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Research Article
Single Nucleotide Polymorphism Genotypes of the Follicle Stimulating Hormone Gene Associated with Egg Production from Tegal and Magelang Ducks with Their Resulting Reciprocal Crosses

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Abstract

Background and Objective: The egg productivity of Indonesian local duck is higher than that of other local poultry. Follicle stimulating hormone (FSH) is part of the glycoprotein hormone that plays an important role in stimulating egg production by the ovaries. The FSH gene can be identified based on its nucleotide sequence by using the single nucleotide polymorphism (SNP) technique. The aim of this study was to identify SNPs of the FSH gene and investigate the SNP associations with egg production characteristics in two native Indonesian ducks, including the Tegal duck, the Magelang duck (F0) and reciprocal crosses named Gallang and Maggal (F1). **Materials and Methods:** This study used 200 ducks, comprising 50 of each breed. FSH gene amplification used the forward primer FSH-*Anas*PF: L 556 5'- TTCAGGCCTCCCCTACTTCT-3' and reverse FSH-*Anas*PR: H 820 5'- GTGCTGCAAGGCTTTTTAGG-3'. The SNP was determined according to the BioEdit v7.2.0 program through the ClustalW menu (accessory application). Allele and genotype frequencies were applied to observe the Hardy-Weinberg (H-W) equilibrium. The SNP genotype gene FSH associated with egg production was subject to quantitative trait loci (QTL) analysis. **Results:** The research successfully amplified a 264-bp PCR product to identify SNPs. SNPs were found at 700-nt (c.700T>C) and 701-nt (c.701G>A) in the H-W equilibrium. The characteristics of egg production were affected by a double allele C (dominant) and A (recessive). Therefore 3 genotype pairs CC, CA and AA for all populations determined the high, medium or low egg production. The effects of the average gene C (α_1) in the Tegal duck, Magelang duck and reciprocal crosses were 15.25; 13.74; 12.23 and 11.01, respectively, whereas gene A (α_2) had a negative effect and led to values of -12.99 -14.30, -11.29 and -11.46, respectively. **Conclusion:** SNP genotypes of the FSH gene in Tegal, Magelang and the reciprocal cross ducks were polymorphic and associated with egg production characteristics. SNP genotypes of the FSH gene (SNP c.700T>C and SNP c.701G>A) were viable candidates for marker assisted selection (MAS) to determine the egg productivity of Indonesian local ducks.

Key words: SNP, FSH gene, egg production, Tegal duck, Magelang duck

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Indonesian local ducks are potential meat and egg producers that are second only to chickens. Ducks contribute 14.72% or 290.10 thousand tons of the national egg demand[1]. In China and South East Asia, the duck is important in egg production for human consumption[2], representing 14.9%[3]. Tegal duck and Magelang duck are two Indonesian local ducks developed in Central Java province and have excellent production[4]. According to Purwantini *et al.*[5], a wide distance of genetic variation is found in Magelang ducks rather than the other local ducks, within 0.000-0.950 compared to 0.000-0.132. The reciprocal crosses are a backcross[6]. Numerous studies on local duck crossbreeding in Indonesia[7,8] and Asia[9,10] have reported excellent results that are typically above the average of the ascendant and were a suitable foundation for QTL mapping. To date, however, research on molecular markers using SNP genotypes of the FSH gene to examine whether it was associated with egg production in Tegal duck, Magelang duck and the reciprocal crosses has not been conducted.

Follicle stimulating hormone (FSH) is part of a glycoprotein hormone produced by the pituitary gland in the brain that serves to stimulate the production of eggs by the ovaries and influences the increase in the female hormone estrogen. Systems in males manage and maintain the process of sperm formation as well as determine the production of gametes and fertility[11,12] and sperm production is a proper indicator of mature testicle function in broilers[13]. Kang *et al.*[14] reported that follicle-stimulating hormone receptor (FSHr) mRNA in the ovaries of the Zi goose was stable and increased as the animals grew from 4 months old to laying age. Studies have been conducted on the FSH gene in poultry, including chickens[15,16], ducks[17] and quails[18] but not in Tegal or Magelang duck.

