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Effect Analysis of Prolactin (*PRL*) Gene Polymorphisms on Chicken Egg Productivity (*Gallus gallus domesticus*) BC$_1$ from Crossbreeding between Pelung and Layer Chicken

Research Article

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ABSTRACT

Prolactin (*PRL*) Gene in chicken was closely related to broodiness and brooding behavior that located at chromosome 2. This research was conducted to detect insertion/deletion (in/del) 24-bp polymorphism in the prolactin promoter gene and its association with the BC$_1$ hybrid chicken egg productivity. Procedures of this research conducted are chicken maintenance, egg collection for 23 weeks of production, eggs proximate quality (water, ash, protein, carbohydrate, fat, and fiber percentage), DNA isolation, *PRL* gene amplification, and visualizing DNA bands on PRL promoters. The results showed the average number of cumulative eggs of BC$_1$ chicken for 23 weeks was 42.9 eggs, lower than layer (104.34 eggs), and higher than pelung (30.17 eggs). Analysis using the pearson correlation test shows the frequency of insertion-deletion polymorphism alleles on the PRL promoter has a signification value of 0.521 and correlation value 0,684, so it is concluded that there is no correlation (P>0.05). Proximate test results of BC$_1$ whole raw egg chicken has characteristics of low fat (3.2%) and high carbohydrate (9.1%) compared to commercial egg and has a calorie content of 98.37/100 g of the sample.

KEY WORDS egg, layer, pelung, promoter gene PRL, proximate analysis.

INTRODUCTION

Prolactin (PRL) is a polypeptide hormone produced in the anterior hypophysis of the pituitary gland. Prolactin has diverse functions for invertebrate species (Freeman *et al.* 2000). The chicken prolactin hormone plays a crucial effect on egg production. The promoter of PRL is located at the start point and become crucial due to its early activation function for transcription of *PRL* gene expression (Lewin, 1997). The mutation that occurs in the promoter region causes the *PRL* gene less optimal to express its product and brooding behavior Therefore, egg production will increase.

The molecular analysis method through the identification of superior molecular markers (biomarkers) becomes a feasible method for selection because it causes the selection of superior traits to broodstock can be faster and more accurate. One of them with marker assisted selection (MAS). MAS is a program that is used to analyze the relationship between the diversity of DNA and expected quantitative characters (Montaldo and Herrera, 1998). Mutations in the *PRL* gene sequence are found in exons and 7 introns. The results of research on the *PRLR* gene in chickens show the significance of the relationship of single nucleotide polymorphism (SNP) on exon 2 to body weight (DOC) when

hatching and the age of ripe. The relationship between exon 5 in the *PRLR* gene and egg count also showed significant results (Rashidi *et al.* 2012). In this study, the selection of egg productivity traits based on the prolactin (*PRL*) gene as a biomarker will be observed. The *PRL* gene in chickens has been sequenced nucleotides and research has concentrated a lot on identifying the polymorphic part of that gene (Table 1). Indonesia is a densely populated country. Based on data submitted by the Indonesian Minister of the Interior (Mendagri) Tjahjo Kumolo, the total population of Indonesia as of 30 June 2016 was 257,912,349 people, an increase compared to 2010 which only had 237,641,326 people (Badan Pusat Statistik, 2017). The increasing population in Indonesia has led to an increase in food needs, including livestock products. The highest livestock products consumed by the community are meat, which is largely supplied from chicken meat because the price of chicken meat is relatively cheap and affordable. The biggest product of chicken meat comes from broilers (Direktorat Jenderal Peternakan, 2017). High market demand and interest in chicken farming results make improving local chicken quantity and quality were necessary. One of the efforts was the chicken crossbreeding program. Cross-breeding between local chickens and broilers can improve the quality of local chickens (Saragih and Daryono, 2012). Previously, according to research by Nataamijaya *et al.* (1993) crossbreeds had been made between groups of free-range chickens from different strains, namely pelung and kampung chickens. This cross produces chicks with a higher weight (1.7 kg) compared to the weight of native chickens (0.875 kg) and pelung chickens (1.46 kg) at 15 weeks old. This research aims to investigate the egg production in BC_1 chicken (female brown layer and male pelung), the polymorphism produced based on PRL molecular markers and the effect of *PRL* gene polymorphisms as markers of egg productivity traits in BC_1 chickens.

