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Short Communication: Prolactin and X-collagen genes polymorphism in Central Javanese local ducks, Indonesia

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Abstract. *Susanti R, Yuniastuti A. 2020. Short Communication: Prolactin and X-collagen genes polymorphism in Central Javanese local ducks, Indonesia. Biodiversitas 21: 605-610.* Genetic conservation is an effective approach to preserve unique and protected species in Indonesia, including indigenous duck (Anas platyrhynchos Linnaeus, 1758). One of the most important things is an identification effort by studying genetic characteristics to support genetic resource conservation programs. This study aim was to analyze the genetic polymorphisms of Central Javanese local duck based on prolactin (PRL) and X-collagen (COLX) genes. A total of 35 local ducks in Central Java was collected as samples, the duck's calamus feathers were taken for tissue DNA isolation. Polymorphism analysis of PRL and COLX genes were performed using single nucleotide polymorphism (SNP) markers and PCR-RFLP method. The amplicons were then digested with DraI restriction enzyme (AhaIII) (5'TTT/ AAA) for PRL gene, and HaeIII (BsuRI) enzyme (5'GG/ CC-3 ') for COLX gene. The genotype was determined based on RFLP PCR amplicon band results from each target gene. For each gene, data on genotype frequencies, allele frequencies, expected heterozygosities (HE), and Hardy-Weinberg equilibrium were analyzed using PopGene32 software. Based on the PRL gene, there were 25 ducks (71.43%) with BB genotype and 10 ducks (28.57%) with AB genotype. While based on the COLX gene, as many as 25 ducks (71.43%) had TT genotypes, 9 ducks (25.71%) had TC types and 1 duck (1.86%) had CC types. Both PRL and COLX genotypes were clustered the Central Javanese local ducks into 5 haplotypes (A-E). The high allele frequency has belonged to B allele (85.71%) of the PRL gene and T allele (84.25%) of the COLX gene that showed high egg production and quality. Allele B (PRL gene) and T allele (COLX gene) could be predicted as weight eggs' alleles marker.

Keywords: Prolactin, X-collagen, duck, PCR-RFLP

INTRODUCTION

In duck husbandry, low productivity and quality of production become most worrying problems. Genetic improvement in terms of reproductive properties aims to improve production efficiency, product quality, and increase economic value. Genetic approaches may have permanent effects and more sustainable, because improved genetic characteristic inherited from generation to generation. Molecular identification using loci-associated genetic that economically important, can be used to enhance genetic quality in livestock (Chang et al. 2012). Identification and utilization candidate of potential genes that associate with reproduction and profitable is important to improve poultry breeding programs (Basumatary et al. 2019).

In fact, maternal factors are transferred from duck to embryo through eggs, so the absorption of maternal factors is limited only before and some short time after hatching. Reproductive efficiency in poultry is determined by factors (i) the high ratio of hatching and fertilized eggs, (ii) fertility duration, (iii) egg weight, and (iv) egg quantity. The egg production is also controlled by genes. In duck reproduction, several genes involved in reproductive efficiency characteristics such as X-collagen or COLX gene (Chang et al. 2012) and prolactin (PRL) gene (Li et al. 2009).

Hormone-coding genes considered as the most prospective candidates for duck reproduction (Kulibaba 2015). Prolactin is an anterior pituitary polypeptide hormone involved in many reproductive pathways and is essential for reproductive performance (Shamsalddini et al. 2016). The single-chain polypeptide hormone prolactin (PRL) plays an important role not only in regulating growth, differentiation, and lactation, but also in affecting the hair growth cycle (Shamsalddini et al. 2016). This gene plays an important role in egg production and reproductive cycle (Reddy et al., 2006). Previous research reports that polymorphism in the PRL gene correlates with egg performance (Cui et al. 2006), it may decrease or even increase egg productivity. Because of that, the important role of PRL gene and its polymorphism are potential to be studied (Alipanah et al. 2010). The PCR-RFLP analysis on intron-1 PRL genes showed that the DraI enzyme is appropriate to recognize T1326C mutation, by producing 3 sequence genotypes AA, BB, and AB. The ducks with BB genotype (eggs weight 30 weeks; EW30) have average egg production higher than AB genotypes (Li et al. 2009).

