Analysis Variation of *Growth Hormone Receptor* (GHR) on the Z Chromosome in Maron Chicken

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Abstract. The growth hormone receptor (GHR) gene plays an important role in regulating growth as a receptor for the growth hormone (GH) gene. This study aims to analyze the variation of the GHR|*Eco72I* gene and its association with body size in the Maron Chicken elders. Ninety DNA samples were extracted from the blood of Kedu, Arab, and Lingnan Chickens from BBPTT Maron Chicken, Temanggung, Central Java. Amplification of the GHR|Eco72I gene in intron 5 using the PCR-RFLP method with a product size of 326 bp. The genotype variations found in the GHR|*Eco72I* gene were AA and AG with two types of alleles: A and G. The analysis showed that the AA genotype frequency was the highest in all populations. The highest allele frequency was the A allele (0.99), and the lowest was the G allele (0.01). The populations of Kedu, Arab, and Lingnan Chickens were in Hardy-Weinberg equilibrium. The observed heterozygosity value (Ho) in Kedu Chicken is higher than the expected heterozygosity value (He). The GHR|*Eco72I* polymorphism is not significantly associated with body weight, chest length, chest width, wingspan, shank length, or shank diameter. The study concludes that the GHR|*Eco72I* gene is polymorphic in the Kedu Chicken population but monomorphic in Arab and Lingnan Chickens.

Keywords: association, genetic variation, GHR gene, native chicken, polymorphism

1 Introduction

Local chickens have the potential to be developed into superior lines to produces animal protein needs for the community. However, lack of genetic improvement in local

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chicken still have a slow growth rate [1]. Applying good breeding practices to develop genetic in breeding farm of local chicken is needed to create good genetic of local chicken. Crossbreeding is one solution to improve genetic quality by mating distantly related groups to obtain a positive heterosis effect [2]. One of the efforts to improve the genetic quality of local chickens is the crossbreeding of Lingnan, Arab, and Kedu chickens to develop superior Maron Chicken lines as local commercial meat and egg chickens. The development of superior commercial chicken lines must be based on local chicken lines identified comprehensively, including morphometry, growth rates, productivity, and genetic information [3]. Gene variations can be identified as a basis for selection to improve genetic quality [4]. The molecular selection method or Marker Assisted Selection (MAS) is a process that is considered effective and has a high success rate in obtaining traits that are considered important. The genetic diversity of livestock can be measured using molecular markers that utilize trait genes. Molecular markers explain genetic variability within and between populations [5].

The *Growth Hormone Receptor* (GHR) gene is a somatotropin gene that is central to regulating growth and development. It functions as a receptor for growth hormone (GH), a peptide hormone secreted by the pituitary gland, and modulates numerous physiological processes [6]. Variations in the somatotropin gene can serve as candidates for evaluating chicken growth and development traits. Previous studies showed that a single nucleotide polymorphism (SNP) of the GHR gene had a significant effect on carcass component growth traits [7]. Growth traits and body composition have a significant relationship with meat quality characteristics, especially in fat deposition and muscle density and size [8]. The GHR gene was chosen because it is known to affect one of the economic traits. The GHR gene is located on the Z chromosome and influences growth traits that can be used as a marker gene [9]. The GHR gene is known to be associated with the growth hormone (GH) gene, which plays an important role in chicken growth performance. The GHR gene marker g.565G>A also known as GHR|*Eco72I* exhibits a correlation with enhanced growth performance in chickens. The GHR|*Eco72I* is located in intron 5 GHR gene. The GHR|Eco72I was found to be significantly linked to adiposity and muscle fiber attributes in native Chinese chicken populations [8]. Furthermore, this variation is correlated with growth-related traits, including live weight, carcass weight, breast weight, thigh weight, breast muscle weight, and drumstick muscle weight in Kampung chickens crossbred with Cobb broilers [7]. These findings show that the GHR gene is an important candidate as a trait in growth traits [7]. Diversity analysis can be the basis for selecting superior parents to obtain uniform production in the next generation. This study aims to determine the genetic diversity of the GHR|*Eco72I* GHR gene in the Maron Chicken elders, namely Lingnan, Arab, and Kedu chickens.

2 Materials and Methods

2.1 Materials

This research used 90 DNA samples from Maron chicken elders, namely Lingnan (age: 6 months; n: 30 samples), Arab (age: 10 months, n: 30 samples), and Kedu chicken (age: 8 months, n: 30 samples). All samples were collected from the Breeding Center for Non-Ruminant Animals (BPBTNR), *Satker Ayam Maron*, Temanggung, Central Jawa, Indonesia. This research followed the ethical ethics for husbandry and animal health, Low of Republic Indonesia Number 18, 2009, and was supervised by a veterinarian.