MATERIALS AND METHODS

One male F_1 (crossed from female Lochmann Brown layer and male pelung chicken) was crossed with four female Lochmann Brown layer chicken in a semi-intensive pen (3 m×3 m×2 m). The floor of the cage was given sand to keep the pens clean. The eggs were collected every day and hatched using an incubator with a temperature of 37-38 °C and humidity 55-65% for 21 days. A total of 19 female BC_1 chickens were produced which was used in this study. The chicken was fed with BRcf pellet type feed and chicken mix feed with concentration bran: concentrate: corn 1: 2: 2 volume. Backcross-1 chickens were kept in battery cages at the Universitas Gadjah Mada (UGM) Center for Agrotechnology Innovation (PIAT). One battery cage slot is used for 1 chicken with a width of 13.75 cm. Eggs were collected

every day in each battery cage and tabulated once a week. The weekly egg production data will be used to graph the egg production for 23 weeks of production.

Egg quality
The egg quality was observed through two stages, including observation of external and internal egg quality. External egg quality observations include: (1) determination of the shape index (2) egg weight, eggshell thickness, and percentage of physical egg. The shape index determination was done to determine the shape of the egg by calculating the length (L) and width of the egg (W) using a vernier caliper. Then the value obtained is entered into the formula:

$$\text{Shape index} = (W/L) \times 100$$

The results obtained from these calculations can determine the shape of sharp shape eggs (if the result was <72), normal shape (if the result was 72-76) and round shape (if the result was >76) (Sarica and Erensayin, 2014).

Internal egg quality was done through proximate observations of whole raw eggs of BC_1 chicken. Egg proximate test was carried out by observing water, ash, protein, fat, and carbohydrate content

DNA isolation
DNA isolation was carried out using chicken blood by first washing with TE buffer by inserting 10 µl of blood with 1 ml of TE buffer (10 mM pH 8.0). Then the centrifugation was carried out at 13000 rpm, with repetition of the process 3 times. Supernatant from centrifugation results was removed and then added 200 µL 5% chelex, 18 µL DDT 0.05 M, and 2 µL proteinase K. After adding the first incubation at a temperature of 56 °C for at least 60 minutes with an inverse every 15 minutes to make sure it was well mixed. After that, the second incubation was continued at 100 °C for 8 minutes. Then homogenization using vortex then centrifugation with a speed of 13.000 rpm for 3 minutes. From the results of centrifugation two phases will be formed, the pellet and supernatant phases. DNA is isolated from the supernatant. The supernatant was transferred to a new microtube and stored at -20 °C (Singh *et al.* 2018).

PRL gene amplification
For gene target amplification, the reaction composition was made with a total volume of 25 µL with the mixture PCR master mix Bioline 12.5 µL, each primer 1.25 µL, ddH$_2$O 5 µL, and DNA sample 5 µL.

The primer used in DNA amplification was '5-TTTAATATTGGTGGGTGAAGAGACA-3' forward and 5′-ATGCCACTGATCCTCGAAAAC TC-3′′ reverse. The PCR program used was a 25× cycle setting.

Table 1 Association of laying characters with polymorphisms in *PRL* and *PRLR* genes in chickens

Gen	Polymorphism location	Associated character
PRL (chromosome 2)	Insertion/deletion position in nucleotide number 358	Egg productivity (Cui *et al.* 2006)
(Miao *et al.* 1999)	Region promoter	Laying character (Jiang *et al.* 2005)
	Exon 2	DOC weight when hatched, mature age (Rashidi *et al.* 2012)
PRLR (chromosome Z)	Exon 2	Egg production, age when first laying eggs (Liu *et al.* 2011)
(Wilkanowska *et al.* 2014)	Exon 6	DOC weight when hatched, mature age (Rashidi *et al.* 2012)
	Exon 5	Number of eggs (Rashidi *et al.* 2012)

The initiation stage was 95 °C for 5 minutes, denaturation was 95 °C for 45 seconds, annealing 55 °C for 60 seconds, elongation 72 °C for 45 seconds, and the last elongation for 5 minutes (Cui *et al.* 2006).

Electrophoresis
The 2.0% agarose gel was made by taking 0.8 g agarose then diluted with 40 mL TBE 1× then dissolved by heating in the microwave for 1 minute. After being heated then allowed cooling a bit then adding 3 μL florosafe DNA staining then poured in an agar mold, waiting for it to become lumpy. The finished gel is placed into the electroporator tank then TBE was poured until the gel was submerged. Then 4 μL PCR product were added to each well. DNA migration was done by turning on the electrophorator with a 50 volt voltage for 50-65 minutes. The visualization of DNA bands seen with UV Geldoc.