Another gene, COLX gene is expressed in isthmus segment of avian oviducts (Fernandez et al. 2001; Wang et al. 2002). COLX gene encodes X-collagen compound, a non-fibrillar protein, short-chain, collagen-forming tissue, and involves cartilage mineralization (Tian et al. 2009). An

unexpected finding that COLX is also existed in the shell membrane (Arias et al. 1991) and protecting the shell membrane from mineralization (Arias et al. 1997). Chang et al. (2012) report that even only one single-nucleotide-polymorphism (SNP) T74C: Val24Ala in coding region of COLX gene able to produce three various genotypes, they are TT, TC, and CC. The results of the study showed that the TT and TC genotypes could contribute to an increase in egg weight in Tsaiya ducks (indigenous Taiwan duck).

Moreover, genetic diversity in indigenous breeds is the main interest reflecting the urgency of protecting what may be a valuable and irreplaceable enrichment, about novel productive requests (Khodabakhshzadeh et al. 2016; Mohammadabadi 2017). Having significant, accurate and profound information about the genetic resources of the specific breed is the basis of conservation (Zamani et al. 2011; Shamsalddini et al. 2016). Therefore, defining native and indigenous breeds applying molecular techniques is very important. Genetic diversity conservation in livestock species needs enough performance of conservation advantages and sustainable management schemes, that are formed on the basis of wide data about the structure of the populations, containing genetic variability origin among and within breeds (Mohammadabadi et al. 2010a). One of the most necessary parts for population survival, evolution, genetic progress and adaptation to variable environments is genetic diversity (Ebrahimi et al. 2017). In addition, determining genes affecting and characterizing polygenic traits is troublous (Mohammadabadi et al. 2010b; Shamsalddini et al. 2016). Utilization of molecular genetics possesses several great benefits (Mousavizadeh et al. 2009), hence genetic polymorphism definition for farm animals breeding is very important (Baghizadeh et al. 2009; Ruzina et al. 2010). Thus, the aim of this study was to analyze the genetic polymorphisms of indigenous Central Javanese duck based on prolactin and X-collagen genes.

MATERIALS AND METHODS

In this study, 35 local ducks from around Central Java, Indonesia were selected. DNA isolation was performed from follicle of five calamus feathers from each wing. The DNA isolation was run using gSYNC™ DNA Extraction Kit followed manufacture procedures and was visualized on 1.5% agarose gel electrophoresis. The isolated DNA was then amplified by PRL and COLX genes primers (Table 1), using GeneAmpR PCR system thermocycler 2400 (Perkin Elmer. Massachusetts, USA). The amplification was completed for 25µl of DNA cocktail that consisted with 1.2µl of 10 µmol forward primer, 1.2µl of 10 µmol reverse primers (Table 1), 2µl of 50 ng DNA template, 2.5µl of PCR master mix and 8.1µl of ddH2O. The PCR process was operated for 35 cycles and using optimized temperature and time (Table 2). All isolated DNA samples were amplified using 2 pairs of primers specific to 2 gene targets (PRL and COLX). Amplification was carried out with annealing temperature optimization results, 61.0ºC and 57.6ºC, respectively (Table 2).

Table 1. Primers sequence for PRL and COLX gene and size of products used in the study based on previous researches

Genes	Primers	Size of PCR product (bp)	References
PRL	PRL-F: 5'-GAATAGAACACTTGACCC TG-3′ PRL-R: 5'-′TAGAGGAGGCAAGCATAG-3′	566	Li et al. (2009)
COLX	COLX-F: 5'-CTGGCAGTGCTGTCATCGAT-3' COLX-R: 5'-GCGTGACCTCCTAAAGGACAT C-3'	220	Chang et al. (2012)