2.2 Methods

Blood collection. Blood sample collection was performed using a 3ml syringe with a 21G needle, following protocols in previous research [10]. Blood was taken from the branchial vein and then put into a 3ml vacuum tube containing EDTA anticoagulant. Homogenization was carried out immediately after the blood was put into the vacuum tube and stored temporarily at 4°C, then stored at -18°C for long-term storage.

DNA extraction and genotyping of GHR gene. DNA extraction was performed at the Animal Production Laboratory, Animal Science Study Program, Faculty of Animal Science, Universitas Sebelas Maret. A total of 50 µl of blood sample was used for DNA extraction following Wizard® Genomic DNA Purification Kit (Promega, USA) protocol adapted to poultry blood, in accordance with previous research [10]. The extraction results were then visualized using 1.5% agarose with EtBr dye. Genotyping of the GHR gene was carried out using the Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) method and genotyping by sequencing with sanger sequencing method for confirmation. The *Eco72I* enzyme was used as the restriction enzyme. The components of the GHR gene fragment amplification are presented in Table 1.

Table 1. List of primers and enzymes for GHR gene genotyping.

Gene	Primer (5' – 3')	Ta* (°C)	PCR product (bp)	Enzyme	Reference
GHR	F: TCTGCAGAGTCGGGATATTTAGCA R: ACTCTCCATCAGAATTTATCCCG	58.0	326	*Eco72I*	[7]
GAPDH	F: GATACAGCAACCGTGTTGTG R: GAGGAAGAAATTGGAGGAGT	51.4	198	-	[10]

* Ta: annealing temperature (°C)

A total of 10 µl Promega GoTaq® Green Master Mix (Promega, USA) was mixed with 7 µl Nuclease Free Water (Promega, USA) in an Axygen® microtube 200 µl (Corning, USA). Then, 1 µl of forward and reverse primers were added to the mixture. A total of 1 µl of the extraction result was added as a DNA template. The mixture was then inserted into the SelectCycler™ II Thermal Cycler machine (Select BioProduct, Taiwan). The PCR process was applied with a configuration of 1 pre-denaturation cycle for 5 minutes at a temperature of 95 °C. Furthermore, 35 cycles with denaturation stages at a temperature of 95 °C for 30 seconds, annealing at a temperature according to Table 1 for 30 seconds extension at a temperature of 72 °C for 30 seconds and finally, 1 final extension cycle at a temperature of 72 °C for 10 minutes. Positive and Negative controls were added to ensure that the amplification process was running well and there was no contamination. The positive control used GAPDH primer with Ta configuration, according to Table 1. Negative control was made by replacing the DNA template with 1 µl Nuclease Free Water. The amplification results were electrophoresed with a voltage of 135V for 25 minutes with 2% agarose with EtBr dye. The electrophoresis results were visualized with a UV Transilluminator. The sequencing process was then carried out. Sequencing was carried out using the Sanger Sequencing method. A total of 30 µl of DNA amplification product was used in the sequencing process. Sanger Sequencing was carried out at PT. Genetika Science, Jakarta. Digestion was carried out by adding 9 µl Nuclease Free Water, 1 µl Tango Buffer (Thermo, USA), 5U of *Eco72I* enzyme (Thermo, USA) and 6 µl of amplification product into a 200 µl microtube. The mixture was then incubated at 37°C in a Thermal Dry Block (Clever Scientific, UK). The digestion results were then electrophoresed at 135V for 25 minutes with 2% agarose and EtBr dye. The electrophoresis results were visualized using a UV Transilluminator. This visualization was utilized for genotyping GHR|*Eco72I* by analyzing the digestion pattern of the PCR products in each lane, where the AA genotype exhibited a single band at 326 bp, the AG genotype showed three bands at 326 bp, 212 bp, and 114 bp, and the GG genotype displayed two bands at 212 bp and 114 bp.

Data analysis. Sequencing results and genotype determination at the GHR|*Eco72I* marker were analyzed using Unipro UGENE v.48 by examining the base-calling outputs, where the AA genotype was identified by an A base-call, the AG genotype by the presence of overlapping A and G peaks (double peak), and the GG genotype by a G base-call at the marker site [11]. Furthermore, the R program with the *Genetics* and *Adegenet* packages was used to analyze genotype frequencies, allele frequencies, and heterozygosity [12–14]. The association of the GHR|*Eco72I* genotype with the phenotype was analyzed using the t-test method with the R program [12].