Data analysis
In this study, data was taken of the number of eggs from BC_1 chicken compared with 3 other groups in previous studies (the eggs productivity of F_1, layer and pelung). The data that will be obtained are (1) data on the average number of eggs per week presented in the graph using the calculation of the number of eggs per week divided by the number of chickens, (2) data on the number of cumulative eggs per chicken group for 23 weeks, egg weight and eggshell weight were presented in the form of histograms and significant differences between groups of chickens were tested with Tukey honest significantly difference (HSD) and duncan multiple range test (DMRT) with the significance level used $P < 0.05$, (3) frequency of the percentage of the physical part of the egg is presented in a pie diagram using the calculation of the weight between the yolk, egg white, and eggshell, (4) shape index data will be calculated based on the average shape index value, the lower limit, the upper and standard deviations and (5) the polymorphism data from the results of the visualization of DNA bands on the PRL promoter insertion and deletion genotype frequency follows Hardy-Weinberg's Equilibrium (HWE).

The relationship of polymorphism of PRL promoter insertion and deletion with egg productivity was tested using Pearson correlation analysis with the SPSS program (SPSS, 2011; $P<0.05$).

RESULTS AND DISCUSSION

In this study, Back Cross-1 (BC_1) chickens were used, which were the result of the crossing of layer hens and F_1 roosters.

F_1 male chickens are obtained from a cross between layer chickens and pelung chickens (Figure 1). BC_1 chickens were used as many as 19 females (Figure 2). In Table 2, the results of crossing between layer females and F_1 males still have high phenotypic variations. Can be seen in the color of chicken feathers BC_1 had a combination of brown, light brown, and white.

The white color was considered dominant because even though parental only has a few white feathers, 5 BC1 chickens have a wholly white coat (26.3%). Besides, the results of the crossing can be seen in two types of foot colors namely white color 52.6% and yellow 47.4%. Whereas parental only has white feet.

This was also found in the study of Utama *et al.* (2018) reported that BC_1 chickens crossing between F_1 hens (♀ pelung×♂ broilers) produced chickens with a non-uniform divergence of feathers, which were dominated by white feathers (male:46% and female:47%) followed by light brown (male:31% and female:26%), brown (male:23% and female:20%) and black (male:0% and female:7%).

The genetic dose effect of multi parallel feathering appears in the coat color of many BC_1 chickens after separation from wild-type (e^+) feathers from male heterozygotes and brown (e^b) heterozygotes of female parent F_1 (Utama *et al.* 2018).

Egg productivity
Egg productivity was the ability of chickens to produce eggs for a certain period. In this study, the productivity of BC_1 chicken eggs was observed for 23 weeks.
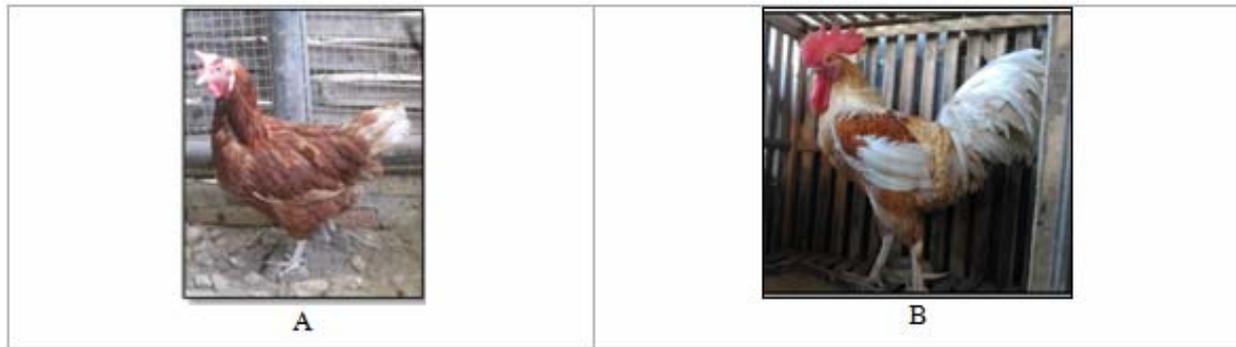
Figure 1 Parental crossbred chickens BC$_1$ (A) layer female (B) filial-1 male rooster (F$_1$ kamper rooster)

Figure 2 BC$_1$ chicken results from crossing layer × F$_1$ kamper chicken

The results of the study were compared with the eggs productivity of F$_1$, layer and pelung eggs carried out in the pre-study, which were referred to as the results of Ernanto's research (Ernanto, 2017).

The data obtained were graphs of egg productivity per week of the BC$_1$ chicken and compared with F$_1$, layer, and pelung chickens. Figure 3 was the data of cumulative egg production per chicken for 23 weeks.

