Novel Mutation of Exon 5 Prolactin Gene in IPB-D1 Chicken

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ABSTRAK

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Gen prolaktin (PRL) merupakan salah satu gen yang mengontrol sifat mengeram dan produksi telur ᴘada ayam petelur. Sifat mengeram akan menurunkan produksi telur dan mengganggu sistem reproduksi pada ayam lokal. Tujuan dari penelitian ini yaitu mengidentifikasi keragaman gen prolaktin pada ayam IPB-D1 dengan menggunakan metode *direct sequencing*. Analisis keragaman pada gen prolaktin ekson 5 dilakukan pada 46 sampel DNA ayam IPB-D1 yang merupakan koleksi Divisi Genetika dan Pemuliaan Ternak, Fakultas Peternakan IPB. Sekuens DNA sebagai acuan untuk merancang primer ekson 5 didapatkan dari *National Center for Biotechnology Information* (NCBI) dengan kode akses GenBank AF288765.2. Ekstraksi DNA dilakukan dengan menggunakan teknik *phenol-chloroform*. Amplifikasi DNA menghasilkan produk PCR dengan ukuran 557 pb. Pada penelitian ini dihitung frekuensi genotipe, frekuensi alel, dan nilai heterozigositas serta keseimbangan Hardy-Weinberg. Hasil penelitian menemukan 5 SNP pada ekson 5, yaitu g.7823A>G, g.7835A>G, g.7886T>A, g.8052T>C, dan g.8069T>C. Seluruh SNPs bersifat polimorfik dan berada dalam keseimbangan Hardy-Weinberg kecuali g.8052T>C. Mutasi basa g.7823A>G, g.7835A>G, g.8052T>C merupakan mutasi *synonymous* yang tidak mengubah asam amino, sedangkan mutasi basa g.7886T>A dan g.8069T>C merupakan SNP *non-synonymous* yang mengubah asam amino sehingga dapat dijadikan sebagai kandidat *marker assisted selection* sifat produksi telur pada ayam IPB-D1.

Kata Kunci: Ayam IPB-D1, Gen Prolaktin, Mutasi, SNP

ABSTRACT

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The prolactin gene (PRL) is a gene that controls the incubation and egg production in laying chickens. The nature of incubation will reduce egg production and disrupt the reproductive system in local chickens. The purpose of this study was to identify the polymorphism of prolactin genes in IPB-D1 chickens using the direct sequencing method. The polymorphism of the exon 5 prolactin gene was carried out on 46 samples of IPB-D1 chicken DNA which was a collection of the Division of Animal Genetics and Breeding, Faculty of Animal Science IPB. DNA sequences as a reference for designing exon 5 primers were obtained from the National Center for Biotechnology Information (NCBI) with the GenBank access code: AF288765.2. DNA extraction was carried out using the phenol-chloroform technique. DNA amplification resulted in a PCR product with a size of 557 bp. In this study, the genotype frequency, allele frequency, heterozygosity value and Hardy-Weinberg equilibrium were calculated. The results of the study found 5 SNPs in exon 5, namely g.7823A>G, g.7835A>G, g.7886T>A, g.8052T>C, and g.8069T>C. All SNPs are polymorphic and in Hardy-Weinberg equilibrium except g.8052T>C. The g.7823A>G, g.7835A>G, g.8052T>C SNPs are synonymous mutations that do not change amino acids, while the g.7886T>A and g.8069T>C SNPs are non-synonymous that change amino acids. Both g.7886T>A and g.8069T>C SNPs are potential as a marker assisted selection for the characteristics of egg production in IPB-D1 chickens.

Key Words: IPB-D1 Chicken, Mutation, Prolactin Gene, SNP

INTRODUCTION

In 2019, the native chicken population in Indonesia was approximately 8.37% of the national chicken population (DJPKH 2019). This contributed 6.01% of the total national meat production in 2018 which decreased compared to those in 2017 (DJPKH 2019).

The low productivity of native Indonesian chicken eggs is one of the challenges of the native Indonesian chicken industry. Sinurat et al. (1992) in: Hidayat & Asmarasari (2015) reported that the egg production of native chicken that is extensively reared is up to 30.20 eggs per chicken per year. Meanwhile, with an intensive maintenance system, the egg production of native

chicken eggs reaches 80.30 eggs per chicken per year (Sinurat et al. 1992). One of the solutions to increase the productivity of local chickens is the invention of a novel crossbreed chicken, namely IPB-D1 (Ministry of Agriculture Reg No. 693/KPTS/PK.230/M/9/2019). IPB-D1 chicken is a result of crossbreeding between F1 ♂Pelung x ♀Sentul and F1 ♂Kampung x ♀Parent Stock Cobb. The superiority of this chicken is its fast growth, which reaches 1.18 ± 0.2 kg (male) or 1.04 ± 0.12 kg (female) slaughter weight at the age of 10-12 weeks. In addition, this chicken also resistant to New Castle disease (Sumantri & Darwati 2017).

