effect of genotype on body weight and shank length. Replications used individual chickens identified by genotype and morphometrics, thus producing unequal replicates. If the genotype had a significant effect on the measured variables, the MC4R gene had an association with body weight and shank length.

RESULTS AND DISCUSSION

Qualitative characteristics of Red and Kampung Kedu chickens

Qualitative characteristics of local chickens included feather color, feather pattern color, feather glimmer, shank color, comb type, comb color, earlobe color, and eye color (see Table 1). Feather color is dominated by various colors and influenced by qi control genes or genotype Ii (Crawford 1990), while white fur marks the absence of melanin pigment (Roulin & Ducrest 2013). There are two main types of melanin responsible for feather pigments: eblack melanin produced by umelanin, red melanin produced by phaeomelanin, and white coloration due to some reflectivity on their surface. While most colors of bird feathers are produced by the presence of pigments (Stevens 1991), white feathers are very rare but very expensive in tropical countries, thus benefiting farmers (Brown et al. 2017).

This study identified that the dominant feather patterns were Columbian and black (Table 1). Three types of feather patterns of birds are Columbian, black, and wild, with distinctive color border on the head, chest, wings, and tail (Ismoyowati et al. 2018). Columbian (Co), as well as Mahogany (Mh) and Columbian Dark Brown (Db), belong to locus E which limits the distribution of eumelanin and affects the color of the primary feather. These three alleles will influence the color of the feathers on the back, wings, femur, and tail. The black gene (E) has black fur all over the body. The wild color gene (e+) has a black stripe on the back and the Colombian (e) has black feathers on the neck, wings, and tail. (Dako *et al*., 2020).

The color of the shank in this study was dominated by black or black and white. While pigments affect the diversity of shank colors in local chickens, the other contributing factors to varied shank color are the interaction of the main modifier genes (Salces et al. 2015), and differences between local free-range chickens in different regions, diverse pigments, and the genes responsible for determining color (Odah et al., 2019). Melanin pigment affects shank color and is found in the dermis and epidermis layers, so the absence of melanin in both layers results in white shank.

Various types of combs in local chickens are attributed to genetic factors. The comb types in this study – ranked from the most to the least dominant – were single, rose, and pea types. According to Brown et al. (2017), the low frequency of pea comb is due to irrelevance to the tropical climate because the adaptive nature of cold climates can reduce body heat. Odah et al. (2019), stated that chickens with a single comb type were significantly more resistant to the effects of heat than their female counterparts.

The colours of comb and earlobe of local chickens were dominated by red and blackish red, while eye color was dominated by orange and brown. When the chicken's eyes hatch, they will be dark in color and will be visible when the chicken is sexually mature, where the pigments melanin and carotene will be fully expressed. (Riyanti *et al*., 2023). The diversity of eye colors is caused by genes that affect blood supply, increased melanin pigment, and environmental effects (Orounladji et al. 2021). The characteristics of eye color depend on carotenoid pigments and blood circulation in the eye (Odah et al. 2019). In addition, eye color correlates with shank color, and can be modified by feather color genes (Salces et al. 2015).