Table 2 Chicken grouping calculations based on the number of eggs for 23 weeks

Item	Chicken group	N	Subset for alpha= 0.05		
			1	2	3
Tukey HSD[a,b]	Pelung	23	1.5848		
	BC$_1$	23	1.8940		
	F$_1$	22		3.6182	
	Layer	21			4.8395
	Sig.		0.781	1.000	1.000
Duncan[b]	Pelung	23	1.5848		
	BC$_1$	23	1.8940		
	F$_1$	22		3.6182	
	Layer	21			4.8395
	Sig.		0.348	1.000	1.000

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size= 22.219.
[b] The group sizes are unequal. The harmonic mean of the group sizes is used.
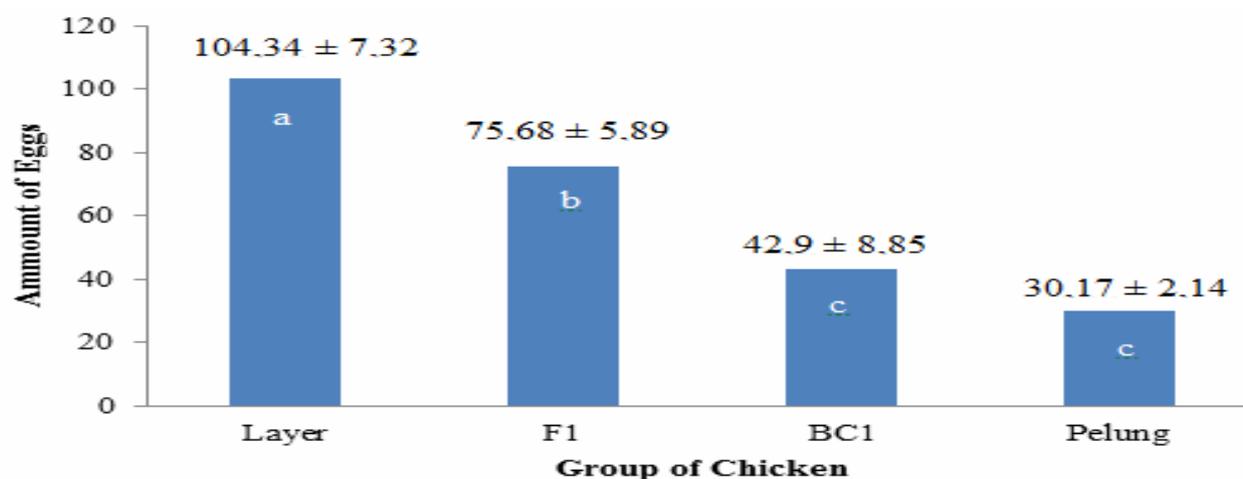
Figure 3 The cumulative egg production of layer, F$_1$ kamper, BC$_1$, and pelung for 23 weeks
Different letters indicate significantly different significance (P<0.05) (Results of F$_1$ kamper egg production, pelung and sulking layers in Ernanto's research (Ernanto, 2017))

The highest layer chicken was 104.34 eggs, the second-highest F$_1$ K amper chicken was 75.68 eggs, the third-highest BC$_1$ was only 42.9 eggs and the fourth-highest pelung chicken was 30.17 eggs.

Egg quality
Eggs were a source of protein, fat, minerals, and vitamins. The nutritional value of eggs was very complete, eggs were a good source of protein, were around 14%, so each egg contains 8 grams of protein. In this study, the observations were made on the external (shape) and internal (nutritional content) egg quality which included egg weight, shape index, shell thickness, and percentage of egg content (egg yolk, egg white, and shell).

Based on Figure 4, it shows that the average weight of layer chicken eggs was the highest, followed by BC1, then pelung chicken eggs.

Layer chicken had a high egg weight because the distribution of nutrients obtained by layer chicken leads more to eggs than to muscle growth and fat formation (Utama *et al.* 2018).