The objective of this research was to identify SNP in the FSH gene and investigate SNP associations with the egg production characteristics of the Tegal duck, Magelang duck and crosses (F1). The significance of the research was to obtain the SNP markers from the FSH gene and correlate them with the specific characteristics of egg production to provide accurate QTL mapping and useful information for breeding Tegal duck, Magelang duck and crosses (F1) that have high egg production at a younger age. The other benefit was to provide basic information on local duck crossbreeding that was expected to have higher economy value.

MATERIALS AND METHODS

Animals and sampling: Two hundred ducks, including Tegal and Magelang ducks, served as the parent generation (F0) and crossbreeds were produced from breeding a male Tegal duck with a female Magelang duck (called Gallang) as well as from breeding a male Magelang duck with a female Tegal duck (called Maggal[19]) were the offspring generation (F1). There were 50 ducks in each group. The populations were maintained under equal conditions according to standards for ethical animal research. Sixteen-unit pen mating cages were used for each generation and each unit consisted of one male with 6-7 female ducks. Feed was given at an amount of 160 g/duck/day and consisted of 50% rice bran, 30% corn and a 20% layer concentrate with 16.95% crude protein (CP), 2993 kcal kg^{-1} metabolic energy (ME), 15.86% crude fiber (CF), 9.12% crude fat (CF), 0.56% calcium (Ca) and 0.97% phosphor (P). The observed quantitative traits were egg weight, egg production and first laying age (FLA). Egg production was recorded from the first to the 90th laying. A 3 mL blood sample was taken from each duck for analysis.

DNA Isolation: Blood samples were taken from vena axillaries and stored in a vacutainer filled with EDTA as anticoagulant. The total genomic DNA was isolated with the DNA Isolation Kit (PT Genetica Science, Jakarta, Indonesia) according to the manufacturer's protocol. The DNA isolation results were used as PCR template without additional refining.

Primer design: The primer design of specific FSH genes was based on the GeneBank DQ232890.1 database[20]. The primer pair was FSH-*Anas*PF: L 556 5'- TTCAGGCCTCCCCTACTTCT-3' and FSH-*Anas*PR: H 820 5'- GTGCTGCAAGGCTTTTTAGG-3', which were selected from a conserved area and analyzed using Software Design Oligoprimer with the Primer3 online program.

PCR amplification: The amplification process was conducted with a PCR technique using the GeneAmp^{R} PCR machine system thermocycler 2400 (Perkin Elmer). The PCR reaction solution consisted of 12.5 μL of KAPA (Kit PCR from PT Genetica Science, Jakarta, Indonesia), 1 μL of primer FSH-*Anas*PF 10 pmol, 1 μL of primer FSH-Anas PR 10 pmol, 9.5 μL of dH_2O free nuclease and a 1 μL DNA template. The PCR cycle included pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec,

elongation (extension) at 72°C for 1 min and post-elongation at 72°C for 5 min. These steps were repeated for 35 cycles. The PCR products were separated by horizontal electrophoresis in a low melting point 1% agarose gel using 1x TBE buffer (Mupid, Japan) at a 50 V voltage for 15 min. The PCR products were visualized using UV light.

Sequencing FSH gene: The PCR products for the FSH gene were sequenced by PT Genetika Science, Jakarta, Indonesia. The sequence products were read using the Sequence Scanner v1.0 software and an electropherogram was produced that consisted of the nucleotide sequence from the FSH of Tegal and Magelang ducks as well as their crosses.

Identifying polymorphism and SNP genotyping: SNP genotyping was determined with the BioEdit v7.2.0 program by aligning sequence products according to the sequence in the GeneBank DQ232890.1 database from the ClustalW menu (accessory application). The alignment result was generated with electropherogram to obtain SNPs in a particular position to use for genotyping. The base sequence of the FSH gene in Mallards (*Anas platyrhynchos*) was at 1901-bp. The SNP was confirmed based on the electropherogram results and used for genotyping. Pearson's Chi-square test was used to verify that the samples did not deviate from the Hardy-Weinberg (H-W) equilibrium. The following model was applied:

$$X^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

where, X^2 is the Chi-square value, O_i is observed frequency, E_i is the expected frequency and n is the number of possible outcomes of each event[21].

