

Single Nucleotide Polymorphism of Prolactin Gene Exon Two in Ducks of Pekin, Mojosari and Pekin Mojosari Crossbred

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ABSTRAK

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Gen prolaktin merupakan gen kandidat yang memerankan fungsi krusial pada reproduksi dan produksi telur pada unggas. Tujuan penelitian ini adalah mengidentifikasi polimorfisme gen prolaktin itik pada ekson dua dan parsial intron. Sampel darah berasal dari 168 itik yang terdiri dari 19 ekor Pekin, 36 ekor Mojosari dan 113 ekor silangan Pekin Mojosari yang dikoleksi dari Balai Penelitian Ternak Ciawi. Primer ekson dua didesain berdasarkan database prolaktin itik (Kode Akses GenBank: AB158611.1). Amplifikasi menghasilkan produk PCR dengan ukuran sekitar 400 pb. Ditemukan satu insersi basa Adenin pada posisi basa 2001 pada daerah intron dua. Hasil uji homologi dengan metode BLAST memiliki tingkat kesamaan 99% dengan database acuan (Kode Akses GenBank: AB158611.1). Komposisi Adenin pada keseluruhan sampel lebih tinggi dibandingkan database prolaktin acuan. Ikatan triplet hidrogen pada pasangan basa Adenin-Timin lebih tinggi dibandingkan ikatan duplet pada pasangan basa Guanin-Sitosin. Semua sampel itik pada penelitian ini homozigot dan monomorfik.

Kata Kunci: Prolaktin, Pekin, Mojosari, Persilangan

ABSTRACT

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Prolactin gene plays crucial role in the reproduction and egg production of birds. The objectives of this study were to characterize single nucleotide polymorphism in partial intron and coding region of duck prolactin gene. Blood samples were collected from 168 ducks consisted of 19 Pekin, 36 Mojosari, and 113 of their crossbreds collected from Indonesian Research Institute for Animal Production (IRIAP). Primer pairs for the coding regions in prolactin gene were self designed based on the duck genomic sequence database (GeneBank: AB158611.1). PCR products based on DNA of prolactin gene exon two was amplified approximately 400 bp. There is one base insertion of Adenin at the position of 2001 bp intron two region of duck prolactin. Homology test based on BLAST method indicated 99% identity with duck reference (Code Access GeneBank: AB158611.1). Adenin composition in all of duck samples was higher than reference. Triplet hydrogen bonds between Guanine and Cytosin pairs was higher than those at duplet hydrogen bonds between Adenine and Thymine. All duck samples were homozygous and monomorphyc.

Key Words: Prolactin, Mojosari, Pekin, Crossbred

INTRODUCTION

Indonesia has many species of local duck such as Mojosari from East Java, Alabio from Kalimantan, Bali duck, Tegal, Cirebon, Magelang, Kerinci, Cihateup, Turi, Pitalah, Talang Benih, Bayang, Rambon and Pegagan. All of Indonesian local duck are layer with small body size and lower meat production. Data from ILAHS (2013) showed that in 2013 Indonesia have produced 272.431 tones of duck eggs, while meat duck only 30.980 tones.

Recently, the demand of duck meat in Indonesia are increasing together with the increasing of product diversification. To supply requirement and demand of

duck meat, meat performance of some native duck breeds remains to be improved. In order to improve meat productivity of local duck and create specific strain for broiler duck type, genetics approach are needed to create superior breed by crossing local ducks with commercial broiler ducks such as Pekin.

Pekin duck is a commercial meat duck from China. In two months age, body weight of Pekin duck can reached 2-3 kg. Mojosari duck is one of the Indonesian native local duck from East Java. In six months age, body weight of Mojosari only 1.7 kg. Genetic approach to cross Pekin and Mojosari ducks for producing Pekin Mojosari crossbred is one of the method to improve meat performance of local duck. Evaluating molecular

genetic on prolactin gene in Pekin Mojosari crossbred and parents are needed to observe the diversity of gene due to the crossing.