Table 2. PCR condition for PRL and COLX genes amplification

Genes	Pre-denaturation	Cycles			Final Extension
		Denaturation	Annealing	Extension	
PRL	95ºC for 5 min	94ºC for 45 s	61ºC for 45 s	72ºC for 1 min	72ºC for 10 min
COLX	94ºC for 5 min	94ºC for 1 min	57.6ºC for 1 min	72ºC for 1.5 min	72ºC for 10 min

Table 3. Restriction used enzymes, restricted fragment size and genotypes for the PRL and COLX genes

Genes	Restriction enzim	SNP	Genotype products
Prolaktin	DraI (AhaIII) (5'TTT/AAA)	T/C mutation at 1,326 bp position	AA: 518bp; and 47bp AB: 518bp; 309bp; 209bp; and 47bp. BB: 309bp; 209bp; and 47bp.
COLX	HaeIII (BsuRI) (5'GG/CC-3')	Non-synonyms (Val, Ala)	TT: 175bp; and 45bp. TC: 175bp; 102bp; 73bp; and 45bp. CC: 102bp; 73bp; and 45bp.

PCR-RFLP was done digesting the PCR products (amplicons) using restriction enzymes DraI (for PRL) and HaeIII (for COLX). The RFLP reaction was carried out by composing a mixture of 5 uL of PCR products, 4U of restriction enzymes, 1 ul of buffer and sterile H2O to a total volume up to 10 µL and placed into microtube. Then, the mixture was incubated at 37ºC overnight. The results were visualized in 3% of agarose gel and genotypes were determined based on the observed fragmented bands (Table 3).

Genotype data of PRL and COLX genes for each duck were compiled and grouped for same haplotypes. The analysis data for each gene, included genotype and allele frequency, expected heterozygosities (HE), and Hardy-Weinberg equilibrium was analyzed using PopGene32 software.

RESULTS AND DISCUSSION

The PRL and COLX gene fragments digested by DraI and HaeIII restriction enzymes, were shown in Figures 1 and 2.

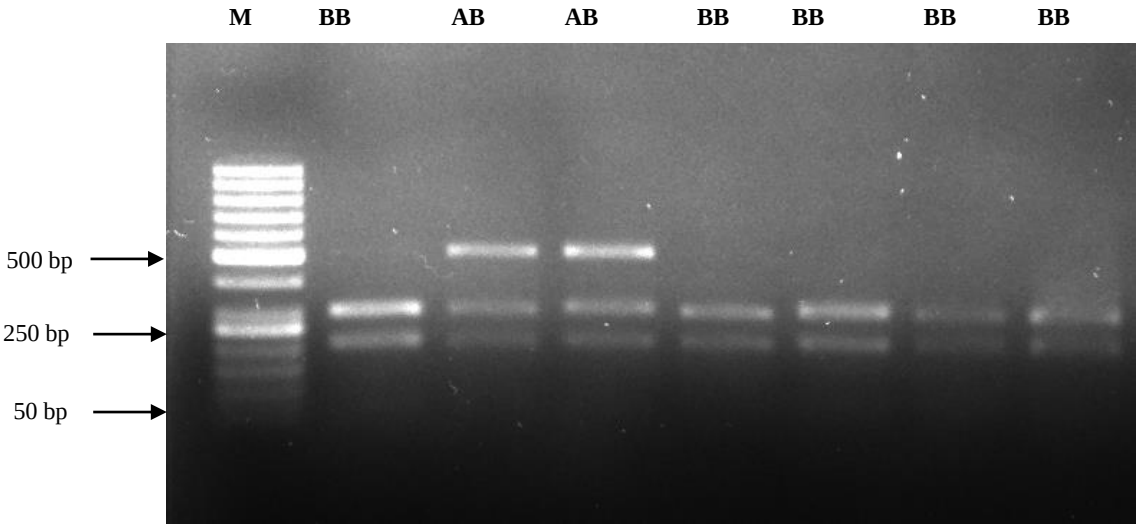

Figure 1. PRL gene fragments restricted using the DraI enzyme. DNA ladder was marker 50 bp (M); homozygous BB has three fragments (309, 209, and 47 bp); heterozygous AB has four fragments (518, 309, 209, and 47 bp)