QTL analysis of the FSH gene was associated with egg production. Quantitative traits were affected by a single locus with 2 alleles, such as SNP c.376C>A, which would produce double alleles between C and A as well as a total of 3 genotype pairs, specifically CC, CA and AA in the whole population data. Individuals whose genotype had the same allele pair are called homozygotes and individuals with different allele pairs are called heterozygotes. The genotype value (G) is formulated as G = A+D, where A is the additive effect and D is dominant deviation[22-25]. Genotype value is calculated based on the point of origin (O) that is the average of the homozygote. The genotype value of the dominant homozygote individual (CC) is a, the heterozygote (CA) is d and the recessive homozygote (AA) is -a. The calculation for a single or double locus is the same and starts by calculating the mean deviation (m) using the following formula:

$$m = a (p-q)+2 pqd$$

Where:

m = Mean deviation
a = Homozygous genotype values
p = Frequency of allele C (dominant)
q = Frequency of allele A (recessive)
d = Heterozygous genotype values

According to Falconer and MacKay[23] and Pirchner[25], mean (m) plus point of origin (O) equals to mean of the population (M).

Effects of the FSH gene on production performance: The effects of each allele on quantitative traits was calculated under the guidelines of previous studies[22-25] that declared:

$$p+q = 1 \text{ so } (p+q)^n = 1 \text{ and } (p+q)^2 = p^2+2pq+q^2=1$$

The effects of the mean allele C are referred to as α_1 and the effect of allele A is α_2 with the formula:

$$\alpha_1 = q[a+d(q-p)] \text{ and } \alpha_2 = -p[a+d(q-p)]$$

The contribution of allele C to egg production was obtained using the formula of Griffith *et al.*[24]:

$$\frac{2p^2G_{CC} + 2_{pq}G_{CA}}{2p^2 + 2pq}$$

Further, the formula to obtain the contribution of the allele A is:

$$\frac{2q^2G_{AA} + 2_{pq}G_{CA}}{2q^2 + 2pq}$$

Where:

G_{CC} = Egg production of genotype CC
G_{CA} = Egg production of genotype CA
G_{AA} = Egg production of genotype AA

The contribution of allele C is the mean of population (M) plus the mean effect of allele C and the contribution of allele A is the mean of population (M) plus the effect of allele A.

Contribution of allele A or C can be calculated using the formula of Griffith *et al*.[24] or from the results of the mean of population plus the effect of each allele.

RESULTS AND DISCUSSION

Quantitative traits: Table 1 shows the means and standard deviations of egg production characteristics for the four genotype groups, including Tegal ducks (F0), Magelang ducks (F0) and the Gallang (F1) and Maggal (F1) reciprocal crosses. Table 1 indicates that egg weight and first laying age were not significantly ($p>0.05$) different but they were higher in Magelang ducks than in Tegal, Gallang or Maggal ducks. Egg production in the parent generation (F0) was significantly different from the offspring (F1) generation ($p<0.05$). Magelang ducks had a relatively higher egg production than did Tegal ducks or their offspring.

PCR amplification from DNA extraction: Total genomic DNA from the blood samples of Tegal ducks, Magelang ducks and their crosses had relatively bright and thick bands, which determined DNA quality. FSH gene amplification with primer FSH-*Anas*PF (L556) and FSH-*AnasPR* (H820) resulted in one bright 264 bp band, Fig. 1. Kang *et al*.[14] amplified a 207-bp fragment of the FSH gene in Zi geese from China.

Identifying polymorphisms and SNP genotyping: The results of PCR product sequencing included a 264-bp product from the FSH gene of Tegal and Magelang ducks as well as the reciprocal crosses and the product aligned with the FSH gene from GenBank (DQ232890.1) in ClustalW and BioEdit Fig. 2. Figure 2 shows that the FSH gene in Tegal and Magelang ducks as well as the reciprocal crosses was found at 700-nt (c.700T>C) and 701-nt (c.701G>A). At the position of 700 nucleotides (nt), there is a thymine (T) base mutation that turns into cytosine (C), whereas there is a guanine (G) base mutation that occurs at position 701 nt that becomes adenine (A).

Chi-square test: SNP results in locus (c.700T>C) and (c.701G>A) were utilized for a chi-square (χ^2) test to assess the genetic equilibrium in populations of Tegal ducks, Magelang ducks and the reciprocal crosses. The Chi-square test was based on allele frequency from the FSH gene polymorphism of each breed. The results are presented in Table 2.