Morphometric characteristics of Red Kedu and Kampung chickens

The results of the t-test showed different performance between male (Table 2) and female Red Kedu and Kampung chickens (Table 3) in terms of body weight, chest circumference, shank length, beak length, and femur length in male chickens, while body weight, wattle length, chest circumference, chest width, shank length, and third digit length in hens ($P<0.05$). Meanwhile, no significant differences ($P>0.05$) were observed on the size of the shank circumference, tibia length, wing length, beak length, and femur length in females, as well as wattle length, chest width, and third digit length in males. The average body weight of male Red Kedu was higher than that of Kampung chicken, namely 2.34 ± 0.36 and 2.2 ± 0.20 kg (Table 2). While varied body weights are due to genetic factors and uniform environmental influences (Henrik et al. 2018), low body weight is the result of poor management and diverse genetic composition (According to Odah et al. 2019). Male chickens had higher body weight than their female counterparts due to different hormones contained in body size. Similarly, gender affects rooster's body weight through androgenic hormones (Febrianto et al. 2018). Dimorphism in chickens is regulated by different genes and hormones (Salces et al. 2015). Chicken body weight is a common morphometric component to analyze species diversity in local chickens (Sophian et al. 2021). In addition to genetic factors, weight gain in chickens is strongly influenced by the quality of the feed given, where a more balanced nutritional component can improve the performance of poultry digestive organs (Utama et al. 2020). The rate of bone growth can be influenced by the protein consumed (Lukmanudin et al., 2018). Increasing the maximum growth rate can be supported by providing efficient feed and adequate nutrition (Hanafi et al., 2021).

The results of the t-test on chest circumference, chest width, femur length, shank length, and third digit length had significant differences ($P<0.05$) in body weight. According to Liyanage et al. (2015), chest circumference and shank length are the best predictors to determine live weight. The shank bone can be measured by the length of the shank and the circumference of the shank. Shank bone has the ability to support the body of livestock. A long shank size will affect body weight more significantly than a shorter shank size (Debes et al., 2015).

The mean chest circumferences of male and female Red Kedu chickens were not significantly different from that of Kampung chickens. According to Putranto et al. (2018), it can be assumed that local chickens are local species that potentially runs dual-purpose as the producers of meat and eggs. Meanwhile, differences in morphometric measurements are attributed to genetic and environmental factors (Rofii et al. 2018).

Table 3 shows that the coefficient of variation of shank length is higher in male than female because male shank is longer than the female. Intensive maintenance can result in a short shank and relatively large body. Meanwhile, the length of femur, length of tibia, and length of the third digit can be used to estimate the

Table 1. Differences in the qualitative characteristics of Red Kedu and Kampung chickens

Qualitative characteristics	Red Kedu chicken		Kampung chicken	
	Male	Female	Male	Female
Feather Colour	Coloured	Coloured	Coloured	Colour and White
Feather Pattern Colour	Black and Columbian	Black and Columbian	Columbian	Columbian and Wild
Feathers	Silver	Silver and Gold	Silver and Gold	Silver and Gold
Shanks colour	Black	Black and HP	Black and HP	White, Yellow, Black, and HP
Comb type	Single	Pea, Single, and Rose	Singles and Rose	Pea, Single, and Rose
Jigger colour	Red	Red and MH	Red	Red
Earlobe Colour	Red	Red and MH	Red and Black	Red and Black
Eye colour	Chocolate	Orange and Chocolate	Orange and Chocolate	Orange and Chocolate

HP= Black and white, MH= Red and black

Table 2. Mean, standard deviation, and coefficient of diversity of body morphometric characteristics of Red Kedu and Kampung male chickens

Parameter	Red Kedu chicken	Kampung chicken	CV (%)	
			Red Kedu chicken	Kampung chicken
Beak length (mm)	33.58 ± 2.87[a]	38.26 ± 3.57[b]	8.56	9.33
Wattle length (mm)	45.82 ± 6.81	34.95 ± 13.20	14.86	37.76
Chest circumference (mm)	350.56 ± 25.30[b]	315.56 ± 20.68[a]	7.22	6.55
Chest width (mm)	66.84 ± 8.94	67.14 ± 10.87	13.37	16.19
Wing length (mm)	206.11 ± 16.35	200.00 ± 29.15	7.93	14.58
Tibia length (mm)	147.78 ± 25.87	155.56 ± 10.14	17.51	6.52
Femur length (mm)	103.89 ± 9.28[a]	116.67 ± 10.00[b]	8.93	8.57
Shank length (mm)	105.56 ± 7.27[b]	98.89 ± 7.82[a]	6.88	7.91
Shank circumference (mm)	49.44 ± 1.67	51.10 ± 5.46	3.37	10.69
3rd Digiti length (mm)	60.56 ± 6.35	65.00 ± 6.12	10.48	9.42
Body weight (kg)	2.34 ± 0.36[b]	2.20 ± 0.20[a]	15.26	8.95

CV= Coefficient of variation. Different superscripts in the same line show significantly different

suitability of body shape and positively correlated with body weight (Febrianto et al. 2018). According to Abinawanto et al. (2021), the sternum length, shank circumference, shank length, and the third digit length have significant differences with body weight and have a higher diversity than other characters.