The IPB-D1 composite chickens also showed the superiority of reproduction and egg production. Cholifah (2018) reported that crossbreeding between pelung, sentul, Kampung, and broiler chickens produced higher fertility, higher hatchability, and lower mortality than Kampung chicken. In addition, the crossbreeding between Kampung chicken with broilers has a low incubation capacity that is able to produce a higher egg production. The production and reproduction of the IPB-D1 chicken in the 3rd generation, in this case, is explained by Sumantri & Darwati (2017). It is mentioned that the age of first laying eggs is 24 weeks with bodyweight of 1.80±0.23 kg. Meanwhile, egg production of the chicken is 45.20% with the egg weight which is 33.50±0.97 grams. Furthermore, the egg fertility of IPB-D1 chicken is 84.25% with 64.70% of hatchability (Sumantri & Darwati 2017). In further research, Habiburahman et al. (2020) mentioned that the egg production of this chicken increased to 49.22% in the 7th generation. The increase in egg production of IPB D-1 G7 chickens was due to the genetic percentage of broilers as much as 25%. The 25% genetic contribution of purebred chickens has led to an increase in egg production of IPB D-1 chicken 7th generation compared to local chickens in general.

To overcome the low production of native chicken eggs among other things, influenced by the nature of the incubation, it can be done by reducing or eliminating the broody nature of the chicken. Molecular selection can be done quickly and as early as possible through the marker assisted selection of natural brooding. The prolactin gene (PRL) is a gene that controls the incubation and egg production in laying chickens. Prolactin is a single chain polypeptide hormone that belongs to a family of growth hormone genes and is generally synthesized in the anterior pituitary in all vertebrate animals (Sharp et al. 1979). In aves, it has been reported that prolactin has many important roles in the regulation of physiological processes, including egg production, stimulating and maintaining the nature of the incubation, osmoregulation, immune-modulation, function and development of gonad cells (Plant & Zeleznik 2014). Sherwood et al. (2005) mentioned that prolactin is a gene that controls the nature of brooding

because prolactin is an important part of the functioning of neuroendocrine which stimulates the occurrence of incubation. This incubation event is caused by the prolactin promoter functioning to activate the initial transcription of the prolactin gene expression. Mutations in the prolactin promoter are reported to affect the activity of the prolactin gene in the incubation process (Sartika 2005). In this case, the prolactin gene (PRL) has a major role in the nature of the incubation and egg production in chickens. Therefore prolactin gene can be used as genetic markers in Indonesian local chicken selection programs, especially in IPB-D1 chickens. This study aims to identify the polymorphism of prolactin genes (PRL) in IPB-D1 chickens.

MATERIALS AND METHODS

Animals

All procedures performed in this study were approved by the Animal Care and Use Committee (ACUC) of IPB University (ACUC No: 163-2019 IPB). A number of 46 IPB-D1 chickens (24 weeks-old, hen) were collected from Genetics and Animal Breeding Division, Faculty of Animal Science, IPB. All chickens were kept under uniform conditions with a uniform feed of protein and energy balance. Feed and water were given *ad libitum*. Blood samples were taken from the pectoralis vein in the wing area. From this process, a total of 1.0-1.5 ml samples were taken using a syringe. Blood is put into a tube containing K3-EDTA *(ethylenediamine tetraacetic)* anticoagulant and then stored in a refrigerator at 4 °C until it is ready for DNA extraction.

Primer designing

The primer sequences were designed using the Primer3 and BLAST Primer applications from NCBI to obtain the primer target according to Ye et al. (2012). It also used the Primer Stats application for primer compatibility test. The length of the PCR product was 557 bp and it is located in exon 5. Sequential data for the primer design were obtained from the National Center for Biotechnology Information (NCBI) with the GenBank access code: AF288765.2. The primer sequence used in this was the author's design with the following primers. The forward primer sequence was (F): 5'-TGGAGGAGGCCAAAAAGAGATG-3' and reverse primer sequence was (R): 5'-GCAGCCCACAGGTACTTAGC-3'.

DNA isolation

Genomic DNA was extracted by using Phenol-chloroform technique (Sambrook & Russell 2001) and

modified by adding cells buffer lysis (250 µl 1 x STE, 40 µl SDS, and 10 µl proteinase-K). The DNA was purified by adding 40 µl 5M NaCl, 400 µl phenol, and 400 µl CIAA (Chloroform Iso Amyl Alcohol) and precipitated by using 40 µl 5M NaCl and 800 µl ethanol absolute. The precipitation was washed once by adding 800 µl of 70% ethanol and centrifuged with a speed of 12.000 rpm for 5 min. The ethanol was discarded and evaporated, then the precipitated DNA was dissolved in 100 µl of 80% TE (Elution buffer).