QTL analysis of the FSH gene associated with egg production: SNP and genotyping results in the FSH gene were obtained through a QTL analysis approach, which indicated that egg production in Tegal ducks, Magelang ducks

Table 1: Mean and standard deviation of egg production in Tegal Ducks, Magelang ducks and the reciprocal crosses (Gallang and Maggal)

	Mean and standard deviation		
Native duck	Egg production (%)	Egg weight (g)^{ns}	First laying age (day)^{ns}
Tegal (F0)	39.08±13.93^{a}	64.46±3.40	156.43±4.23
Magelang (F0)	58.91±20.63^{b}	65.39±3.24	160.10±3.39
Gallang (F1)	54.58±18.86^{b}	63.20±2.62	152.67±16.96
Maggal (F1)	48.34±17.25^{c}	64.40±3.26	159.43±28.17

*Different superscripts (a, b) within columns indicate significant differences in an HSD test ($p<0.05$), ** ^{ns}Non-significant

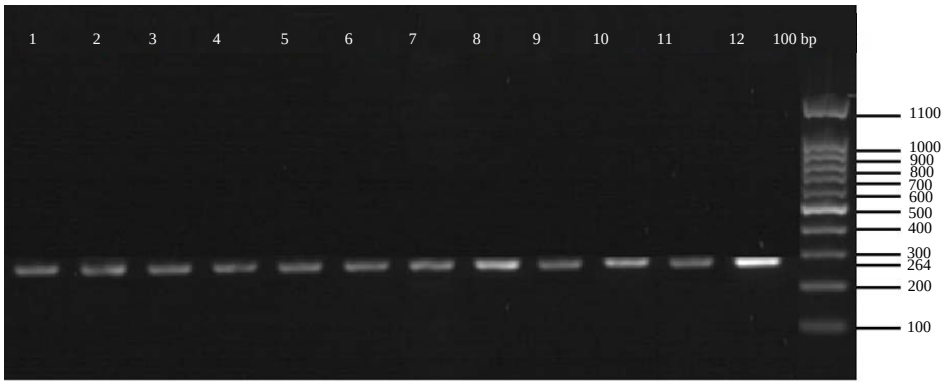

Fig. 1: PCR Product-FSH gene in ducks (264-bp) generated with the primer pair FSH-Anas PF (L556) and FSH-*Anas* PR (H820) using a 1.0% Agarose gel

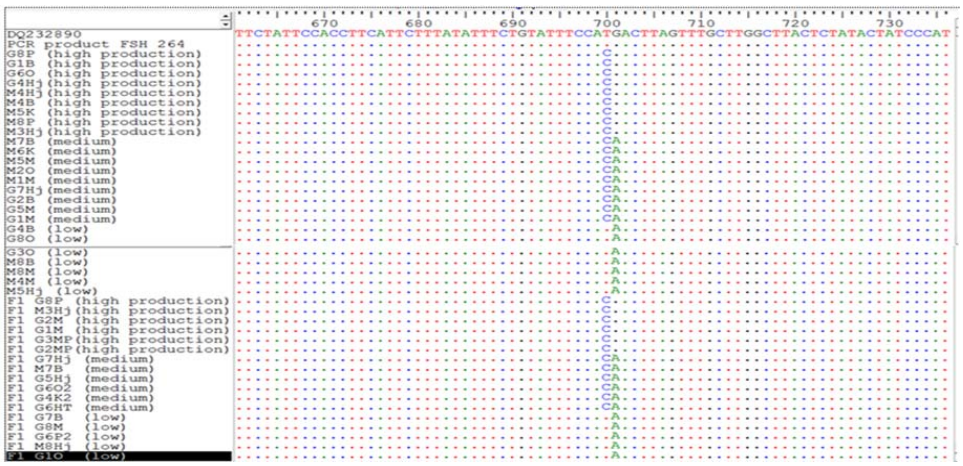

Fig. 2: Alignment of the FSH gene from GenBank (DQ232890.1) with the sequence of the PCR product of the FSH gene of Tegal ducks, Magelang ducks and the Gallang and Maggal reciprocal crosses using Clustal W program and BioEdit after editing

Table 2: Chi-square test of the FSH gene in Tegal ducks, Magelang ducks and the reciprocal crosses (Gallang and Maggal)

