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Identification of Polymorphism Growth Hormone Gene in Local Chickens Resulting from Three Breed Crosses

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Abstract

Local chicken has an important role as a source of animal protein producing eggs and meat. Local chicken has the advantage of being able to adapt to hot weather and be more resistant to disease. However, there are still weaknesses, namely relatively slow growth and relatively low egg production. Therefore, one way to overcome these weaknesses is by holding selection and crossing. Local chickens used for crossing in this study were sentul, kampung and kedu chicken. In connection with the growth of chickens that are slow and low in productivity of local chickens, it is necessary to examine the factors that influence growth and productivity. The hormones that have an effect on growth are GH (Growth Hormone), so that the characteristics of the gene of growth in local chickens from the cross can be known.The purpose of this study was to detect the characteristics of the Growth Hormone gene in local chickens resulting from Three Breed Crosses . The research material used in this study was forty local chickens produced by cross-sentul, kampung and kedu chicken. To find out GH gene polymorphism is by PCR-RFLP with cutting enzyme *Eco*RV.The results of the GH gene study in sentul, kampung and keducrossed chickens are polymorphic and are in the Hardy-Weinberg equilibrium state. Kampung chicken cross population was polymorphic for GH|*Eco*RV loci and the GG genotype was predominant in this population. G allele frequency in kampung,kedu and sentul chicken cross population is higher than the A allele. The highest genetic diversity was observed at kampung,kedu and sentul chicken cross and the heterozygosity value was lowcategory.

Keyword:GH gene, local chicken cross, polymorphism

Introduction

Indonesian local chicken is a commodity that is most maintained by the community, especially in rural areas. Thus, local chicken has a very important role for the people of Indonesia because it can meet economic needs, increase the income of farmers and sources of animal protein. Another advantage of local chickens is that they have the ability to survive in a hot environment, surviving in conditions of low feed quality and disease resistance. This advantage is not found in broiler chickens, so that local chickens have the potential to be developed with the advantages possessed, then expected to have rapid growth.

However, the productivity of local chickens is generally still low due to the slow growth and low of egg production and extensive maintenance systems (Suryana and Hasbianto 2008). In an effort to increase the growth of local chickens can be done through selection and crossing. The crossing of local chickens is expected to increase productivity through crossing between clumps to get a high level of heterocyst which can further improve genetic quality. Crossbreeding is one of the tools for exploiting genetic variation. (Saadey *et al*., Siwendu *et al*., 2012).

The selection of kampung, kedu and sentul chicken were crossed in this study because the chicken is a local chicken family that has the potential to be developed, both as an egg and meat producer. Kampung chicken has the genetic distance closest to sentul chicken, then followed by kedu chicken and pelung chicken (Sartika and Iskandar 2007). FurthermoreSulandari*et al*. (2007) stated that the benefits and advantages of free-range chicken are producers of meat and eggs and are resistant to disease. Kampung chicken also has a higher body weight than sentul and kedu chickens. Sentul chicken is a local chicken that has high potential as an egg-producing livestock and relatively fast meat growth (Sartika 2010).

Regarding the growth of chickens that are slow and low in productivity of local chickens, this study needs to be studied in the factors that influence growth and productivity. Among the hormones that influence growth in addition to GH (Growth Hormone), so that the characteristics of the gene of growth in local chickens from the cross can be known. GH gene is a candidate gene for growth in livestock because it affects development and growth, which regulates somatic growth including muscles, bones, epithelium and fibroblast cells (Abbasi and Kazemi 2011). According to Vasilatos-Younken*et al*. (2000), that the GH gene or Growth Hormone is considered as the gene that most determines the performance of chickens because it functions in growth and metabolism in chickens. Furthermore, Nie et al. (2005) stated that mutations in the GH gene also had an effect on chicken growth, carcass characteristics, egg production and fat deposition. Research on the genetic diversity of GH genes in crossbred chickens is still rare, so that the quality improvement of cross-breed chickens can be accelerated by selecting these genes. The purpose of this study was to identify polymorphism of GH gene in local chicken using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method.