Prolactin is a single chain polypeptide protein secreted in the cephalic lobe of the anterior pituitary gland and acts on peripheral target tissues as hormone (Kansaku et al. 2005). It is coded by prolactin gene and involved in a broad variety of biological functions in all vertebrates (Forsyth & Wallis 2002). Many polymorphism-trait association studies have been reported using prolactin gene such as in bovine (Brym et al. 2005, 2007; Schennink et al. 2009; Lu et al. 2010), pigs (Korwin-Kossakowska et al. 2009), goat (Lan et al. 2009), sheep (Chu et al. 2009; Staiger et al. 2010) and chicken (Jiang et al. 2005; Cui et al. 2006; Liang et al. 2006, Battacharya et al. 2011a, Battacharya et al. 2011b, Battacharya et al. 2011c, Xu et al. 2011). However, to date, no such studies have been reported in Indonesian local ducks.

In birds, the role function of prolactin believed to be manifested during incubation and feeding of nestling and egg formation in follicular development (Chang et al. 2007). Wang et al. (2011) showed that prolactin gene exon two (A-1842-G) in duck is mutated. In partial intron one (T-1326-C), mutation is related with egg performance in Chinese duck (Li et al. 2009). Therefore, the objectives of this study was to characterize single nucleotide polymorphism of prolactin gene exon two and partial intron in ducks of Pekin, Mojosari and crossbred of Pekin Mojosari.

MATERIALS AND METHODS

Blood samples

Blood samples were collected from 168 ducks, consist of Pekin, Mojosari, and Pekin Mojosari crossbreds (Table 1). A total 1-2 ml blood samples were taken by veinipuncture into a syringe. Blood samples were collected in 1.5 ml tube containing EDTA as anticoagulant.

Table 1. Description of duck samples

| Breed | Sex | | Total |
|-----------|--------|-------|-------|
| | Drakes | Ducks | |
| Pekin | 4 | 15 | 19 |
| Mojosari | 12 | 24 | 36 |
| Crossbred | 15 | 98 | 113 |
| Total | 31 | 137 | 168 |

DNA extraction

Extraction of genomic DNA by using Genomic DNA Mini Kit (GeneAid™ DNA Isolation Kit) according to the manufacturer's protocol. Kit contained RBC lysis buffer, cell lysis buffer, protein removal buffer and DNA hydration buffer.

DNA quality test

DNA qualities were measured by 260 and 280 nm wavelengths in spectrophotometer (purity and concentration). Formula of DNA concentration = $A_{260} \times DF \times 50$. DF is dilution factor. Visualization of genomic extraction was performed in gel electrophoresis. Gel electrophoresis contained agarose gel 0.8% in TBE 0.5X with 2µl fluorosave. Universal ladder (KAPPA™) was used as a DNA marker. Gels were run out at 100V for 30-45 min.

DNA amplification

Amplification of prolactin gene was carried out using Polymerase Chain Reaction technique with thermocycler machine. Final volumes was performed in 50 µl containing: 1 µl DNA sample, 24 µl destilated water, 25 µl PCR kit and 1 µl primer. Primer designed based on duck prolactin gene (Code Access AB158611.1) using *primer3* (Table 2).

Table 2. Forward-reverse primer and annealing temperature

| Code | Primer sekuens | Region Amplification | TM (°C) | Size (bp) |
|------|--|----------------------|---------|-----------|
| PF2 | 5'-CCT GAC TGT TTA CAT CCA CAGC-3' | 1774-1955 | 60 | 399 |
| PR2 | 5'-GGG ACT ATC ACT GC CTG TGC -3' | | 60 | |

PCR condition were set on: pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation step at 94°C for 10 sec, annealing at 60°C for 20 sec and, extension at 72°C for 1 min. Final extension stage at 72°C for 5 min.