DNA amplification

The amplification of PRL gene fragments was done by PCR machine. The DNA sample to be amplified was transferred into a 0.2 ml tube with a sample volume of 0.5 µl. After the sample was added the 14 µl premix containing 0.4 µl primers, 12.5 µl Green Master mix, and 11.1 µl Nuclease Free Water. Then the sample was homogenized and put into the ESCO Swift Maxi Thermal Cycler machine. The PCR technique was carried out through 3 stages. The first stage was the cycle of the temperature predenaturation process of 95ºC for 5 minutes and the second stage, it was carried out 35 cycles consisting of denaturation (95ºC) for 10 seconds, annealing (57ºC) for 20 sec, and extension (72ºC) for 30 seconds. Meanwhile, the third stage was in the form of a final extension (72ºC).

Direct sequencing and genotyping

Direct sequencing was carried out from two directions, forward and reverse. Sequence products were obtained by using services from 1st Base Selangor, Malaysia. The sample sequencing results were then verified through alignment by multiple alignment ClustalW in MEGA-X (Kumar et al. 2018). The alignment of IPB-D1 chicken samples was carried out using BioEdit programs to identify the polymorphism in this study (Alzohairy 2011). IPB-D1 chicken sequences were compared with GenBank from the National Center for Biotechnology Information (NCBI) with access code of the GenBank AF288765.2. The sequencing chromatogram results are scanned using FinchTV programs to identify heterozygous of the polymorphic positions which is marked by a double peak on the chromatograms (Treves 2010).
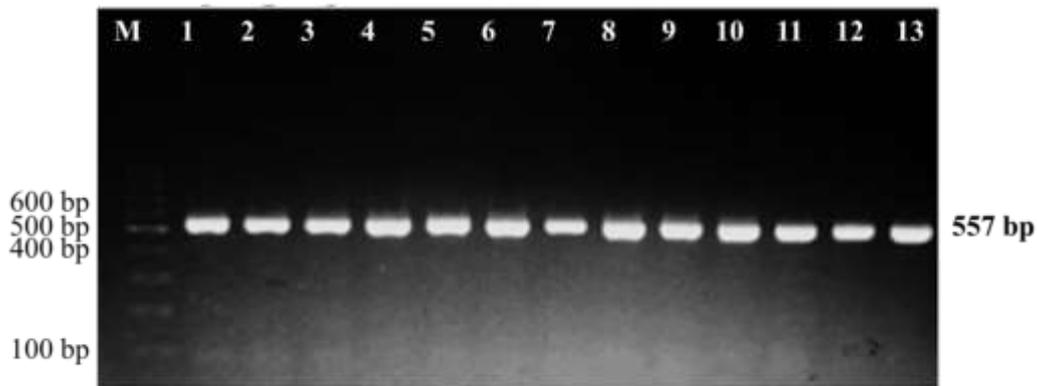

Figure 1. PCR amplification product of PRL gene (557 bp); M= DNA ladder 100 bp; 1-13 = IPB-D1 chicken samples

Data analysis

Genetic diversity analysis was performed by calculating genotype frequency and allele frequency. In addition, the chi-square value and heterozygosity values were also calculated. The formula for calculating allele frequencies and genotype frequencies according to Nei and Kumar (2000) is as follows.

$$X_i = \frac{(2N_{ii}+N_{ij})}{(2N)}; \qquad X_j = 1-X_i$$

Where, X_i = Frequency of the ith allele; X_j = Frequency of the jth allele; N_{ii} = Number of samples with genotype ii; N_{ij} = Number of samples with ij genotypes; and N = Number of samples.

Meanwhile, for the genotype frequency was calculated using the following formula.

$$X_{ii} = \frac{N_{ii}}{N} \times 100\% \qquad X_{ij} = \frac{N_{ij}}{N} \times 100\% \qquad X_{jj} = \frac{N_{jj}}{N} \times 100\%$$

Where, X_{ii} = Frequency of the ii^{th} genotype; X_{ij} = Frequency of the jth genotype; and X_{jj} = Frequency of ij^{th} genotype.

Furthermore, genetic diversity was calculated using the frequency of observed heterozygosity (Ho) and expected heterozygosity (He) was calculated using the Weir (1996) formulas as follows.

$$H_o = \sum_{i \neq j} \frac{n_{ij}}{N} \; ; H_e = 1 - \sum_{i=j}^{q} X_i^2$$

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Where, Ho= heterozyegosity observations (population); He = value of expected heterozygosity; nij= number of heterozygous individuals; N = number of individuals observed; Xi = allele frequency; and q = number of alleles.

Genotype frequency deviations that arise from Hardy Weinberg's equilibrium were analyzed using the chi-square test (x^2) based on the Nei and Kumar (2000) formula as follows.

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

Where, X^2= chi-square test; O = Frequency of observed sample genotypes; and E = frequency of expected genotype.

RESULTS AND DISCUSSION

Discovery single nucleotide polymorphisms of exon 5 prolactin gene

The results of prolactin gene sequencing are shown in Figure 2. Sequencing results indicated the same mutation position based on Osman et al. (2018), Erehehuara (2003), and Li et al. (2013), i.e. g.8052T>C. Furthermore, 4 novel of mutation points were also found, namely g.7823A>G, g.7835A>G, g.7886T>A, and g.8069T>C.