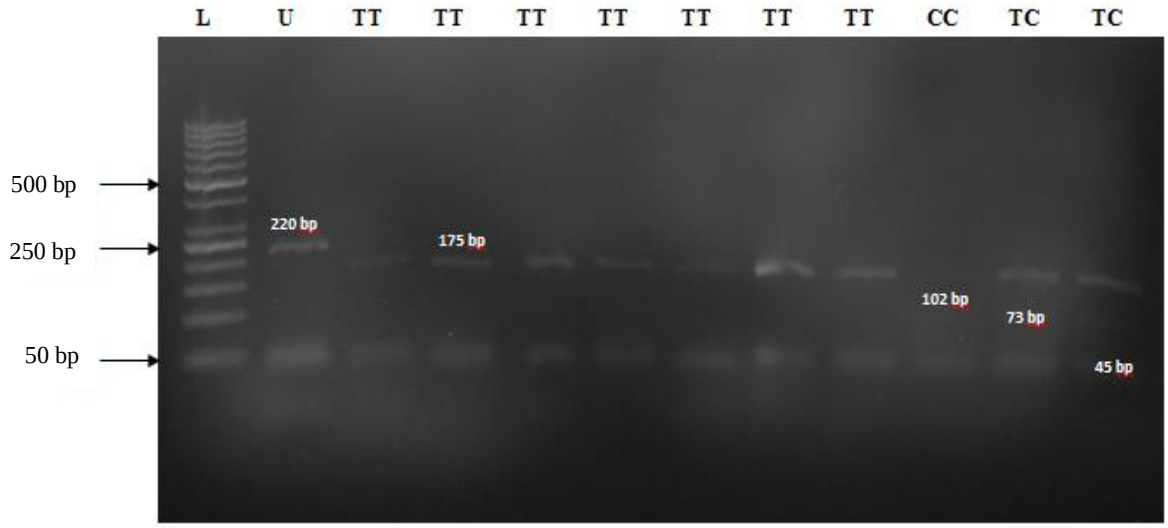

Figure 2. COLX gene fragment restricted using the HaeIII enzyme. DNA ladder marker was 50 bp (L); 220 bp (U) PCR products; homozygous TT has two fragments (175 and 45 bp); homozygous CC has three fragments (102, 73, and 45 bp); TC heterozygotes of four fragments (175, 102, 73, and 45 bp)

Table 4. Distribution of PRL and COLX genes genotype and alleles in Central Javanese duck

Gene	Genotype frequence			Alleles frequence	
Intron I PRL	AA	AB	BB	A	B
	0.00 (0)	0.2857 (10)	0.7143 (25)	0.1429	0.8571
COLX	TT	TC	CC	T	C
	0.7142 (25)	0.2571 (9)	0.0285 (1)	0.8429	0.1571

Table 5. Duck's haplotype based on PRL and COLX genotype

Haplotype	Genotype		Duck sample	Amount (%)
	PRL	COLX		
Haplotype A	BB	TT	M1, M2, M4 PK1, PK2, PK4 PG2, PG3, PG4 TB1, TB2, TB4, TB5 TBr1, TBr2 TL1, TL4, TL5	18 (51.43%)
Haplotype B	AB	TT	TB3 TJ2, TJ3 TL2 M3 PK3, PK5	7 (20.00%)
Haplotype C	BB	CC	TBr3	1 (2,86%)
Haplotype D	BB	TC	TBr4, TBr5, TJ1, TJ4, TJ5, M5	6 (17,14%)
Haplotype E	AB	TC	PG1, PG5 TL3	3 (8.57%)
Total				**35** (100%)

Table 6. Genetic diversity of Central Java local duck based on the PRL and COLX genes

Statistics parameters	PRL	COLX
N_A	2.0000	2.0000
N_E	1.3243	1.3604
I	0.4101	0.4349
Observed homozygosity	0.7143	0.7429
Observed heterozygosity	0.2857	0.2571
Expected homozygosity	0.7516	0.7313
Expected heterozygosity	0.2484	0.2687
Average heterozygosity	0.2343	0.2257

Note: N_A: observed number of alleles, N_E: effective number of alleles, *I*: Shannon's information index

Based on the analysis, the digested DNA fragments from each sample were different, even in the homologous DNA. It can be detected by difference length fragments appear after DNA restriction by endonucleases for specific sites. Previous research has shown PCR-RFLP technique is effective to be used for duck's genotypes selection based on different various genes, such as avian influenza resistance gene (*Mx* gene) (Susanti et al. 2018), and hatchability (*ovalbumin* gene) (Susanti et al. 2019).