Gel documentation and DNA sequencing

Visualization of PCR product was analyzed by gel electrophoresis. Gel electrophoresis contained agarose gel 1.5% in TBE 0.5X with 2µl fluorosave. Universal ladder (KAPPA™) was used as a DNA marker. Gels were run out at 100V for 30-45 min. Individual banding

patterns were determined under visible light by using UV transilluminator. A total of 30 µl of PCR product from each PCR samples were sequenced for forward sekence in *First Base*, Malaysia, by using BIOTRACE 3730.

Analysis of single nucleotide polymorphisms

Single nucleotide polymorphisms were identified by comparing individual alignment to current prolactin published sequence for *Anas platyrinchos* (Code Access GenBank: AB158611.1) using *Molecular Evolutionary Genetics Analysis* (Tamura et al. 2007) and chromatograms were individually examined via *BioEdit Sequence Alignment Editor*

Analysis of homology

Analysis of sample sequence identity with database reference and others avian species in gene bank were used online using *Basic Local Allignment Search Tool* (BLAST) available online at <https://blast.ncbi.nlm.nih.gov>. Percentation of homology showed in identity value for each database description and code access numbers.

Analysis of nucleotide composition

Analysis of nucleotide composition was used with *BioEdit Sequence Alignment Editor* and *Molecular Evolutionary Genetics Analysis* (Tamura et al. 2007)

RESULTS AND DISCUSSION

DNA quality

DNA purity and concentration from blood method were high, there were 1.81 and 169 µg/ml respectively.

Marero et al. (2009) reported that DNA molecule classified as pure if ratio A_{260} and A_{280} more than 1.8. Its means this DNA extracts from duck genomic is pure. The purity was caused by lower contaminants such as blood protein and organic compounds (Tataurov et al. 2008).

DNA concentration in this research were low. Viljoen et al. (2005) recommended genomic concentration 250 µg/ml for best result in amplification process. DNA concentration from minimum 2 picograms samples also can be used for PCR (Verkuil et al. 2008). Lower DNA concentration with higher purity in this research was used for further process. DNA extracts from blood showed in light bands (Figure 1).

Prolactin gene amplification

A total of 113 individuals of duck were successfully amplified by primers PR2 and PF2 using 60°C annealing temperature. Amplification of the prolactin gene in this research resulted an amplicon with the length of 399 bp (Figure 2). PCR products contained 181 bp of target fragment (exon two), 218 bp of introns one and two. Duck prolactin consisted five exon and four intron. Exon two in duck prolactin covered 1744-1955 bp region between intron one (271-1773 bp) and intron two (1956-2358 bp) (Kansaku et al. 2005).

Forward and reverse primers were succes annealing at 1694-1715 bp location at intron one and 2074-2093 bp at intron two of prolactin. These two primers resulted 1694-2093 bp of prolactin gene or approximately 400 bp PCR product (Figure 3). Uppercase is target fragment (exon two) while lowercase is primer region (underlined) on prolactin intron.