Single Nucleotide Polymorphism (SNP) is a form of differences in genetic material variation between two individuals in the form of a single nucleotide in a series of DNA nucleotide base sequences at specific locations in the genome (Murray et al. 2014). The mutation point positions on exon 5 found in this study are g.7823A>G, g.7835A>G, g.7886T>A, g.8052T>C, and g.8069T>C which all of them are classified as substitution mutations. In Figure 3, a partial sequence of prolactin gene mutations is described. Nucleotide base substitution mutations are divided into transition and transversion substitution mutations. The base mutations of g.7823A>G, g.7835A>G, g.8052T>C, and g.8069T>C classified as a transition mutation, which changes purine with other purine (A>G) or pyrimidine with other pyrimidine (C>T). Whereas transversion are purine substituted with pyrimidine bases or vice versa (Luo et al. 2016). Transition mutations are common, although most of them repaired by various proof-reading function (Dubey 2014).

Adenine-Thymine in DNA conformation has two hydrogen bridges (Muladno 2010). Hydrogen bonds are non-covalent interactions that have small free energy of 2-6 kj mol^{-1} in water, so that the strength of a weak bridge will be easily split and put back together (Ferst 2017). Changes in the structure of hydrogen bonds can affect DNA transcription and changes in the structure or shape of protein molecules produced. According to Luo et al. (2016), transversion mutations are more influential on changes in the structure and function of regulation of a gene than with transition mutations.

Each mutation that appears will affect 1 or more roles of the encoded protein. The role of proteins that may be affected by mutations is stability or folding protein, ligand binding protein, catalysis, regulation with allosteric and other mechanisms as well as post-translational protein modification (Nagasundaram et al. 2015). Overall changes in amino acids from the prolactin gene mRNA sequence as a result of translation are presented in Table 1.

Table 1. Amino acids changes from the prolactin gene mRNA sequence as a result of translation
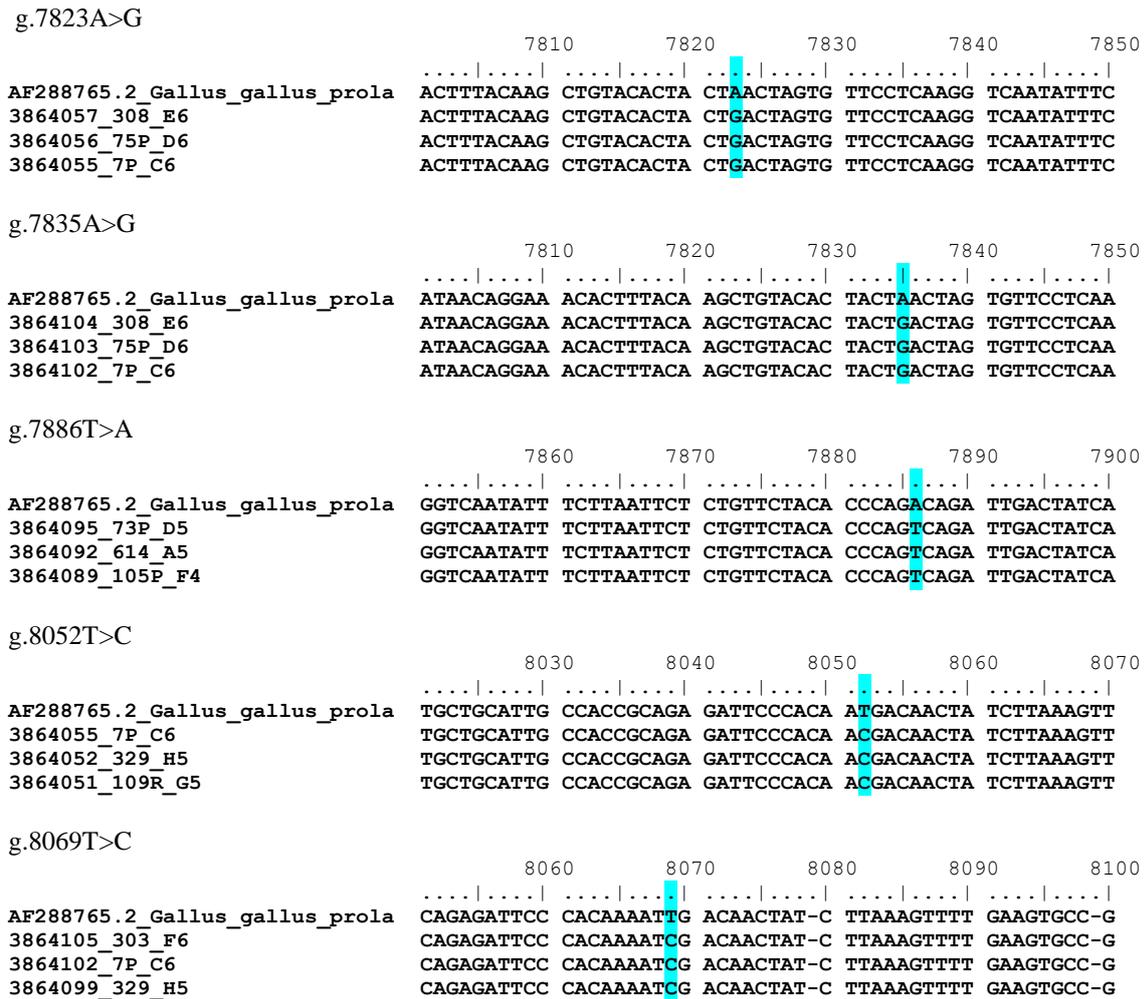
SNPs Position	Amino Acids Changes		
g.7823A>G	Lysine	>	Lysine
g.7835A>G	Leucine	>	Leucine
g.7886T>A	Aspartate	>	Valine
g.8052T>C	Isoleucine	>	Isoleucine
g.8069T>C	Leucine	>	Serine