Method

The research methods should elaborate on the method utilized in addressing the issues including the method of analysis. It should contain enough details allowing the reader to evaluate the appropriateness of methods as well as the reliability and validity of findings.The research material used in this study was forty local chickens produced by crossing of sentul, kampung and kedu chicken.

Animal and Phenotypic Data Collection

Hatched eggs from the crossing of sentul, kampung and keduchicken are collected every morning and evening. The eggs are hatched inside the hatching machine every 1 week. The hatched eggs are weighed using a digital scale and given a wing band for identification. DOC maintenance is carried out in a colony enclosure equipped with lights, places to eat and drinking water.

Providing chicken feed is done twice a day in the form of a commercial mixture and rice bran. Commercial feed is given from 1 day to 3 weeks. Mixture of commercial feed and rice bran with a ratio of 80%: 20% given to chickens aged 3 weeks. 4-week-old chickens are fed with a ratio of commercial feed: bran which is 70%: 30% and a ratio of 60%: 40% given to chickens aged 5-12 weeks. Drinking water is given in ad libitum. Measurement of chicken body weight gain is carried out every 1 week.

DNA Extraction

Total DNA extraction from blood sample was basically performed according to Sambrook& Russel (2001). Each 20 µL of whole chicken blood sample was added with 800 µL Red Blood Cell lysis buffer, homogenized, and centrifuged (800 rpm) for 5 min. The supernatant part was then removed. The pellet in the bottom of the tube was added with 40 µL 10% SDS, 10 µL Proteinase K 5 mg/mL, and 300 µL 1 x STE, and slowly shaken at 55^{o}C for 2 h. Then, each sample was added

with 400 µL phenol solution, 400 µL CIAA, and 40 µLNaCl 5M, and slowly shaken at room temperature for an hour, and centrifuged (12000 rpm) for 5 min. About 400 µL liquid from top layer was carefully removed into a new tube and added with 800 µL 96% EtOH and 40 µLNaCl 5M. Then, the sample was stored in -20ºC for 12 h, was centrifuged (12000 rpm) for 5 min, and the supernatant part was discarded. The pellet was dried in the clean open air for 3-4 h and added with 100 µL 80% TE. The DNA sample was ready for further analyses.

Amplification and Genotyping

Chicken GH specific fragments were produced with the polymerase chain reaction (PCR) method using thermocycler machine (Mastercycler, Eppendorf AG, Hamburg, German). The primers used to amplify the GH gene on chromosome 1 base 2248 were designed based on the NCBI Primary Designing Tool reference sequence in GenBank (Access number: AY461843.1). Primers are designed to have the sequence shown in Table 1.

Table 1 Primary GH Gene

Target Genes		Primary Sequence	Sequence Length
GH	Intron 3	F: 5'- ATGTCTCCACAGGAACGCAC -3' R: 5'- GCTCTGTAAGCTGAGCACCAC -3'	339 pb

Information : F= *Forward*, R= *reverse*

A total 35 cycles of PCR process consisted of denaturation (95ºC for 10 s), annealing (60ºC for 20 s), and extension (72ºC for 30 s). Amplification was performed with a total volume of 25 µL containing 50 ng/mL DNA sample, 0.5 pmol primer, 0.5 unit GoTaq Green Master Mix (Promega, Madison, USA), and Nucleotide-free water (NFW). The structural reconstruction of the GH gene primary design in Gallus gallus is presented in Figure 1.