Egg Shape Index was defined as the ratio of width to egg length which was an important criterion in determining egg quality. Usually, local chicken eggs have an unusual shape, such as being too long and narrow, round or flat side, cannot be classified in AA grade (almost perfect) or A (slightly worse than AA). Eggs with round and shape have a bad appearance and do not fit when placed in the egg carton. This was related to eggs with such a form that will be more easily damaged when shipping, compared with eggs that have a normal shape (Sarica and Erensayin, 2014). In this study shape index observations were carried out on 3 groups of chickens: layer, BC$_1$ and pelung with 32 eggs, 32 eggs, and 29 eggs respectively.
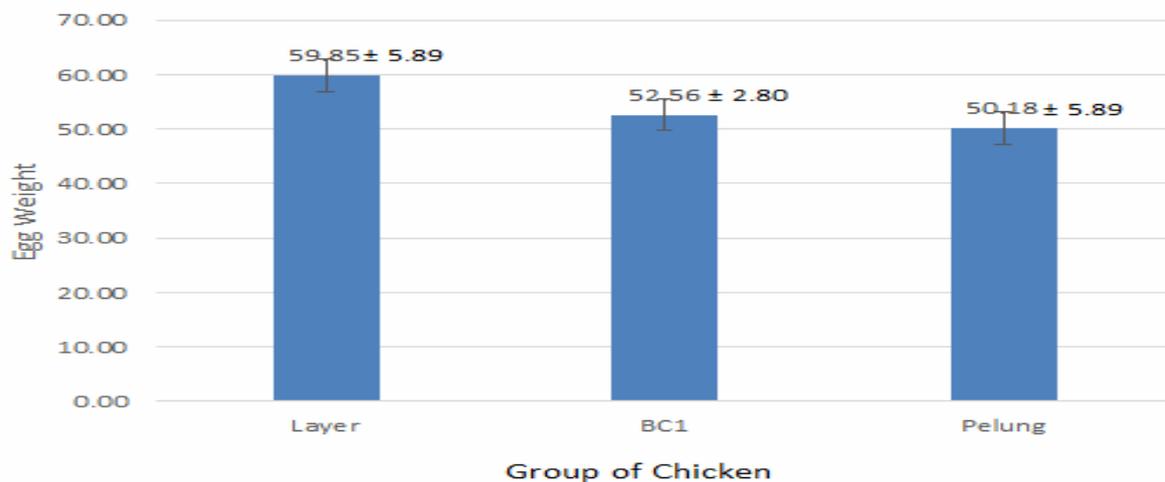
Figure 4 The average weight of layer, BC_1 and pelung eggs (n=34)

Layer chicken eggs and BC_1 eggs do not have a sharp shape. While pelung chicken eggs only have 1 egg that had a sharp shape. But based on Table 3, eggs that have normal shape only have a percentage of 31.25% for layers, 28.12% for BC1 chicken eggs, and 10.3% for pelung eggs. This indicates that most of the chicken eggs observed were not included in the egg quality standards that were good for sale (Liu *et al*. 2011).

Egg shell thickness
Shells are the outer layers of the egg and weigh up to 9-12% of the total weight of the egg. Shells are useful as the first defense from bacterial contamination. Shells consist mostly of calcium carbonate and a little magnesium carbonate and calcium phosphate. Eggshells are the outermost layer of eggs to prevent contamination from microorganisms, physical damage and evaporation. Shell thickness can be influenced by nutrients and mineral content and the same temperature. The thickness of the eggshell has an inverse relationship with the ambient temperature, high temperatures will affect the quality of the egg whites and affect the strength and thickness of the eggshell.

Shell thickness was very influential on egg damage. Factors that influence the quality of eggshells were influenced by drugs, diseases, and genetics. The normal thickness of the shell is 0.35-0.40 mm where if the thickness of the shell is better to minimize decomposition. The strength of the eggshell was very dependent on the mineral content and vitamins in the feed (calcium, phosphorus, manganese and vitamin D). When the feed was low in calcium the chicken would automatically produce eggs with thin shells or even without shells. In this study the thickness of chicken eggshell was observed using calipers.

Data collection was carried out on 10 chickens from each group layer, BC_1 and pelung, and presented in a graph (Figure 5).

Based on Figure 9, it can be observed that the thickness of the layer chicken eggshell is the highest, the second is BC1 and the third is pelung. With an average thickness of 0.5, 0.46, 0.41 mm, respectively. Thin eggshells were relatively had more and larger porous, thus accelerating the decline in egg quality that occurs due to evaporation (Lin *et al*. 2016). The thickness of the eggshell was influenced by chicken strains, age of the mother, feed, stress, and disease in the mother (Lin *et al*. 2016).

Chicken egg nutritional content
Proximate levels were the results of chemical analysis to identify the nutritional content of food and feed. Proximate observations were divided into six nutritional factions including water content, crude fat, crude fiber, crude protein, ash and extract material without nitrogen. Determination of egg proximate levels to determine the chemical composition of eggs. Protein, carbohydrates, fats, vitamins, minerals, and water are important nutrients that, when combined in the right proportion will support healthy living (Haryono, 2000).

Based on Table 4, the results of proximate analysis were observed including water, ash, fat, protein and carbohydrate observations. The results of the proximate analysis of BC_1 chicken eggs compared with the results of proximate chicken egg from the market. From the results of proximate analysis of BC_1 eggs, different values were obtained in the percentage of fat and carbohydrate. At the percentage of BC_1 egg fat obtained a value of 3.2% while in the chicken egg market 11.8%.