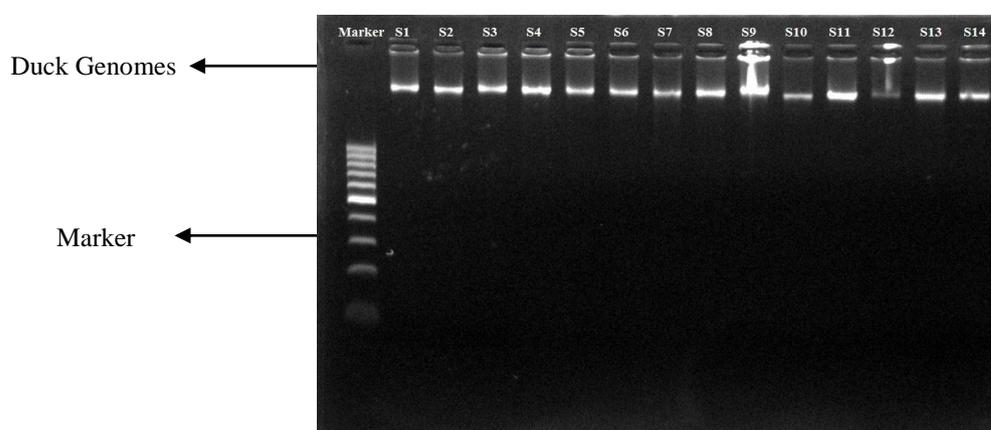


Figure 1. Duck genomic extraction in agarose gel 0.8%

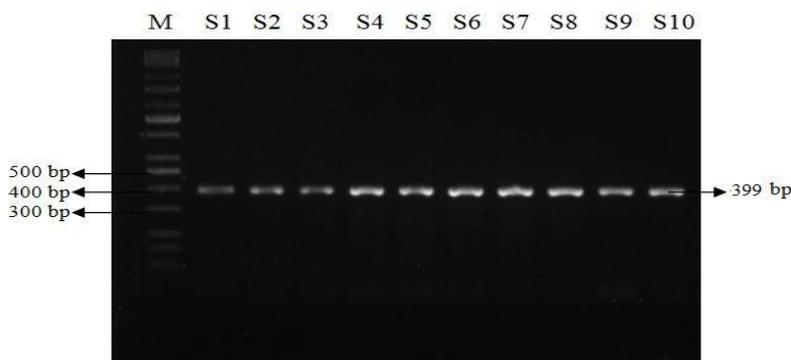


Figure 2. PCR products of duck prolactin gene on agarose gel 1.5% (M=Marker, S=Samples)

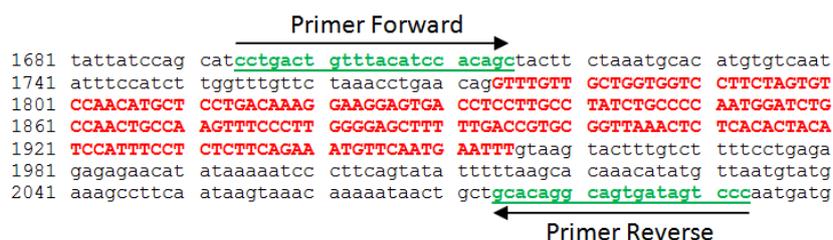


Figure 3. Target fragment and primers location on amplification process

Homology of duck prolactin

Online BLAST method indicated 99% homology with duck prolactin gene of *Anas platyrhynchos* (Access Code AB158611.1, KF487305.1 and JQ677091.1) (Table 3). The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between protein or nucleotide sequences. The program compares nucleotide or protein sequences to sequence in a database and calculates the statistical significance of the matches (Wheeler & Baghwat 2007). There are three kinds of duck breeds (Pekin, Jingxi and Linwu) in National Center for Biotechnology Information (NCBI) database. High similarity indicated that inputted sequence on BLAST is duck prolactin fragment.

Pekin sequence on database (Access Code AB158611.1) different 1% with Pekin sequence sample in this research due to mutation. Alignment analysis and chromatogram sequence showed adenine base insertion at 2001 bp location in intron two in Pekin (Figure 4 and 5). This insertion also occurred in Mojosari and Pekin Mojosari both duck and drake.

Identification of mutation

Total of 113 PCR products sequenced contained a part region of intron one (1693-1773 bp), exon 2 (1774-1955), and intron two (1956-2093 bp). Insertion of Adenine base have been found in all of samples (Pekin, Mojosari and Pekin Mojosari crossbred, both of drakes and ducks). The location of insertion at 2001 bp in intron two showed in Figure 6.

Wang et al. (2011) found mutation A-1842-G at exon two and in this research, we did not find any mutation on those exons. This insertion of adenine at intron two did not change amino acids composition (silent mutation or synonym) and the location far from splicing sites (donor, acceptor, and branch site). Hartl (2000) reported that synonym rate of prolactin gene is faster than non synonym mutation. Effect on this insertion compared with duck which no insertion evidence to the phenotypic traits are still needed further research. Although intron did not participate in protein synthesis, the variance of the intron may affect translation process (Li et al. 2011).