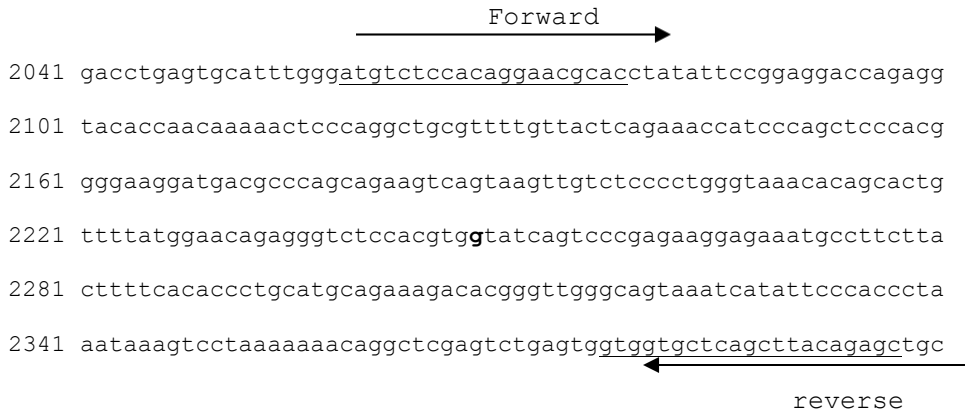

Figure 1. Fragment target of the chicken GH/*Eco* RV locus in exon 3. Arrows show forward and reverse primer annealing position; box shows *Eco* RV restriction site; asteriskshows mutation site. GenBank accession number: AY461843.1.

The restriction fragment length polymorphism (RFLP) analysis was performed for genotyping. PCR product and *Eco*RV restriction enzyme (Thermo Fisher Scientific, EU, Lithuania) were incubated at 37ºC for 12 h. Genotype was visualized through 2% agarose gel electrophoresis (v/w), which was stained with Fluoro Safe DNA Staining (1st Base, Singapore) above UV Transilluminator (Alpha Imager, Alpha Innotech, Santa Clara, USA). Gel was made of 0.45gr of

agarose gel and 30 mL of 0.5 x TBE which was heated in a microwave at medium-high temperature for 3 minutes. Homogenization was carried out with a stirrer, and then added 2.5 µL of 10% EtBr.

The gen was printed on the printer tray and allowed to harden. 5 µL amplicons was separated (running) through electrophoresis at a voltage of 10 V for 35-45 minutes, until the DNA fragments finished migrating in the gel. The bands would be visible with the help UV light so that the genome was carried on a long basis of visible DNA fragments.

Genotyping

Genotyping was done by RFLP technique. A total of 4 µL of amplicon was added with 0.9 µL of DW, 0.7 µL of buffer, and 0.4 µL of restriction enzymes.*Eco*RV enzymes on the Growth Hormone Intron gene cuts 3 were incubated at 37°C temperature for four hours. 5 µl DNA results later electroporetically are returned to 100 V voltage over 35 to 45 minutes on agrose gel 2%. DNA samples of electroporetics then lifted and observed under UV light. DNA fragment that appear from electroporetical results compared to the marker's long known fragments. DNA positions formed identified as allel to determination of genotype every sample.

Data Analysis

Polymorphism parameters (genotype frequency, allele frequency, the Hardy-Weinberg Equilibrium,and degree of heterozygosity) were analyzed according to Nei and Kumar (2000).

Allele Frequency and Genotype

The allele frequency is calculated using the formula according to Nei and Kumar (2000)as follows:

$$xi = \frac{(2nii + \Sigma i \neq jnij)}{2N}$$

The genotype frequency is calculated by the following formula (Nei and Kumar 2000):

$$xii = \frac{nii}{N}$$

Information:
xi = frequency of the ith allele;
xii = frequency of genotype i;
nii = number of individuals of genotype ii;
nij = number of individuals with genotype ij; and
N = total number of samples.