3 Results and Discussion

3.1 Result of GHR gene amplification

Based on the visualization of the electrophoresis results of the PCR DNA template products, blood samples from Kedu, Lingnan and Arab chickens were successfully amplified. Figure 1 shows a visualization of the DNA amplification stage with an annealing temperature of 58°C using a 2% agarose gel to produce a product of 326 bp.

Amplification produced the same product size as in previous research conducted by [7] with a length of 326 bp. The positive control roles to prove that the primer used amplifies the selected target, while the negative control roles to determine whether there is contamination in the reagent mix used.

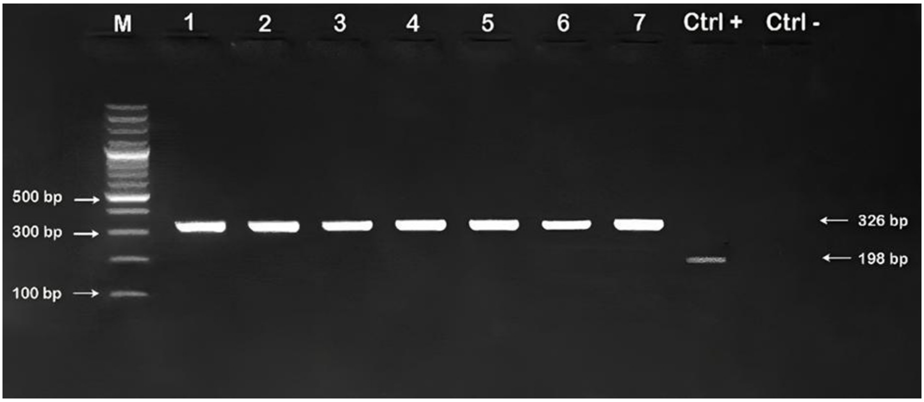

Fig. 1. Visualization of GHR gene amplification; M: Marker 100bp; 1-7: PCR products; Ctrl +: positive control; Ctrl -: negative control

3.2 Confirmation of PCR targets by sequencing method

This study performed the sequencing method to confirm the DNA sequence resulting from PCR by comparing the sequencing data with the sequence in NCBI's GenBank database. Based on the BLAST results, the PCR sample's sequence similarity was 99.56% with the Gallus gallus partial GHR gene for growth hormone receptor, exons 5 and 6, with accession number AJ506750.1. The BLAST results are the same as those of previous research by [15], which refers to the sequence reference accession number AJ506750.1 (Table 2).

Table 2. Comparation of PCR sequencing products with NCBI Genbank.

PCR Samples	Sim. (%)	Accession Number	Description
Kedu	99.56	AJ506750.1	*Gallus gallus partial GHR gene for growth hormone receptor, exons 5 and 6*
Arab	99.56		
Lingnan	99.56		

* Sim. = similiarity

3.3 Genotyping GHR|*Eco72I*

The PCR-RFLP method was used to perform GHR|*Eco72I* genotyping and continue PCR product analysis. The GHR intron 5 gene fragment was cut using the *Eco72I* restriction enzyme, which cuts at the 5’-CAC|GTG-3’ site. The G allele has an *Eco72I* restriction point, so it will be cut while the A allele does not, so there will be a difference in the size of the DNA fragment. The restriction process produces 2 genotype variations, namely AA and AG. The AA genotype produces a DNA band of 326 bp, while the AG genotype produces DNA bands of 326 bp, 212 bp, and 114 bp, as shown in Figure 2. This result similar with previous research conducted by [7].

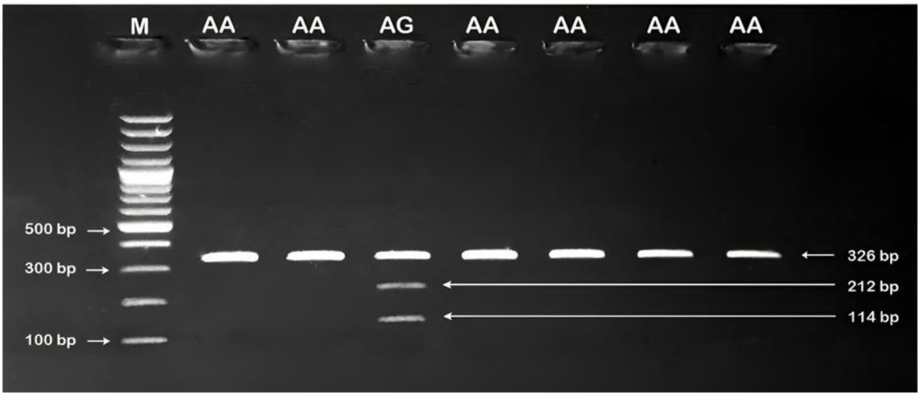

Fig. 2. Visualization of GHR|*Eco72I* digestion; M: Marker 100bp; AA: Genotype of AA; AG: Genotype of AG