Table 3. Homology of duck prolactin sequence based on BLAST

| Access Code | Description | (%) |
|-------------|---|-----|
| AB158611.1 | Prolactin gene of duck <i>Anas platyrhynchos</i> (Pekin) | 99 |
| KF487305.1 | Prolactin gene of duck <i>Anas platyrhynchos</i> (Jingxi) | 99 |
| JQ677091.1 | Prolactin gene of of duck <i>Anas platyrhynchos</i> (Linwu) | 99 |

Mutation at T-1326-G at intron one associated with egg weight at the age of 30 weeks and double yolk in egg ducks (Li et al. 2009). Song et al. (2010) found mutation at A-383-A in intron 1. Wang et al. (2011) found mutation C-381-A and A-412-G in intron 1 of duck prolactin. Mutation at C-381-A associated with shell strength in egg duck. Haplotype analysis in T-295-C, G-309-T and C-381-T showed association with reproductive traits in Tsaiya duck (Chang et al. 2012). Those research also showed that mutation in intron was greater than exon in prolactin fragment.

Some researchers found that mutation in intron have significance function in gene regulation. Some introns had translatable nucleotide sequences that in the absence of splicing can result in production of novel peptides fused to the peptide encoded by N-terminal exon (Li et al. 2009). Introns have wide function in

transcription initiation, transcription termination, genome organization, time delays in transcribed intron, transcription regulation, alternative splicing, expressing non coding RNAs, non sense mediated decay in exon junction complex, nuclear export, cytoplasmic localization, and translation of yield (Chorev & Carmel 2012).

Nucleotide composition

Composition of Adenine percentages in this experiment was higher than those of reference due to the insertion at 2001 bp location in intron 2 (Table 4). Its affected on A-T composition which higher than duck reference. Higher adenin occurred in all of duck samples (Pekin, Mojosari and Pekin Mojosari crossbred).