Hardy-Weinberg Balance

The Hardy-Weinberg equilibrium was tested by the chi-square (2) formula according to Nei and Kumar (2000), namely the following formula:

$$\chi 2 = \frac{\Sigma(O - E)2}{E}$$

Information:
$\chi 2$ = chi-squared;
O = observed value; and
E = expected value.
X2 table (α; db), if X2 count> X2 table = real, X2 count <X2 table = not real
db = 3.84

Degree of Heterozygosity

Genetic diversity is carried out by calculating the heterozygosity value observation (Ho) according to Weir (1996):

$$H0 = \frac{\Sigma N1ij}{N}$$

Information:
H0 = observed heterozygosity;
N1ij = number of heterozygous individuals at locus 1; and
N = number of individuals analyzed.

The expected heterozygosity is calculated using a formula based on Nei and Kumar (2000), namely:

$$He = 1 - \sum_{i=1} xi2q$$

Information:
He = heterozygosity of expectations;
xi = expected frequency; and q = number of alleles.

Result

Polymorphisms of GH/*Eco*RV Locus

The partial fragment of GH gene in 40 chickens were successfully amplified and showed a 339 bp bands. The lengths of PCR products were in good agreements with the reference sequences (GenBank accession number: AY461843.1.). This genotyping identified two alleles (G and A) and two genotypes (AG and GG).

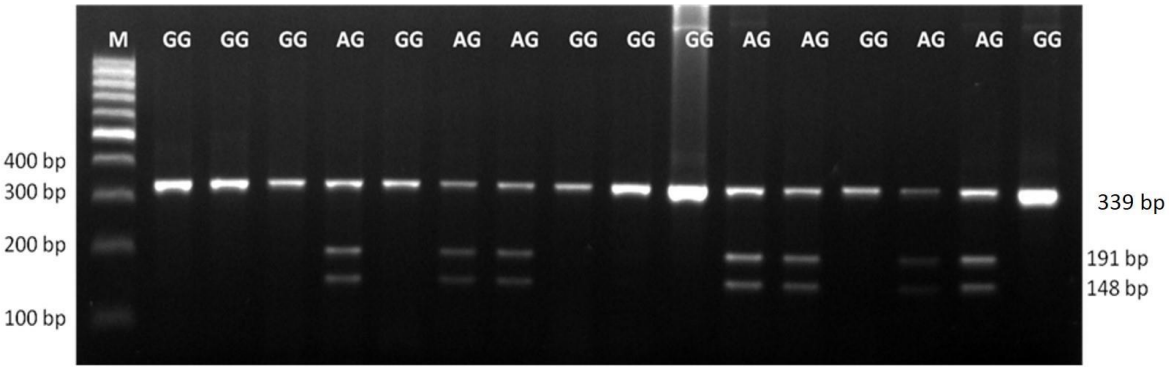

Figure 2. Visualization of GH|*Eco*RV genotyping in 2% agarose gel (M=marker; AG, GG = genotype).

Genotype Frequency and Alel Frequency GH Gen

Kampung, sentul and kedu chicken cross population was polymorphic for GH|*Eco*RV loci (Table 2). The GG genotype was predominant in this population. Similar result was reported in

Kampung chicken population with GG as the highest genotype frequency among all genotypes (Krisdianto, 2016).

Table 2. The polymorphism information of the GH|*Eco*RV in Kampung chicken cross

Population	n	Genotype Frequency			Allele Frequency		x^2
		AA	AG	GG	A	G	
Kampung x Kedu x Sentul	40	0.0	0.35	0.65	0.18	0.82	1.800
KampungSukabumi*	56	0.0	0.16	0.84	0.08	0.92	-

Note: n= number of sample, *Krisdianto (2016)

The Hardy Weinberg balance

The Hardy-Weinberg equation (x^2) of Kampung chicken cross population showed that this population is in equilibrium(x^2hitung < $x^2$0.05) (Table 2). Similar result was reported in KampungSukabumi and KampungCawi chicken population showed that this population is in equilibrium(x^2hitung < $x^2$0.05) (Table 2) (Krisdianto, 2016). This balance shows that there is no deliberate selection, especially the selection made on the GH gene. This indicated that the allele and genotype frequencies were stable from generation to generation in this population and there is no factor in causing genetic change (Allendorf *et al.*2013).