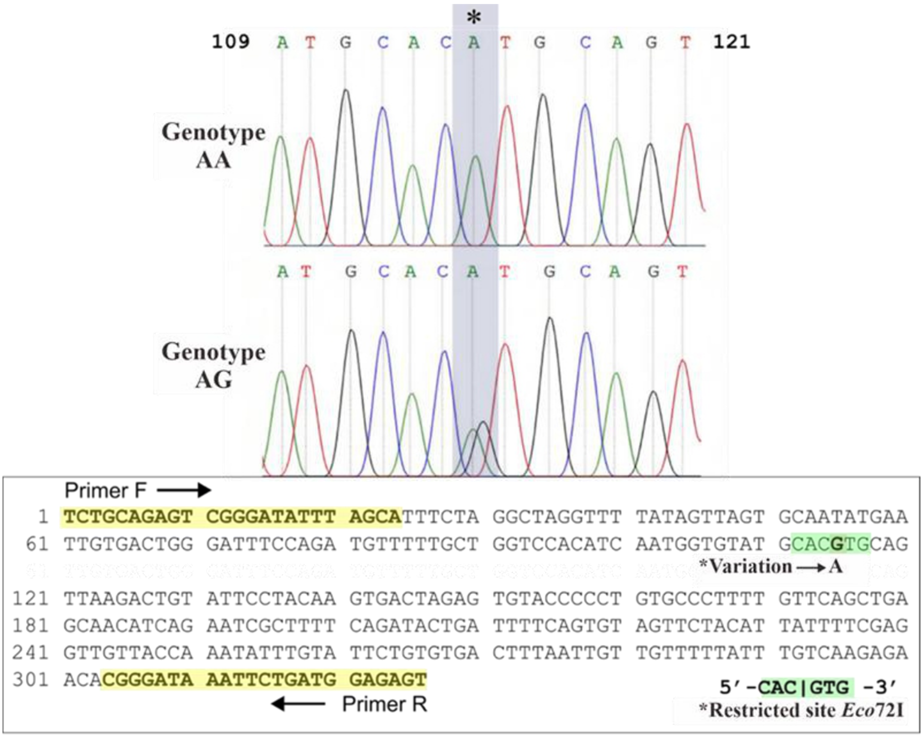

Fig. 3. Chromatogram of GHR fragment sequence results; (*): the point location of mutations A>G

The sequencing analysis results are shown in the chromatogram in Figure 3. A transition substitution mutation occurred in one of the nucleotide bases at the SNP G565A GHR point, namely a change in the guanine (G) base with adenine (A). Transition substitution is a change in the same nitrogen base pair between a pair of purine bases (adenine or guanine) with purine or a pair of pyrimidine bases (thymine or cytosine) with pyrimidine [16]. The occurrence of this mutation or variation produces the A and G allele types. The AA genotype has one peak in calling the A base due to the combination of identical alleles. In contrast, the AG genotype has two peaks in calling the A and G bases because of a combination of A and G alleles. This variant is located on chromosome Z within the GHR gene, designated as NC_052572.1:g.14083690G>A at the genomic level and ENSGALT00010035078.1:c.356-521G>A at the intronic level. It is registered in dbSNP and the European Variation Archive (EVA) under the accession number rs315964687 [17].

3.4 Hardy-Weinberg equilibrium analysis, Allele and Genotype frequencies, GHR|*Eco72I* gene

The diversity of the GHR gene was analyzed based on allele frequency, genotype frequency, heterozygosity and Hardy-Weinberg equilibrium in each population of Kedu, Arab, and Lingnan chicken, presented in Table 3. Genetic variation can be explained by allele frequency, so allele frequency is an essential parameter in viewing genetic changes in a population. Alleles at the locus indicate the DNA sequence, while genotype is when two alleles are in the same locus [16]. Identification of GHR gene variation shows two allele variations, namely allele A and allele G. Based on the results of genotype variation, it is only found in Kedu Chicken, namely genotype AA and AG, while in Arab and Lingnan chickens there is only genotype AA. Previous research by [9] found only genotype AA and AG in native chickens, with the highest frequency of genotype AA. The allele frequency values in all populations show the highest allele frequency, namely allele A and the lowest frequency of allele G. In Kedu chickens, there are allele A (0.93) and allele G (0.03), while in Arab and Lingnan chickens, there is only allele A. In a population, it can be said to be polymorphic if the allele has a frequency below 0.99 and if otherwise, the population is said to be monomorphic [18]. According to the analysis results, the Kedu chicken population is polymorphic, while the Lingnan and Arab chickens are monomorphic.