The body weights of male Red Kedu and Kampung chickens were significantly different (see Table 2). Red Kedu chickens have a higher body weight than Kampung chickens, even though the two chicken lines have the same origin. Red Kedu and Kampung chickens descend from the same ancestor, namely the Red Junglefowl (Gallus gallus) (Sulandari et al. 2008)

Based on the results of this study, chicken body size affected bone length. Febrianto et al. (2018) stated that differences in body size are caused by genetic, environmental, and feed factors. In addition, maintenance factors, treatment, and health conditions also contribute to differences in body size (Abinawanto et al. 2021). Phenotypic diversity is caused by differences in body shape and size through morphometric measurements (Febrianto et al. 2018). High diversity and geographic differentiation affect the phenotypic and morphometric characteristics of chickens (Otecko et al. 2019). The highest coefficient of diversity on Red Kedu chickens was found in the tibia length of males (17.51%), and the wattle length of females (29.61%). In both male and female Kampung chickens, the highest coefficient was found in the length of the wattle (Table 2). The interaction between genes is one of the factors for the emergence of new phenotypes through body weight and morphometric characteristics (Henrik et al. 2018).

Table 3. Mean, standard deviation, and coefficient of diversity of body morphometric characteristics of Red Kedu and Kampung female chickens

Parameter	Red Kedu chicken	Kampung chicken	CV (%)	
			Red Kedu chicken	Kampung chicken
Beak length (mm)	33.05 ± 2.42	34.18 ± 3.27	7.31	9.56
Wattle length (mm)	17.51 ± 5.19[b]	14.38 ± 2.87[a]	29.61	19.97
Chest circumference (mm)	320.98 ± 17.17[b]	297.78 ± 25.19[a]	5.35	8.46
Chest width (mm)	61.46 ± 6.65[b]	55.04 ± 6.20[a]	10.82	11.26
Wing length (mm)	168.03 ± 17.10	175.08 ± 20.38	10.18	11.64
Tibia length (mm)	126.70 ± 9.12	127.15 ± 12.72	7.20	10.01
Femur length (mm)	106.25 ± 15.47	104.30 ± 14.74	14.56	14.13
Shank length (mm)	92.63 ± 9.47[b]	85.88 ± 8.84[a]	10.23	10.29
Shank circumference (mm	43.95 ± 5.22	42.33 ± 4.65	11.88	11.00
3rd Digiti length (mm)	56.40 ± 6.03[a]	58.43 ± 4.57[b]	10.70	7.83
Body weight (kg)	1.93 ± 0.21[b]	1.61 ± 0.24[a]	10.66	15.21

CV= Coefficient of variation. Different superscripts in the same line show significantly different

MC4R gene polymorphism PCR-SSCP results

The PCR product obtained a target of 221 bp as expected, as in GenBank access number AB012211 (Figure 2). The successful PCR was evident from a clear band. PCR results that have a constant concentration and purity are considered as good because they are able to amplify and produce several products even in only one cycle.