The g.7823A>G (Lys>Lys), g.7835A>G (Leu>Leu) and g.8052T>C (Ile>Ile) SNPs are synonymous mutations that do not change amino acids. Synonymous mutations can encode sequences with the same amino acid composition, but the structure and function of the protein can change by influencing the mechanism of RNA transcription, mRNA structure, and translational speed (Supek et al. 2014; (Sauna & Kimchi-Sarfaty 2011).

The g.7886T>A (Asp>Val) and g.8069T>C (Leu>Ser) SNPs are non-synonymous mutations that convert amino acids. The g.7886T>A changes the aspartate amino acid to valine. The structure of these amino acids was changed where valine is one of three branched-chain amino acids (the others are leucine and isoleucine) that enhance energy, increase endurance, and aid in muscle tissue recovery and repair (NCBI 2020). This group also lowers elevated blood sugar levels and increases growth hormone production in chicken (Nascimento et al. 2016). The g.8096T>C changes the leucine amino acid to serine. This amino acid changes involved in the functioning of RNA and DNA, in the muscle formation as well as in the maintenance of a proper immune system (NCBI 2020b).

Based on the mechanism of the prolactin gene action, mutations in DNA sequences can change the structure of the resulting protein so that it can affect the signaling pathway of the prolactin gene, especially in the process of attaching prolactin to its receptors. The prolactin pathway is initiated by attaching prolactin to its receptors. Prolactin receptors will induce signals and binds to the signal transduction also activates the protein transcription factor (Radhakrishnan et al. 2012).

Stat proteins (signal transducers and activators of transcription) will dissociate with their receptors and