Table 3 The egg shape index of BC$_1$, layer, and pelung during 23 weeks production

Group of chicken	N	Round	Normal	Sharp	Min	Max	Mean	Sem
Layer	32	22	10	-	72.7	81.5	78.1	2.58
BC$_1$	32	23	9	-	72.7	83.9	78.5	2.98
Pelung	29	25	3	1	71.7	82.0	77.5	2.38

N: number of eggs; Min: lowest shape index value; Max: highest index shape value; Mean: average value of shape index and Sem: standard error of the average shape index.
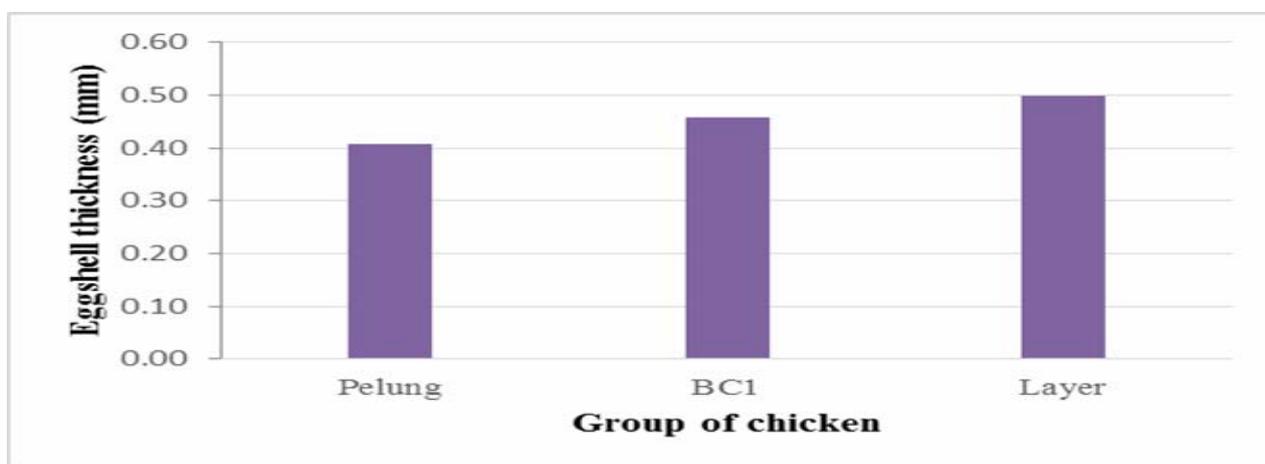
Figure 5 The thickness of pelung, BC$_1$, and layers of chicken eggshells

Table 4 The eggs proximate analysis of chicken BC$_1$

No	Type of analysis	Content	
		BC$_1$	Commercial chicken eggs (Romanoff and Romanoff, 1997)
1	**Water (%)**	76.1	73.6
2	**Dry component (%)**	23.9	26.4
A	Inorganic material:ash (%)	0.8	0.8
B	Organic material	23.1	25.6
a	Fat (%)	**3.2**	**11.8**
b	Protein (%)	10.8	12.8
c	Carbohydrates (%)	**9.1**	**0.7**
3	Calorie	**98.37 kal/100 g**	

Similarly, the percentage obtained on the value of carbohydrates. From the results of the proximate analysis test, the percentage of BC$_1$ chicken carbohydrate was higher, reaching 9.1% compared to the chicken eggs on the market, which was only 0.7%.

Insertion and deletion polymorphism of 24 bp on the PRL promoter

Insertion and deletion (indel) mutations are mutations caused by the addition and / or deletions (cutting of nucleotides) which caused the DNA to be frameshifted or shifted in the length of DNA band Kondrashov and Bogozin, 2004). In this study, the amplified target was that of the PRL promoter section which contained a 24 bp indel mutation in the 358th nucleotide using a specific primer. DNA targets that can be amplified are a band sized 130 base pairs (if deletions occur) and 154 base pairs (Figure 6).