In this research, the results of the genotype analysis based on the RCP-RFLP PRL gene showed that 25 ducks (71.43%) had BB type, and 10 ducks (28.57%) had AB type. While based on the COLX gene, as many as 25 ducks (71.43%) had TT type, 9 (25.71%) had TC type and 1 duck (1.86%) had CC type (Table 4). The high B allele (85.71%) and T allele (84.25%) correlated with egg production quality. Allele B (PRL gene) and T allele (COLX gene) are high egg-weight-allele markers. Alleles variations in functional genes are the result of nucleotide modification caused by such as single nucleotide polymorphism (SNP), insertions or deletions (Fulton 2008).

Based on the restriction site, there were 5 haplotypes (A-E) formed in each gene (Table 4). Then, the individual was grouped by their allies haplotype and clustered into five different groups (Table 5).

The duck samples were dominated by haplotype A followed by Haplotype B and haplotype D. The haplotype A has BB genotype and TT, then, haplotype B has genotype AB and TT. While haplotype D has BB genotype and TC, this information confirms various genotype was observed from the local Javanese ducks' PRL and COLX genes.

Diversity analysis was performed by Shannon's information index for each locus were 0.4101 for PRL and 0.4349 for COLX (Table 6). The expected heterozygosity (He) scores for PRL locus was 0.2484 and at COLX locus was 0.2687. However, even there were differences in duck genotype, the results of Chi-Square test (Table 7) show that the observed and expected genotype was not significantly different from one another. It suggests that the allele and genotype frequencies of PRL and COLX locus from Javanese duck population in this research, are still in the Herdy-Weinberg equilibrium ($p<0.05$), and particularly locus is not changing. The alleles frequencies in single-locus change from generation to generation caused by growing population. The changing frequency occurs because of the growing population increasing opportunity of panmixia (random mating) among population members in low migration circumstances (Eichie 2018).

The samples were dominated by haplotype A (51.43%), with BB and TT genotypes (high egg weight). This shows that the haplotype A ducks can be selected for improving genetic programs related to the duck egg weight. Identification and utilization of potential candidate genes related to reproduction and economic traits are very important in poultry breeding programs (Basumatary et al. 2019) and can be used to enhance genetic preservation of domestic animals (Chang et al. 2012; Indriati et al. 2016).

Table 7. Chi-square test for Hardy-Weinberg equilibrium in the population of PRL and COLX genes

Gen	Genotypes	Observed (O)	Expected (E)	$(O-E)^2/E$	X^2
PRL	AA	0	0.6522	0.6522	0.864407*
	AB	10	8.6957	0.1957	
	BB	25	25.6522	0.0166	
COLX	TT	25	24.7971	0.0017	0.070815*
	TC	9	9.4058	0.0175	
	CC	1	0.7971	0.0516	

Note: star mark (*) is representing statistically significant gene equilibrium in population. X2 calculation of PRL and COLX gene is lower than X2 table (22.164) it means p-value > 0.05

The PRL gene polymorphism was reported to be significantly related to egg production traits in Erlang Mountainous chickens and was found to be a strong candidate for genes that affecting egg production (Zhang et al. 2012). The PRL gene is a candidate gene that plays crucial function in the reproduction and egg production in poultry (Irma et al. 2014). Prolactin is involved in regulating important physiological functions, ranging from mammalian reproduction to osmoregulation in fish and nesting behavior in birds (Fathi and Zarringhobaie 2014).