g.7823A>G

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                         7810       7820       7830       7840       7850
                         ....|....| ....|....| ..:|....| ....|....| ....|....|
AF288765.2_Gallus_gallus_prola  ACTTTACAAG CTGTACACTA CTAACTAGTG TTCCTCAAGG TCAATATTTC
3864057_308_E6                  ACTTTACAAG CTGTACACTA CTGACTAGTG TTCCTCAAGG TCAATATTTC
3864056_75P_D6                  ACTTTACAAG CTGTACACTA CTGACTAGTG TTCCTCAAGG TCAATATTTC
3864055_7P_C6                   ACTTTACAAG CTGTACACTA CTGACTAGTG TTCCTCAAGG TCAATATTTC
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g.7835A>G

```
                         7810       7820       7830       7840       7850
                         ....|....| ....|....| ....|....| ....|....| ....|....|
AF288765.2_Gallus_gallus_prola  ATAACAGGAA ACACTTTACA AGCTGTACAC TACTAACTAG TGTTCCTCAA
3864104_308_E6                  ATAACAGGAA ACACTTTACA AGCTGTACAC TACTGACTAG TGTTCCTCAA
3864103_75P_D6                  ATAACAGGAA ACACTTTACA AGCTGTACAC TACTGACTAG TGTTCCTCAA
3864102_7P_C6                   ATAACAGGAA ACACTTTACA AGCTGTACAC TACTGACTAG TGTTCCTCAA
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g.7886T>A

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                         7860       7870       7880       7890       7900
                         ....|....| ....|....| ....|....| ....|.:..| ....|....|
AF288765.2_Gallus_gallus_prola  GGTCAATATT TCTTAATTCT CTGTTCTACA CCCAGACAGA TTGACTATCA
3864095_73P_D5                  GGTCAATATT TCTTAATTCT CTGTTCTACA CCCAGTCAGA TTGACTATCA
3864092_614_A5                  GGTCAATATT TCTTAATTCT CTGTTCTACA CCCAGTCAGA TTGACTATCA
3864089_105P_F4                 GGTCAATATT TCTTAATTCT CTGTTCTACA CCCAGTCAGA TTGACTATCA
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g.8052T>C

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                         8030       8040       8050       8060       8070
                         ....|....| ....|....| ....|....| .:..|....| ....|....|
AF288765.2_Gallus_gallus_prola  TGCTGCATTG CCACCGCAGA GATTCCCACA ATGACAACTA TCTTAAAGTT
3864055_7P_C6                   TGCTGCATTG CCACCGCAGA GATTCCCACA ACGACAACTA TCTTAAAGTT
3864052_329_H5                  TGCTGCATTG CCACCGCAGA GATTCCCACA ACGACAACTA TCTTAAAGTT
3864051_109R_G5                 TGCTGCATTG CCACCGCAGA GATTCCCACA ACGACAACTA TCTTAAAGTT
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g.8069T>C

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                         8060       8070       8080       8090       8100
                         ....|....| ....|.:.| ....|....| ....|....| ....|....|
AF288765.2_Gallus_gallus_prola  CAGAGATTCC CACAAAATTG ACAACTAT-C TTAAAGTTTT GAAGTGCC-G
3864105_303_F6                  CAGAGATTCC CACAAAATCG ACAACTAT-C TTAAAGTTTT GAAGTGCC-G
3864102_7P_C6                   CAGAGATTCC CACAAAATCG ACAACTAT-C TTAAAGTTTT GAAGTGCC-G
3864099_329_H5                  CAGAGATTCC CACAAAATCG ACAACTAT-C TTAAAGTTTT GAAGTGCC-G
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Figure 2. Alignmented of prolactin gene at exon 5 in IPB-D1 chicken showing 5 Single Nucleotide Polymorphisms (SNPs). Blue highlights show mutation point of each SNP

translocation will occur to the nucleus to bind to the target gene for the promoter prolactin. Attachment to the regulatory part of prolactin will regulate the active and inactive mechanism of the prolactin gene (Radhakrishnan et al. 2012). Thus, when a DNA sequence mutation occurs that changes the structure of the protein, it can cause inactivation of the prolactin gene

Genotype frequency, allele frequency, and prolactin gene heterozigosity

In this study, the chi-square analysis (χ^2) was conducted to determine whether the population is in Hardy-Weinberg equilibrium. In addition, this analysis was also carried out since in an experiment, often the results of crossing carried out offsprings that are not in accordance with Mendel's law. The population is mentioned to be in equilibrium if the calculated value of χ^2 is smaller than χ^2 table. A population that is in equilibrium means that the population has not yet experienced mutations, migration, directed marriages, selection, and large sample sizes (Castle 1903).