The PCR-SSCP results revealed that the MC4R gene was polymorphic, meaning that 3 genotypes were found, namely GG, GC, and CC (Figure 3), which indicated the presence of a single nucleotide polymorphism (SNP) located on CDS (Coding DNA Sequens) at the 54th base sequence from G>C (Figure 4). According to Li & Li (2006), this base mutation leads to changes in Glutamine (Gln) to Histidine (His). Changes in protein configuration result in differences of biological functions between wild and mutants. The GG and CC genotypes are homozygous because individuals inherit alleles from their parents. The GC genotype is called the heterozygous which occurs when a pair of alleles at a locus are not identical, due to the influence of incomplete dominance or additive genes. The results of sequencing the samples obtained 36 GG genotypes, 30 GC genotypes, and 7 CC genotypes, and the rest did not show any genotype. This was because the DNA band did not appear nor smear.

Genetic diversity based on the MC4R gene locus

The results obtained on the alignment of the nucleotide base sequences indicated a mutation at the 54th base, namely G>C. The sequencing results showed a polymorphism due to a mutation of guanine (G) to cytosine (C) at base 54 with a length of 221 bp of PCR product. The values of genotype frequency, gene frequency and heterozygosity of the MC4R gene in Red Kedu and Kampung chickens are presented in Table 5.

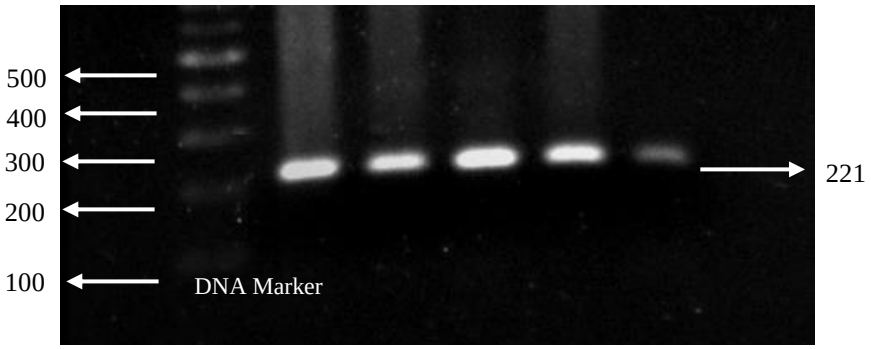

Figure 2. Visualization of 221 bp target DNA PCR products with a marker size of 100-3000 bp

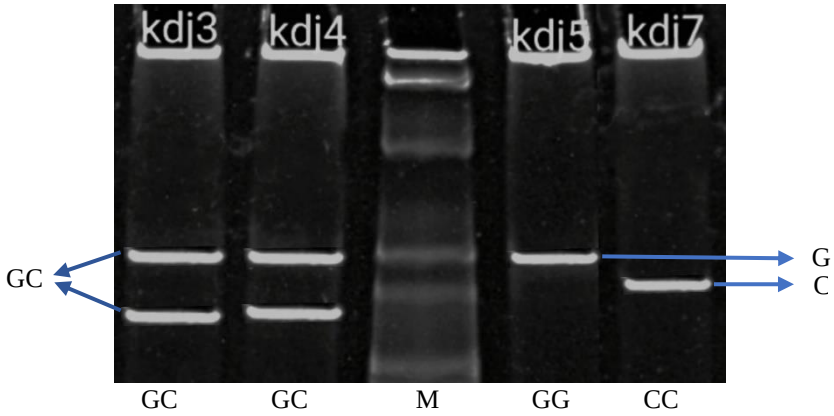

Figure 3. Visualization of the PCR-SSCP results of the MC4R gene with M: marker 100-3000 bp.

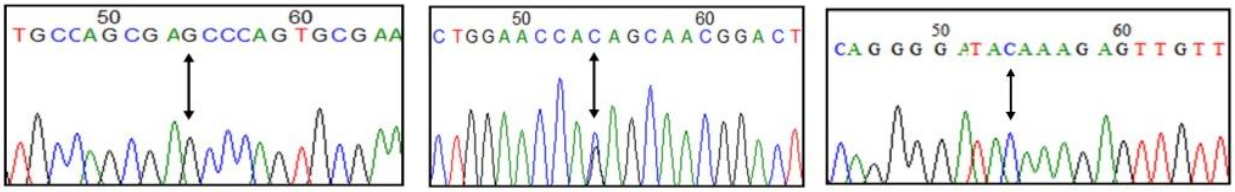

Figure 4. Electropherogram of the MC4R SNP at 54 bp (GG, GC, and CC genotype)