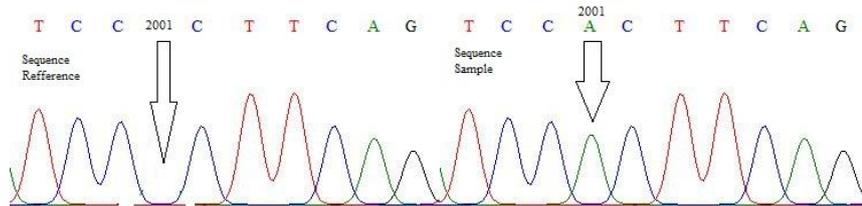


Figure 4. Insertion of Adenin base between prolactin reference and sample

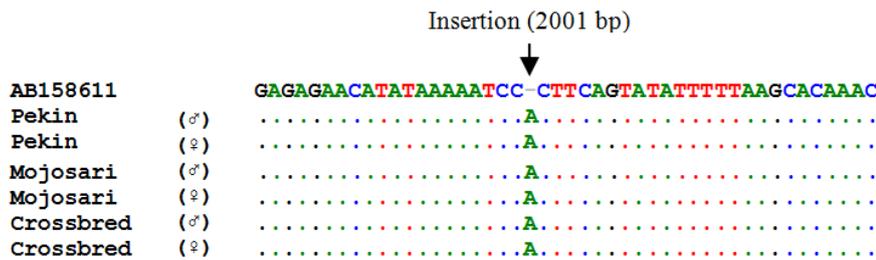


Figure 5. Insertion of Adenin base (A) at 2001 bp location among breeds

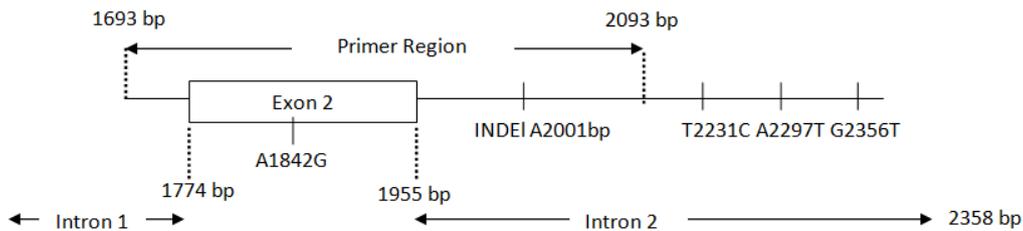


Figure 6. Location of adenine insertion in duck prolactin

Table 4. Nucleotide composition of duck prolactin fragment

| Samples | % | | | | | |
|-----------|-------|-------|-------|-------|-------|-------|
| | T | C | A | G | A+T | C+G |
| AB158611 | 29.91 | 22.74 | 28.97 | 18.38 | 58.88 | 41.12 |
| Pekin | 29.81 | 22.67 | 29.19 | 18.32 | 59.01 | 40.99 |
| Mojosari | 29.81 | 22.67 | 29.19 | 18.32 | 59.01 | 40.99 |
| Crossbred | 29.81 | 22.67 | 29.19 | 18.32 | 59.01 | 40.99 |

Adenine-Thymine in DNA conformation have two hydrogen bridges (Muladno 2010). Hydrogen bond is non-covalent interaction which have small free energy 2-6 kJ/mole in water so the strength of bridge is weak and easy to cleavage and reunited (Petsko and Ringe 2004). It gives weaker bond dissociation than Cytosin-Guanine which has three hydrogen bridges. Weaker bond give a more chance to mutation such as insertion because the bridges are easy to cleavage by enzymatic activity (Yusuf 2001).

Frequency of insertion.

Mutation in all of duck samples only showed ones genotype and allele (100%). This uniformity indicated that allelic variants in duck samples were monomorphic condition (Table 5). Uniformity of insertion were occurred in Pekin, both of drake and duck. Pekin drake is grandparent for producing Pekin Mojosari crossbred in IRIAP. Allele of Pekin ducks and drakes in IRIAP have this insertion.

Table 5. Number of samples in Adenin base insertion at 2001 bp

| Sex | INDEL-2001CAC | | | Total |
|-------|---------------|----------|-----------|-------|
| | Pekin | Mojosari | Crossbred | |
| Drake | 4 | 12 | 15 | 31 |
| Duck | 15 | 24 | 98 | 137 |
| Total | 19 | 36 | 113 | 168 |

Uniformity of adenine base insertion also occurred in Mojosari population, both drake and duck. Mojosari duck is grandparent for producing Pekin Mojosari crossbred in IRIAP also have contributes to the heritage of this insertion. As a result from crossing Pekin and Mojosari, all of the progeny Pekin Mojosari have this insertion. This indicated that mutation in homozygous condition. The flow of insertion occurred from grandparent to next generation of F0, F1, F2, F3 and F5 in homozygous and monomorphic.

In most higher organism such as ducks in this research, each cell of blood samples had DNA resources contained two copies of each chromosome, one inherited from Mojosari through the egg, and one inherited from Pekin through the sperm. All of Pekin

Mojosari crossbred having the same nucleotide sequence along DNA in this locus of prolactin fragment with grandparent. From a biochemical view, gene flow as a specific sequence of nucleotides along of molecule of DNA were genetic materials.

CONCLUSION

Mutation of adenin insertion was found at 2001 bp location in intron two in all of duck samples. Homology test based on BLAST method indicated 99% identity with duck references. Composition of adenine in all of duck samples was higher than those references. Triplet hydrogen bonds between Guanine-Cytosin were higher than those duplet hydrogen bonds between Adenine-Thymine. All of duck samples in this research were homozygous and monomorphic.

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