In heterozygous samples, the two bands of DNA should be visualized, namely the short band/deletion and the long band/insertion, but the results show a pattern of four DNA bands. This was thought to be due to the occurrence of a mismatch when annealing PCR amplification resulting in heteroduplex DNA. A heteroduplex is a double-stranded DNA fragment when the two strands do not stick together perfectly due to a mismatch so that a loop will form in a double strand due to SNP or indel (Suceveanu *et al.* 2014) DNA double strand was not always stick perfectly. Different sequence variations in the pair can cause loops to form strands of DNA that interfere with the structure of the double helix (Figure 7). A loop on the DNA strand that interferes with the mobility of the strand as it passes through the gel matrix (Upchurch *et al.* 2000). Further analysis might perform a Sanger sequencing to see the position of the base sequence that undergoes recombination.
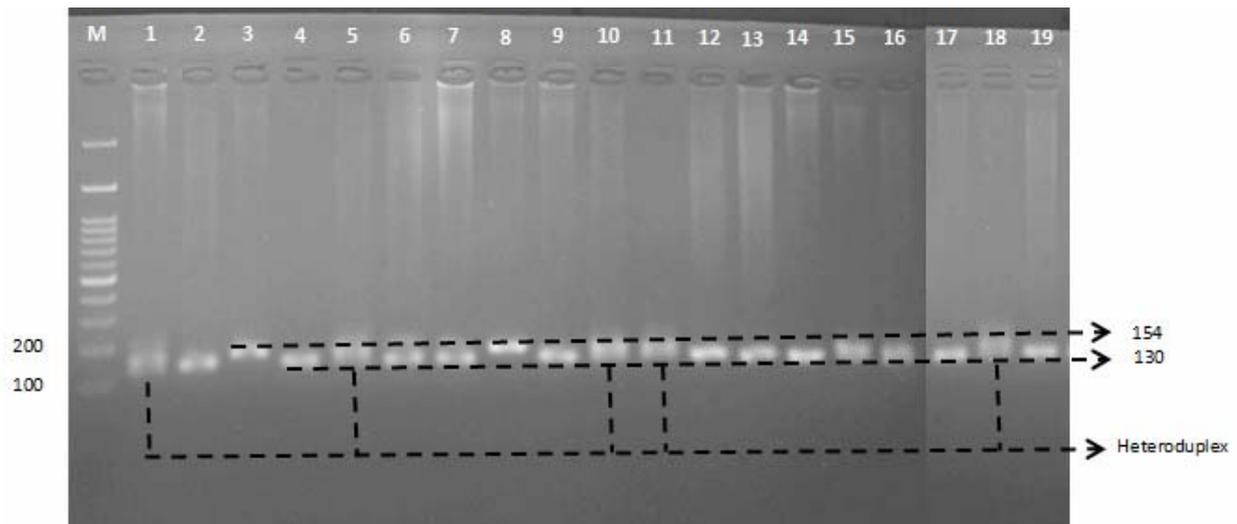
Figure 6 The indel polymorphism on PRL promoter (information M: marker; sequential 1-19: B34, B74, B33, M10, M67, P12, B4, M39, M31, P16, K42, K32, B36, K37, K43, X1, K35, K45, K34)
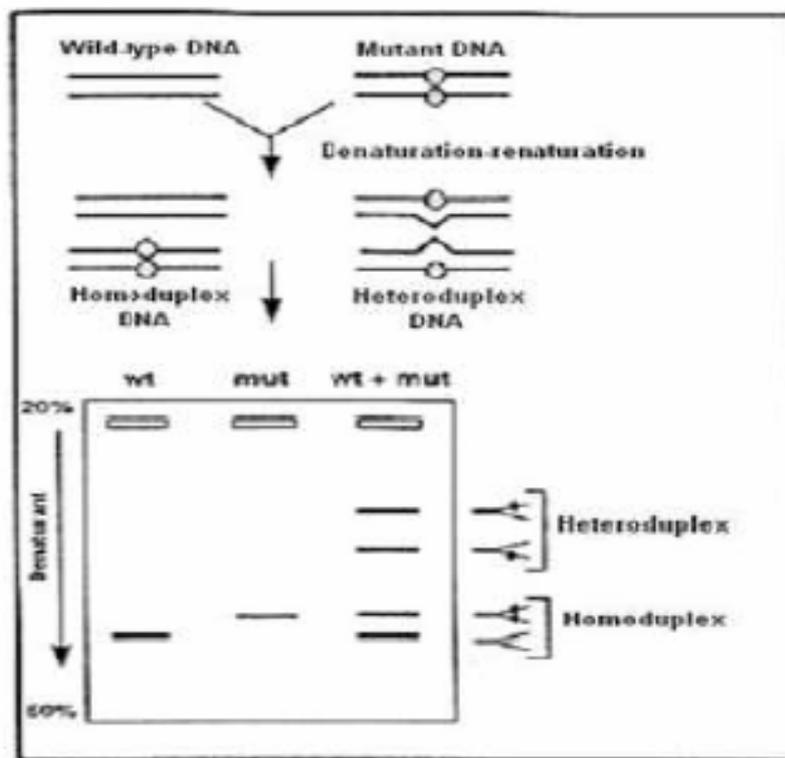

Figure 7 The illustration of normal and mutants DNA migration (Upchurch *et al.* 2000)

Based on Figure 8 and Table 5, there were three types of allele variations namely insertion homozygotes (II), deletion homozygotes (DD) and insertion deletion heterozygotes (ID).