Central Java local ducks in this study have two genotypes, there were AB and BB. While the AA genotype was not found in this study, it possibly caused by small sample size or indeed, the AA genotype actually does not exists. In the first case, the AA genotype has likely existed in the duck population, but too small sample size may not representing the gene distribution of its population. Another study from Li et al. (2009) showed that BB genotype ducks had significantly produced eggs higher compared to AB genotype duck. The report tells that the BB genotype duck produces EW30 (egg weight at 30 weeks). However, among whole three genotypes; AA, AB, and BB were not showed significant difference in produced eggs, longest clutch days, and duck body weight at the beginning of spawning (the bodyweight at first egg). Further research is needed to find out genotype stability and distribution by increasing sample size. Another research is also needed to describe exact regulatory mechanism of PRL gene expression

The T/C mutations at position 1326 bp, intron 1 of the PRL gene (Accession Number: AB158611) was fragmented the gene into three different genotypes, AA, AB, and BB that may also affect the expression of the PRL gene (Li et al. 2009), even though, intron does not play a role in translation during protein-synthesizing, but has broad functions in transcription, from initiation until termination (Chorev and Carmel 2012). The intron 1 variation may contribute to gene expression by affecting the mRNA sequence and even further in functional protein structure. The intron I polymorphisms of the PRL gene have also been reported to be related to the shell strength of duck eggs (Wang et al. 2011). Sequence variations in the PRL gene promoter area can cause changes in the promoter binding site and change the expression of the PRL gene. Polymorphisms in the promoter area, especially those that produce changes in the promoter binding site, will most likely affect mRNA expression, so that in the case of polymorphisms this can influence the behavior of chicken incubation and egg production (Cui et al. 2006).

In this study, there were identified two alleles: T and C, and three genotypes (TT, TC, and CC) were detected at the Central Javanese local duck COLX gene locus. The PCR products digested with HaeIII demonstrated two fragments (175 and 45 bp) for T alleles and three fragments (102, 73 and 45 bp) for C alleles. The frequency of CC genotypes is lower than TT and TC genotypes. This might occur because of the random gene drift, so that C alleles (in the COLX gene) and A alleles (in the PRL gene) frequencies is too low (Liu et al. 2010).

Even, a SNP in coding region (T74C: Val24Ala) of the COLX gene may change the structure of X-type-collagen proteins, the protein structure could be substituted by different amino acids (data not shown). The SNPs might regulate protein function by altering or changing the protein structure. Chang et al. (2012) showed that the TT and TC genotypes contributed to the increase in egg weight in Tsaiya ducks (local ducks in Taiwan), but there was no significant relationship between the COLX-T74C genotype and egg number, hatchability, fertility rate, and or maximum fertility ages.

The COLX protein is a marker of chondrocyte hypertrophy that exclusively expressed in hypertrophic chondrocytes and involved in bone mineralization process. The protein encoded by the COLX gene is non-fibrillar tissue collagen forming, as a major component of hypertrophic zone depositing other matrix molecules. Furthermore, COLX involved in hematopoiesis, mineralization, and endochondral ossification. The COLX is stored in the hypertrophic zone and is removed during the endochondral ossification process (Chang et al. 2012; Gu et al. 2014; Coghlan et al. 2017). Type X-collagen production increases progressively during the development of the embryonic framework, thereby accelerating embryo growth (Prickett 2014).

The Hardy-Weinberg equilibrium test as represented from Chi-square test for the PRL and COLX genes showed that the observed and expected genotype did not significantly different (p>0,05) (Table 7). This shows that the frequency of alleles and genotypes at the COLX locus in the study population are in the Hardy-Weinberg equilibrium. The alleles frequency of one single locus and its equilibrium may change because of independent segregation and assortment. This change occurs due to large population sizes with fewer individual migrations and random fertilization (Eichie 2018).

In conclusion, Central Java local duck genotypes showed genetically good egg production quality, characterized by high frequency of B allele (85.71%) in PRL gene and T allele (84.25%) in COLX gene. The B allele (PRL gene) and T allele (COLX gene) are the genes related to good egg productivity. The genes themselves are also relatively stable in the Central Java local duck population. This information is important to be considered for breeder to preserve the profitable gene, rather than trying to cross-breed with other varieties.

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