		Genotype frequency			Allele frequency		
Native duck	Amount	CC (p^2)	CA (2pq)	AA(q^2)	C (p)	A (q)	χ^2
Tegal (F0)	Observed	9	28	13	0.46	0.54	-0.13
	Expected	10.58	24.84	14.58			
Magelang (F0)	Observed	10	31	9	0.51	0.49	-0.24
	Expected	13.01	24.99	12.01			
Gallang (F1)	Observed	11	26	13	0.48	0.52	-0.04
	Expected	11.52	24.96	13.52			
Maggal (F1)	Observed	12	27	11	0.51	0.49	-0.08
	Expected	13.01	24.99	12.01			

$\chi^{2\,0.05} = 67.51$

and the reciprocal crosses were affected by double C (dominant) and A (recessive) alleles with a total of 3 genotype pairs, CC, CA and AA, for the whole population. Based on Table 1, the means and standard deviation of egg production percentage associated the genotype CC with high productivity, CA with medium productivity and AA with low productivity. These categories were used to analyze SNP genotypes of the FSH gene associated with egg production in Tegal ducks, Magelang ducks and reciprocal crosses, as presented in Table 3.

Important production characteristics in egg-laying birds include egg production, egg weight and first laying age (FLA)[26]. Tegal ducks had low productivity but according to Prasetyo and Ketaren[27], they are capable of consistent production despite low protein feed compared to other native ducks. The production capability of Gallang and Maggal ducks, which are lower than Magelang ducks but higher than Tegal ducks suggests that the capability of offspring is determined by the two parents. Falconer and MacKay[23] as well as Noor[28] stated that the results of crosses between different genotype groups had an advantage between the two parent strains called heterosis.

The age for first laying of Gallang and Maggal ducks was relatively younger than the Tegal and Magelang ducks, which correlates with the results of a previous research conducted by Prasetyo and Susanti[29] that indicated crossbreeds often lay eggs earlier than the parent strains did. The first laying age is also associated with egg weight in that a younger first laying age tends to be associated with a lower egg weight. According to North[30], a younger first laying age will produce more eggs but the eggs tend to be smaller.

Single nucleotide polymorphism c.700T> C of the FSH gene in Tegal ducks, Magelang ducks and the reciprocal crosses is found in individuals with high and medium production capability, whereas c.701G> A is found in individuals with moderate and low production ability. Thus, it can be stated that SNP c.700T> C causes high and medium egg production and SNP c.701G> A causes medium and low egg production. The electropherogram results show that each nucleotide creates a different color peak. The nucleotide A is green, G is black, C is blue and T is red (Fig. 3). The follicle stimulating hormone gene polymorphism has been reported in several previous studies, including studies on chickens[2], geese[14], ducks[17], muscovy ducks[31] and quail[18].

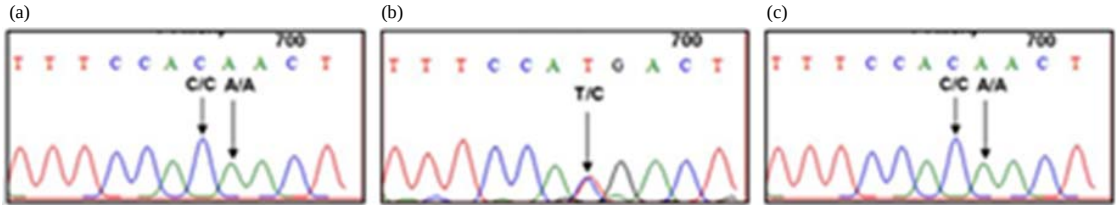

Fig. 3(a-c): Results of the electropherogram of FSH gene sequences for Tegal ducks, Magelang ducks and reciprocal crosses (Gallang and Maggal) after editing. Egg production (1) Is high for the CC genotype, (2) Moderate for the CA genotype and (3) Low for the AA genotype

Table 3: SNP genotypes of the FSH gene associated with egg production in Tegal ducks, Magelang ducks and the reciprocal crosses (Gallang and Maggal)