Table 5. Genotype frequency, gene frequency, and heterozygosity in the MC4R gene

Sample	Number of samples (N)	Genotype frequency			Gene frequency		Heterozygosity
		GC (N)	GG (N)	CC (N)	Allele G	Allele C	
Red Kedu chicken	35	0.51 (18)	0.49 (17)	0	0.74	0.26	0.38
Kampung chicken	38	0.32 (12)	0.50 (19)	0.18 (7)	0.66	0.34	0.45

Table 6. Genetic distance in several lines

Strain	*Gallus gallus*	Red Kedu chicken	Kampung chicken
Gallus gallus			
Red Kedu Chicken	0.71	-	
Kampung chicken	0.41	0.42	-

The calculation resulted in three genotypes, namely GC, GG, and CC with respective values of 0.51 and 0.49 (Red Kedu chicken) and 0.32, 0.50, and 0.18 (Kampung chicken). The frequencies of the G and C genes of Red Kedu chicken were 0.74 and 0.26, while in Kampung chicken the values were 0.66 and 0.34, respectively. According to Harahap et al., (2017), the allele frequency value ranges between 0.45-0.63 indicating polymorphism. A population is polymorphic if it has multiple alleles and the frequency value is below 0.99. At the same, it is monomorphic if the frequency value for one of the alleles reaches 1.00 at the same mutation point position.

Based on Table 2, the heterozygosity values for Red Kedu and Kampung chickens were 0.38 and 0.45. According to Serrote et al., (2020), The heterozygosity value ranges from 0 to 1 (if the number of alleles is high with the same frequency value). he genetic variation increases with the heterozygosity number. The heterozygosity in this study (0.50) was still relatively low, thus affecting a low level of genetic diversity (Tamzil & Indarsih 2020). According to Henrik et al. (2018), a low level of genetic diversity in a population indicates a very small possibility of genetic mutations.

Genetic distance and kinship of Red Kedu and Kampung chicken

The results of calculating genetic distance using MEGA11 software showed that the Red Junglefowl (*Gallus gallus*) had a close kinship with Kampung chickens with a genetic distance value of 0.41 (Table 6).

If the value of genetic distance is closer to 0.00, the kinship relationship between lines is closer. The genetic distance between Kedu and Kampung chickens is close (0.42) compared to that between the Red Junglefowl and Red Kedu chickens (0.71). It is in accordance with Abinawanto et al. (2021) that the genetic distance between Kampung chicken and Red Junglefowl is closer than with Green Junglefowl. Genetic distance is the level of gene diversity in a population, which is measured based on a numerical score and calculated based on its genetic frequency. Different genetic distances can be caused by differences in genes and analytical methods (Febrianto et al. 2018). Genetic distance values support the grouping and closeness of each individual in a population, between groups, and nucleotide differences (Abinawanto et al. 2021).

Genetic distance as the basis for the reconstruction of a phylogenic tree. Based on the results of the phylogenic tree, it was shown that the Red Junglefowl and Red Kedu chicken lines had a closer relationship than the Kedu chicken (Figure 6).

The phylogenetic relationship in the phylogenic tree shows that Red Junglefowl (*Gallus gallus*) has the same cluster as the Kampung chicken. That is, the kinship between Red Junglefowl (*Gallus gallus*) and Kampung chicken is very close because its branch length is 0.21. When compared with the Red Kedu chicken, it looks very far from the Red Junglefowl (*Gallus gallus*) with a branch length of 0.28. This may be due to the high genetic variation in the Red Junglefowl and Kampung chickens. According to Blanchette et al. (2012), the close distance in branch length between clusters is probably caused by the absence of genetic mutations. According to Abebe et al. (2015), uniform grouping in one cluster indicates the genome fraction in an individual has the same ancestor.