Table 3. Hardy-Weinberg gene balance of GH | EcoRV based on chi-square test (X^2)

Sample	N	X^2Count	X^2Table (0.05)
Kampung x Kedu x Sentul	40	0.18	3.84
KampungSukabumi*	57	0.42	3.84
KampungCiawi*	49	0.71	3.84

Note: n= number of sample, *Krisdianto (2016)

Heterozygosity value

. The expected heterozygosity (He) values found in this study were also below 0.5.Similar result was reported in KampungSukabumi and KampungCawi and layer chicken population showed that this population were also below 0.5 (Table 4) (Krisdianto, 2016).

Table 4. Estimates of the heterozygosity value of the GH | *Eco*RV gene

Population	N	Ho	He
Sentul ,Kampung ,Kedu	40	0.35	0.29
KampungSukabumi *	57	0.16	0.15
KampungCiawi*	49	0.29	0.32
Layer*	10	0.10	0.10

Note: n= number of sample, *Krisdianto (2016)

The results of the study the observed heterozygosity value (Ho) was 0.35 while the expected heterozygosity (He) value ranged from 0.29.

Discussion

The growth hormone gene (GH | EcoRV) was amplified using the PCR technique with a target at the 2248 bp, position located at the intron 3. Visualization of PCR-RFLP products using the *Eco*RV enzyme shows the enzyme cut point. Enzym *Eco*RV restriction cut the PCR product of

339 bp GH gene. The cutting occurs because the restriction enzymes recognize the GA | TATC cut site. PCR products based on Gen Bank access number AY461843.1 indicate that at the 2248 point mutations have occurred.The A allele was indicated by 256 and 139 bp bands (restricted), while the G allele was indicated by a single 339 bp (unrestricted, Figure 2). According to GenBank (accession number: AY461843.1)..

The G allele frequency in Kampung, sentul and kedu chicken cross population is higher than the A allele. This finding is contrary to KampungSukabumi population reported by Krisdianto (2016). . The frequency of GG genotypes has a higher chance of occurrence compared to other genotypes in the population of the kampungxkedu and sentul crossbreed chicken. The frequency of A and G alleles of GH gene in this chicken cross was 0.18 and 0.82. This shows that the population is polymorphic. Alleles are polymorphic if they have an allele frequency of less than 0.99 (Hartl and Clark 1997). Likewise, Nei (1987) explained that if a gene has an allele frequency less than or equal to 0.99 then the gene is polymorphic..

Calculations using the chi-square are often performed to determine the condition of the Hardy-Weinberg balance. This is in accordance with the opinion of Rodriguez (2014). That chi square is often used to assess Hardy Weinberg's equilibrium in random samples. The factors that influence Hardy Weinberg's condition are random mating, no gene mutation, no migration, and no selection.This is in accordance with the opinion of Weigend and Romanov (2001) which states that the factors that influence population balance are mutation and recombination, genetic drift, selection, and migration. Furthermore, according to Noor (2010), a large population will not change from one generation to another if there is no selection, migration, mutation, and genetic drift.

The observed heterozygosity value (Ho) in this study was found to be below 0.5 (50%). it can be indicated that the diversity value in the population is low. This is based on the statement of Javanmard*et al*. (2005) that if a heterozygosity value is found in a population below 0.5 (50%), then this indicates that the diversity value in the population is low. It is also not much different from the value found in the expected heterozygosity value (He). Heterozygosity values differ due to the number of populations and the frequency of alleles used in each population there are differences. This is in accordance with the opinion of Allendorf and Luikart (2006).The heterozygosity value is influenced by the number of samples, the number and frequency of alleles and genetic markers used.

Conclusion

The population of the crossed chickens of SentulKampung and Kedu are polymorphic and was in the Hardy-Weinberg equilibrium state. The heterozygosity value of local chickenscross of SentulKampung and Kedu is in the low category.

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