							Gene contribution		Gene effect	
Native duck	Egg production (%)	Category	SNP genotype	Genotype value	m	M (m + O)	C	A	C (α_1)	A (α_2)
Tegal (F0)	58.75	High	CC	17.57	-1.30	39.88	55.13	26.90	15.25	-12.99
	39.71	Medium	CA	-1.47						
	23.61	Low	AA	-17.57						
Magelang (F0)	85.06	High	CC	28.09	1.52	58.49	72.24	44.19	13.74	-14.30
	58.89	Medium	CA	1.92						
	28.89	Low	AA	-28.09						
Gallang (F1)	80.1	High	CC	23.60	-1.97	54.54	66.77	43.25	12.23	-11.29
	54.61	Medium	CA	-1.90						
	32.91	Low	AA	-23.59						
Maggal (F1)	69.63	High	CC	22.49	1.15	48.29	59.31	36.84	11.01	-11.46
	48.55	Medium	CA	1.41						
	24.65	Low	AA	-22.49						

m: Mean and M: Mean of population

Figure 3 shows the change of base T-C. GenBank DQ232890.1., 2006 shows a T base but samples with high egg production have a C base. This outcome indicates that individuals with high egg production have the CC genotype in one electropherogram peak C. Figure 3 also shows the change of base T-C and base G-A. This outcome indicates that the individuals with medium egg production have a heterozygote genotype CA that is shown with two electropherogram peaks for CA. Figure 3 further shows the change of base G-A. This outcome shows that individuals with low egg production also have a homozygous genotype AA with one electropherogram peak for A. Li *et al.*[2] found a genetic marker for the production of Beijing chicken eggs at G.-181A> T and G.-310A>C loci that were associated with an increase in the expression of FSHR mRNA with the genotypes AA, AT and TT and AA, AG and GG.

The calculated value of χ^2 (Table 2) is smaller than the table value of χ^2 (67.51). This result indicates that the distribution of the FSH gene genotype in the population of Tegal ducks, Magelang duck sand the reciprocal crosses was in H-W equilibrium. H-W equilibrium status in the population showed that allele and genotype frequency in the duck population would remain constant from generation to generation if there was no random selection, mutation, migration or crossbreeding within the population. These results agree with the results of Hardjosubroto[32] and Warwick *et al.*[33].

Sequence variation of the FSH gene in SNPs caused amino acid changes at 234 nt, which included a change of tryptophan (Trp/W) to glutamine (Gln/Q) where the amino acid UGA turned into CAA (homozygote) and tryptophan (Trp/ W) to arginine (Arg/ R) where the amino acid UGA turned into CGA (heterozygote), as presented in Fig. 4. These changes altered the amino acid sequence. Similarly many researchers[34-38] stated that the alterations from base A-G changed amino acids such that isoleucine (AUC) turned into valine (GUC). Rahayu *et al.*[39] reported an amino acid alteration in SNP c.376A>G and c.409G>A in which isoleucine changed into valine/valine (homozygote) and isoleucine/valine (heterozygote), alanine into threonine/threonine (homozygote) and alanine/threonine (heterozygote), respectively, from the MC1R gene with plume color and the production performance of Magelang ducks.

Table 3 shows the association between SNP genotypes of FSH genes and high (CC), medium (CA) and low (AA) egg production of Tegal ducks, Magelang ducks and the reciprocal crosses. Analysis revealed genotype value, mean (m), the

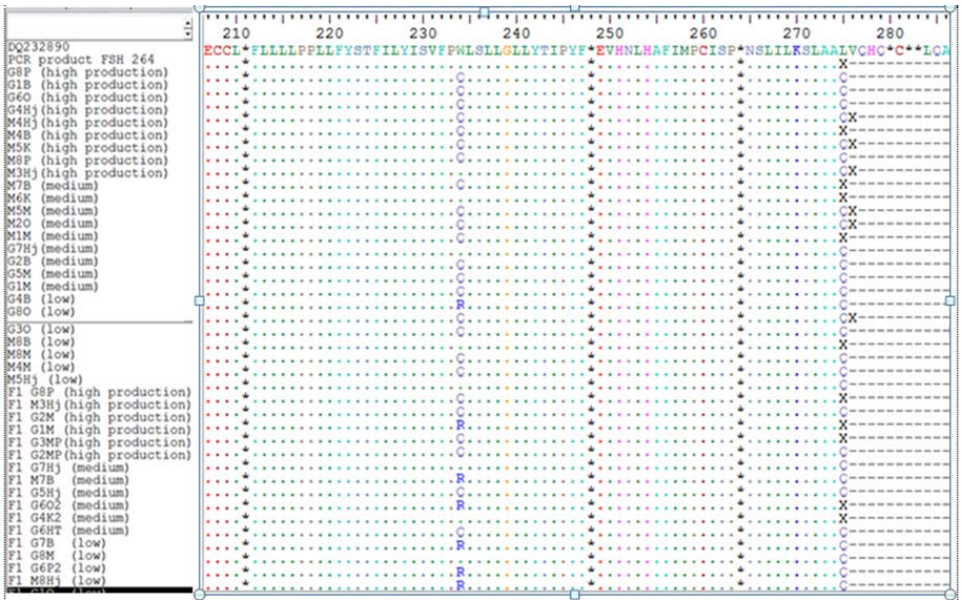

Fig. 4: Amino Acid changes from the FSH gene of Tegal ducks, Magelang ducks and the reciprocal crosses (Gallang and Maggal)

mean of the population (M) and the contributions and effects of gene C and A. Different genotype values of each duck breed were associated with different levels of egg production.