The kinship between Red Kedu and Kampung chickens has a genetic distance value of 0.42 or is closely related. This can be expected because the Red Kedu chicken has undergone a process of genetic quality improvement. According to Febrianto et al. (2018), the closeness of kinship is due to a specific genetic composition for interactions in environmental conditions. It can be concluded that any effort made to improve genetic quality will affect kinship.

Association of the MC4R gene with body weight and shank length

The relationship between the MC4R gene on body weight and shank length was calculated using analysis of variance. The calculation results showed that the genotype had no significant effect on body weight and shank length (P>0.05). This is probably because the chickens in this study were not selected and the MC4R gene did not change the configuration of the protein base. Therefore, the MC4R gene cannot be used to detect body weight and shank length of local chickens. This is consistent with the findings of Molee et al. (2018) that the genotypes were not significantly different across all measured characteristics because the chickens came from relatively unselected populations and each genotype trait was largely varied; therefore, the observed differences were not significant. Based on the correlation value between the genotype and body weight of 0.171, which means the correlation value is low. According to Asmara et al. (2020), the value of the correlation coefficient is between -1 and +1, the +1 value indicates a perfect positive correlation, while the -1 value indicates a perfect negative correlation. Based on the analysis results, the correlation value between the genotype and the shank length is 0.043, indicating a very low correlation..

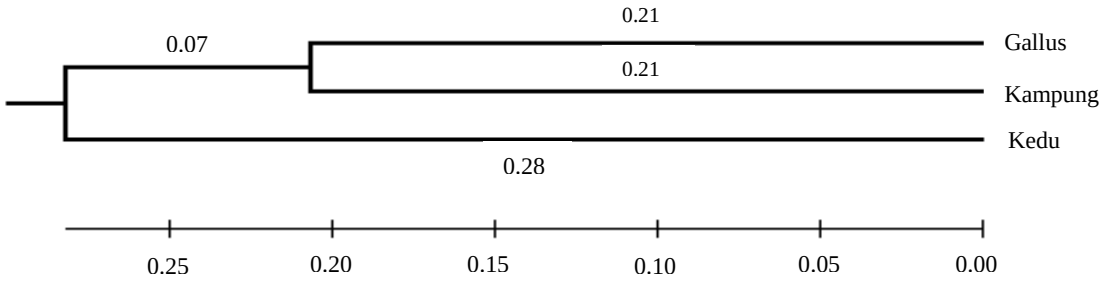

Figure 5. Phylogenic tree using MEGA11

Table 7. Genotype association on body weight and shank length of local chickens

Genotype	Number of samples (N)	Body weight (g)	Shank length (mm)
GG	36	1791.67 ± 343.41	90.28 ± 14.24
GC	30	1736.67 ± 291.53	90.03 ± 10.46
CC	7	1600.00 ± 152.75	88.00 ± 9.33

According to (Hastuti et al., 2021), shank length has the lowest coefficient compared to body weight. A strong relationship between body weight and shank length could possibly be used as a selection criterion, but the genes must be regulated by the same gene action. The results of this study were different from those of Li & Li (2006), which indicated the possibility of identifying the MC4R gene as a marker for selecting body weight and carcass weight. This can be seen in the effect of the MC4R gene on poultry, which contributed 12.02% and 26.97% to body weight and shank length, respectively.

CONCLUSION

There were significant differences in male and female Red Kedu and Kampung chickens in terms of body weight, chest circumference, shank length. The MC4R gene in Red Kedu and Kampung chickens was polymorphic, and the kinship relationship between the two strains was very close with a genetic distance value of 0.42. The MC4R gene was not associated with body weight and the shank length of Red Kedu and Kampung chickens, and therefore, not applicable as a Marker Assisted Selection.

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