Heterozygosity is a parameter used to measure the level of genetic diversity in a population based on the allele frequency of each locus (Wang et al. 2015). Objective heterozygosity (Ho) is the average number of individuals with polymorphic locus based on observations, while expected heterozygosity (He) is an estimate of genetic diversity based on the results of allele frequency calculations. The observed heterozygosity value is greater than the expected heterozygosity value. These findings indicate that the population is diverse. High genetic diversity has heterozygosity values of more than 0.50 (Karabağ et al. 2016). The results of genotype frequency, allele frequency, heterozygosity, and chi-square analysis (χ^2) of the prolactin gene at various locus are presented in Table 2.

Genetic diversity in populations is illustrated by 3 diversity indices, namely the number (percentage) of polymorphic loci in the population, average.
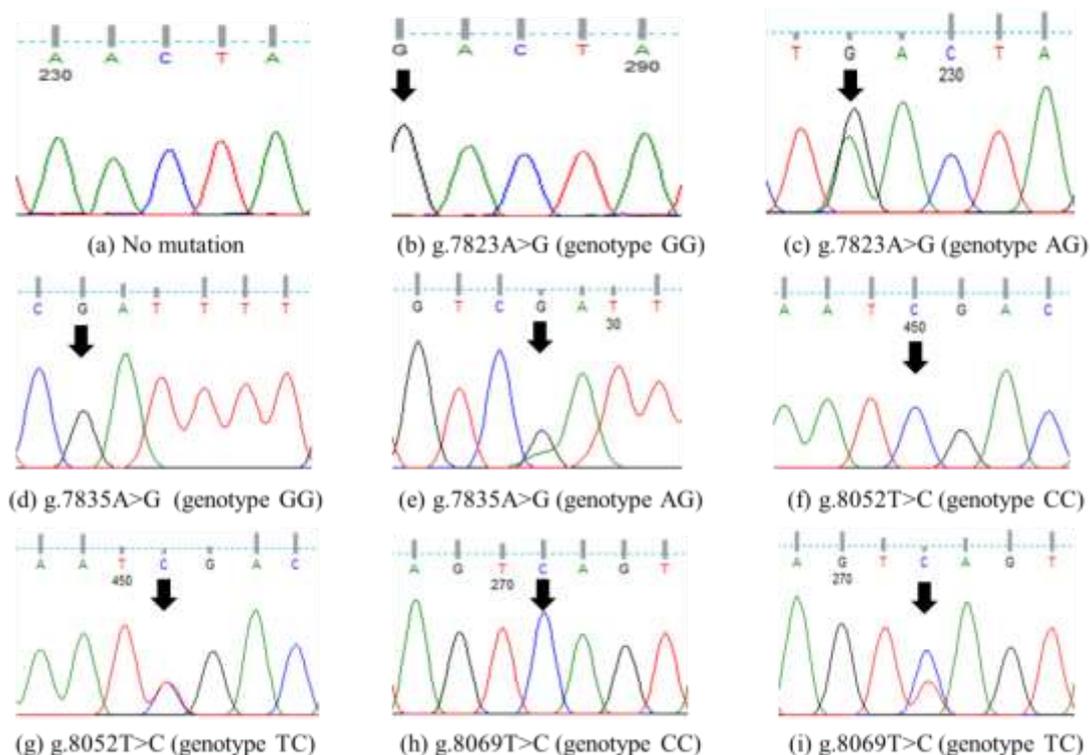
Figure 3. Partial sequencing maps of exon 5 prolactin gene showing transition mutations in IPB-D1 chicken. Arrows show mutation point.
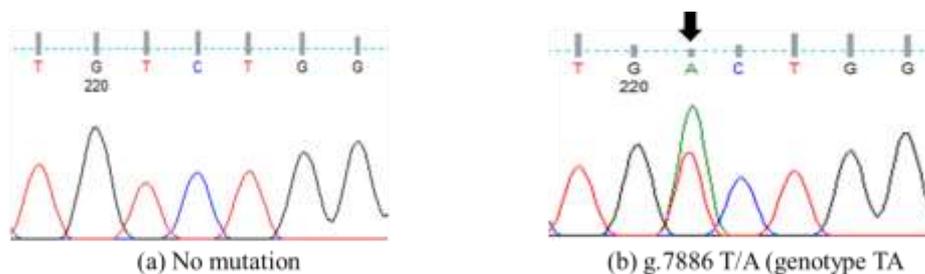

Figure 4. Partial sequencing maps of exon 5 prolactin gene showing transversion mutation in IPB-D1 chicken. Arrow shows mutation point.

Table 2. Genotype frequency, allele frequency, heterozygosity, and chi-square test (χ^2) prolactin gene values at various locus in IPB-D1 chicken

SNP	N	Genotype frequency (n)			Allele frequency		χ^2	H_o	H_e
g.7823 A>G	46	AA 0.50 (23)	AG 0.37 (17)	GG 0.13 (6)	A 0.68	G 0.32	0.95^{ns}	0.43	0.37
g.7835 A>G	46	AA 0.22 (10)	AG 0.57 (26)	GG 0.22 (10)	A 0.50	G 0.50	0.78^{ns}	0.57	0.50
g.7886 T>A	46	TT 0.91 (42)	TA 0.09 (4)	AA 0.00 (0)	T 0.96	A 0.04	0.14^{ns}	0.09	0.08
g.8052 T>C	46	TT 0.69 (32)	TC 0.22 (10)	CC 0.09 (4)	T 0.80	C 0.20	4.27^{*}	0.22	0.31
g.8069 T>C	46	TT 0.26 (12)	TC 0.57 (26)	CC 0.17 (8)	T 0.46	C 0.54	0.89^{ns}	0.57	0.50

N = total sample; (n) = number of samples on the genotype. *=significantly different at P<0.05 ($x^2_{0.05}$=3.84), ns=non significant

heterozygosity at Hardy-Weinberg equilibrium, and allele frequencies (Nei & Kumar 2000). Allele frequency is a measure of the relative frequency of an allele in a population that shows genetic diversity. An allele can be said to be polymorphic if it has the same frequency or less than 0.99 (Hartl & Clark 2000).

The results of the study found five points mutation on exon 5: i.e g.7823A>G, g.7835A>G, g.7886T>A, g.8052T>C, and g.8069T>C. The g.7823A>G and g.7835A>G SNPs produced 3 genotypes: AA, AG, and GG. The g.7823A>G had a higher AA (50.00%) genotype frequency compared to the AG (36.96%) and GG (13.04%) (Table 2). The high frequency of the AA genotype resulted in a higher range of the A allele frequency (0.68) compared to the G allele frequency (0.32) which indicated that the locus was polymorphic. Allele frequency is a measure of the relative frequency of an allele in a population that shows genetic diversity. An allele is classified as polymorphic if it has a frequency equal to or less than 0.99 (Hartl & Clark 2000). The results of the Hardy-Weinberg equilibrium test showed that the results were not significantly different (P> 0.05) or were in the Hardy-Weinberg equilibrium. This result is in line with the results of the heterozygosity value in IPB-D1 chickens which showed that the observed heterozygosity value (Ho) was higher than the value of expected heterozygosity (He), which means that the population that has mutations at the locus g.7823A>G varied.

The g.7835A>G locus also showed polymorphic properties in IPB-D1 chickens. The frequency of the AG genotype (57.00%) was higher than the AA (22.00%) and GG (22.00%) genotypes (Table 2), resulting in balanced A (0.50) and G (0.50) allele frequency. The results of the x^2 were in the Hardy-Weinberg equilibrium (P>0.05) which indicated that the observed heterozygosity value was higher than the expected heterozygosity value, which meant that the population that had mutations at locus g.7835A>G varied.

SNP at locus g.7886T>A was found only 2 genotypes, namely TT (91.30%) and TA (8.70%). The high frequency of the TT genotype resulted in a higher range of the T allele (0.96) than the A allele (0.04), and the frequency magnitudes of these two alleles showed polymorphic. The genotype frequency of SNP locus g.7886T>A based on the chi-square value (χ^2), the IPB-D1 chickens were in Hardy-Weinberg equilibrium (P>0.05). This is reinforced by the results of the calculation of the heterozygosity value obtained in IPB-D1 chickens, that the observed heterozygosity value is greater than the expected heterozygosity value which means that the population at the locus is polymorphic.