The effect of each allele on the quantitative traits was calculated according to the method described by Falconer and MacKay[23] and Pirchner[25]. Egg production in this research was affected by genes C and A from the FSH gene. Mean value of gene C (α_1) was positive, whereas the mean value of gene A (α_2) was negative. This outcome indicated that gene C was dominant, favorable and led to high egg production, while recessive gene A contributed to low egg production. Follicle stimulating hormone gene affected secretion of estrogen and progesterone hormones that played a role in oviduct development[40]. The more follicles grew, the more estrogen was produced, which improved egg-forming substances contributing to the weight and length of the oviduct that eventually improved egg production.

The mean effects of gene C (α_1) in Tegal ducks, Magelang ducks and the reciprocal crosses (Gallang and Maggal) were 15.25, 13.74, 12.23 and 11.01, respectively, whereas, the mean effects of gene A (α_2) were -12.99; -14.30; -11.29 and -11.46, respectively. Gene contributions to quantitative traits of animal population were determined by the mean of the population plus mean effects of genes[24]. Contributions of gene C from the FSH gene in egg production of Tegal ducks (F0) was 55.13, which was obtained from a population mean (39.88) plus a mean effect of gene C (15.25). The gene A contribution was 26.90. The contribution of the FSH gene is assumed to be related to the performance of FSH, which affects the secretion of steroids such as estrogen and progesterone. Progesterone also plays a role in the growth of the reproductive tract (oviduct) and egg laying process. Only 7-10 ova enter rapid development. For approximately 10 days, the first ovum matures followed by egg laying. The estrogen from this ovary will stimulate the growth of the oviduct to prepare for egg formation[40]. The more follicles that develop, the more estrogen is produced, which in turn can increase the egg-forming agent. Thus, estrogen affects the weight and length of the oviduct. Nalbandov[41] stated that oviduct development can occur due to the stimulation of estrogen and progesterone produced by ovarian follicles.

The highest contribution of gene C or A from the FSH gene to egg production was 72.24 and 44.19 in Magelang ducks (F0) while the lowest was 55.13 and 26.90 in Tegal ducks (F0). The largest amount of gene A and C contribution occurred in Gallang ducks (F1) and Maggal ducks (F1) with values of 66.77 and 43.25 as well as 59.31 and 36.84, respectively. These results were likely related to the inherited heterocyst gene, which led to excellent hybrid traits (offspring) that were superior to those of both parental breeds. The influential genes would be inherited from the parent (F0) by the offspring (F1), which was confirmed when the same SNP genotypes of the FSH gene were found in the offspring (F1). These results agree with Falconer and MacKay[23], who stated that heterosis led to better average production in offspring compared with the average production of the parents.

This study discovers the SNP genotypes of the FSH gene (SNP c.700T>C and SNP c.701G> A) which are related to acquire egg production traits that can be beneficial for genetic markers and genetic improvement of basic selection based on biomolecules, especially in local Indonesian ducks. This study will help researchers to determine criteria for local duck standardization with local partners (including breeders) as well as national standards for Indonesian ducks that many researchers have yet to explore. Therefore, a new theory on conventional breeding technology combined with SNP-based molecular techniques using the follicle stimulating hormone (FSH) gene and QTLs, especially in local ducks from Indonesia, could be developed in future studies.

CONCLUSION

Single nucleotide polymorphism (SNP) genotypes of the FSH gene were polymorphic and associated with egg production characteristics. Individual ducks that are local to Indonesia with the genotypes CC, CA and AA had high, medium and low egg production, respectively. The obtained SNP genotypes for the FSH gene (SNP c.700T>C and SNP c.701G>A) were viable candidates for marker assisted selection (MAS) to determine the egg productivity of local Indonesian ducks.

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