From the results of allele frequency calculation, it was found that the heterozygous variation of insertion deletion genotype had the highest value, 0.4662, followed by the frequency of homozygous insertion allele with a value of 0.3969 and homozygous deletion had a frequency of 0,1369. However, the highest number of eggs was in the deletion homozygous genotype, which was 282 eggs, the second-highest number of eggs in the heterozygote genotype was 207 eggs and the number of eggs in the third-highest homozygous insertion genotype was 153.
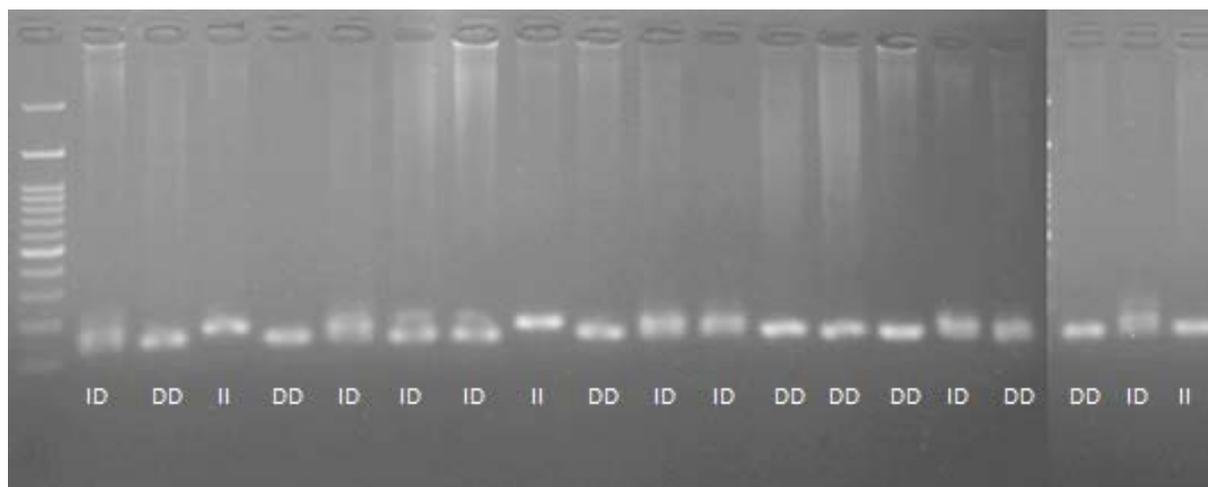
Figure 8 The variation of indel allele on PRL gene (information M: marker; sequential 1-19: B34, B74, B33, M10, M67, P12, B4, M39, M31, P16, K42, K32, B36, K37, K43, X1, K35, K45, K34)

Table 5 Frequency of genotype population of each group of chickens against Indel on PRL promoters

Type of chicken	Type of mutation	Allele code	Number of eggs (eggs)	Frequency of genotype	Conclusions of analysis results
BC$_1$	Insertion	II	153	0.3969	P > 0.05 Does not correlate
	Deletion	DD	282	0.1369	
	Heterozygotes	ID	207	0.4662	

After pearson correlation analysis, it was known that 24 bp prolactin promoter indel polymorphism did not correlate with the productivity of BC$_1$ chicken eggs resulting from crossing between F$_1$ male kamper and female layer. The results of the correlation analysis reached 0.521, this may be due to the amount of variation obtained in this study unbalanced between one type of mutation with another type of mutation, so the calculation deviation affects the P value on the correlation. Another allegation, namely maybe the indel part of the prolactin promoter does not directly affect egg production in a single gene (single gene) but there are other gene factors including external. However, from the analysis results obtained a degree of relationship value of 0.521 indicates there was no correlation between PRL polymorphisms and egg productivity. Similar results were also reported in previous studies conducted by Ernanto (2017). The results showed no correlation between PRL promoter polymorphism and kamper F$_1$ egg productivity. The study of Emamgholi-Begli *et al.* (2010) also found no correlation between 24 bp indel polymorphisms and the productivity of local chicken eggs from Yazd province, Iran. However, in the study of Cui *et al.* (2006) it was found that the productivity of chicken eggs from the crossing of Nongdahe and Taihe Silkies correlated to the 24 bp indel polymorphism of *PRL* gene. This indicates that the correlation between PRL promoter polymorphism and egg productivity was related to the observed chicken breeds.

CONCLUSION

In the study, it was obtained that *PRL* gene polymorphism has three types of allele (homozygous insertions, deletions, and heterozygous) with Pearson Correlation analysis obtained that there is no correlation between egg productivity and *PRL* gene polymorphism. In this case, need to be carried out the addition of observation time for egg productivity. In some samples, there is heteroduplex that also has allele I and D indicate that there are two forging in that sample.

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