SNP at locus g.8052T>C found 3 kinds of genotypes: TT (69.57%), TC (21.74%), and CC (8.70%) (Table 2). The high frequency of TT genotypes

resulted in higher T allele (0.80) compared to C allele (0.20) so that the g.8052 locus was also polymorphic in IPB-D1 chickens. The results of the Hardy-Weinberg balance test with the chi square test (χ^2) indicate that they are in Hardy-Weinberg equilibrium (P>0.05). The g.8069T>C locus also produced 3 genotypes: TT (26.09%), TC (56.52%), and CC (17.39%) (Table 2). The frequency of the C allele (0.54) is greater than the T allele (0.46) and shows the polymorphism of the prolactin gene in IPB-D1 chickens. Allele frequencies in the five SNPs identified have a value ≤0.99. Therefore it can be concluded that the prolactin gene in chicken IPB-D1 is polymorphic. An allele is classified as polymorphic if it has an allele frequency ≤0.99 (Hartl & Clark 2000).

In this current study, based on allele frequencies, the PIC values calculated the result ranged from 0.08 (g.7886) to 0.50 (g.7835 and g.8069) and the average PIC values were calculated from overall locus as 0.37. DNA markers showed an average 0.25 < PIC value < 0.50, which confirms that the marker is reasonably informative (Botstein et al. 1980). The Hardy-Weinberg equilibrium test results, which have been carried out, are known to be in Hardy-Weinberg equilibrium (P> 0.05) which means that the observed heterozygosity values are higher than the expected heterozygosity values. From these findings, it can be said that the population that has mutations at the locus is diverse. This is possible because of several factors, including the lack of optimal selection efforts for chickens from IPB-D1. Castle (1903) states that a large population will not change from one generation to another if there is no selection, migration, mutation, and genetic drift.

The SNP of the exon 5 of the prolactin gene has actually been previously studied and found SNP at position g.8052T>C in local Chinese chickens, namely Qinyuan Patridge chickens and in recessive white chickens (Erehehuara 2003; Li et al. 2013). In addition, non-synonymous SNP position g.7921C>T was found which changed the amino acid Ser into Pro and SNP synonymous at position g.8187C>T in the F15 hubbard chicken (Osman et al. 2018). The results of SNP identification at g.8052T>C in this study are in accordance with the SNP findings of Li et al. (2013) and Erehehuara (2003). Li et al. (2013) showed in the results of his study that the position of SNP g.8052T>C was significantly related to the first age of the chicken laying eggs and the amount of egg production at the age of the first 300 days laying eggs. The g.8052T>C SNP located in the coding region of the gene, but does not change the amino acid sequence, although the egg production traits between two chicken breeds were significantly different, the allele frequencies of the two sites in both breeds were close and only significant genotypic effects existed. The association analysis suggested that there were significant associations

between T8052C and G8113C genotypes of PRL gene and the egg production traits of AFE and EN 300. Moreover, the breed effect and the interaction effect between breeds and genotypes are not significant, and therefore, it was concluded that there may be a relationship between these two SNP sites and egg production traits in chickens. In this study, the H2H3 diplotype was also found to be associated with more egg production traits than other diplotypes (H2H2, H2H4 and H3H3), indicating that H2H3 diplotype may be the most advantageous haplotypes for egg production. Erehehuara (2003) also mentioned that SNP g.8052T>C and g.8113G>C greatly influenced egg production in white leghorn and brown hy-line chickens, that the frequency of A1A1 genotype, was 1.0 and 0.84 in white leghorn and brown hy-line egg layers, respectively.

CONCLUSION

Five SNPs have been found in the exon 5 of prolactin gene fragments in IPB-D1 chicken. The five SNPs including g.7823A>G, g.7835A>G, g.7886T>A, g.8052T>C, and g.8069T>C. The g.7823A>G, g.7835A>G, and g.8052T>C are synonymous mutations, whereas g.7886T>A and g.8069T>C are non-synonymous mutation which alters change amino acids. Both g.7886T>A and g.8069T>C can be used as a candidate of marker-assisted selection for egg production in IPB-D1 chicken. Further studies are needed to confirm this hypothesis.

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