Table 3. Allele and genotype frequency, HWE, and heterozygosity of GHR|*Eco72I* gene.

No	Sample	n	Genotype freq. (n)			Allele freq. (n)		He	Ho	χ^2	P-value
			AA	*AG*	GG	A	G				
1.	Kedu	30	0.93 (28)	0.07 (2)	0.00 (0)	0.97 (58)	0.03 (2)	0.06	0.07	0.03	1

2.	Lingnan	30	1.00 (30)	0.00 (0)	0.00 (0)	1.00 (60)	0.00 (0)	-	-	-	-
3.	Arab	30	1.00 (30)	0.00 (0)	0.00 (0)	1.00 (60)	0.00 (0)	-	-	-	-
	Total	90	0.98	0.02	0.00	0.99	0.01				

* χ^2: *chi-square value*; P<0.05: significant

Heterozygosity values are used to determine the level of genetic diversity of the GHR|*Eco72I* gene in each chicken population. Heterozygosity ranges from 0 to 1, with two measures: observed heterozygosity and expected heterozygosity. Genetic variation is considered high when the heterozygosity value approaches or exceeds 0.5 and low if it is below this threshold [19]. In the Kedu chicken population, the observed heterozygosity is higher than expected, indicating that no inbreeding has occurred within this population. All populations in this study exhibited low diversity, with values below 0.05.

The results of the Hardy-Weinberg equilibrium analysis are presented in Table 3. The Hardy-Weinberg analysis ensures that the population is under random mating, where allele frequencies should remain unchanged from generation to generation, except in evolution driven by mutation, migration, or selection processes. A population is considered in Hardy-Weinberg equilibrium if the P-value exceeds 0.05 [18]. The analysis results for the Kedu chicken population showed a non-significant P-value (P > 0.05) with a value of 1. This P-value indicates that the genotype distribution in the Kedu chicken population is in Hardy-Weinberg equilibrium. The Arab and Lingnan chicken populations were not eligible for chi-square testing because they only had one type of allele and genotype, resulting in zero degrees of freedom.

3.5 Association of GHR|*Eco72I* gene with body measurements in chicken

The association of the GHR|*Eco72I* gene with the body size of chickens was only carried out on male Kedu chickens because only one genotype appeared in the Arab and Lingnan chicken populations. Based on Table 4, the association of GHR|*Eco72I* gene with body size in male Kedu chickens, the results were not significantly different (P>0.05). The results show that the GHR|*Eco72I* gene was not associated with the parameters used: body weight, chest length, chest width, wingspan, leg length, and leg diameter. The GHR|*Eco72I* gene genotypes AA and AG were not associated with body size and carcass in local chickens [7]. The GG genotype has a positive effect on body composition and carcass in crosses between local chickens and the Meat Breed. The results show that the G allele strongly affects chickens' body weight and carcass.

Table 4. Association of GHR|*Eco72I* gene with body measurements in chicken.

Traits	Genotype of GHR|*Eco72I* gene		*P-value*
	AA	AG	
Body weight	2.06±0.15	2.57±0.46	0.36
Chest length	13.78±0.90	13.70±1.78	0.95

Chest width	5.45±0.59	5.34±0.82	0.88
Wingspan	43.53±1.39	47.00±1.41	0.14
Shank length	4.25±0.36	4.69±0.30	0.24

* P<0.05 = significant

The results show that genotype GHR|*Eco72I* was allegedly not associated with chickens' body measurement parameters due to their growth age. The male Kedu chickens used in this study were 32 weeks old. The results of the study [20] reported that the GHR gene has a positive effect on the body weight of chickens at the growth age. According to [21] chicken growth peaks at 26 weeks of age, and according to [22], chicken growth experiences a stationary phase. In adulthood, GHR expression experiences a stationary phase along with decreasing GH gene expression [22].

4 Conclusion

The results found two allele variations in the GHR|*Eco72I*, A and G, and two genotype variations, AA and AG. The AA genotype has the highest frequency in all populations, namely in Kedu, Arab, and Lingnan chickens. Based on the Hardy-Weinberg equilibrium analysis, the Kedu chicken population is in equilibrium. The level of GHR|*Eco72I* diversity in all populations shows a low level of diversity. The AA and AG genotypes in this study were not positively associated with the body measurements in male Kedu chickens.

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