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Research article

Prolactin haplotypes and their effect on body weight and egg production in the KU line of Betong chicken

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Abstract

Body weight and egg production need to be considered for the selection of parents in the KU line of Betong chicken. The allele, genotype and haplotype frequencies were determined of single nucleotide polymorphisms in the 5'-flanking region (5FA) of the prolactin (*PRL*) gene and the effect was estimated of haplotypes on body weight at age 16 wk (BWT), hen weight at first egg (WFE), age at first egg (AFE), first egg weight (FEW) and egg number at age 42 wk (ENO) in Betong chicken (KU line). Data and blood samples were collected from 297 hens. Haplotypes in 5FA of the *PRL* gene were identified using polymerase chain reaction and restriction fragment length polymorphism with the *CViQI* and *AluI* restriction enzymes. The results showed that at site -358 in 5FA of the *PRL* gene, the frequency of the deletion (D) allele was higher than that of the insertion (I) allele, at site -2161 the frequency of the G allele was higher than that of the C allele and at site -2402 the frequency of the T allele was higher than that of the C allele. Five haplotypes were found and haplotype DDGGTT had the highest frequency (0.667). The effects of *PRL* haplotypes were not significant for all traits. Therefore, the five haplotypes identified would not be useful for selection programs aimed at improving body weight and egg production in the KU line of Betong chicken.

Introduction

Betong chicken, a famous native chicken in the southern part of Thailand, can grow faster than other Thai native chicken breeds (Gongruttananun and Chotesangasa, 1996) and they can be sold at high prices because of their preferred meat quality (Thepparat et al., 2016). However, their low egg production due to their broodiness behavior (4.70 clutches/hen/year; Chanjula et al., 2004) limits the number of one-day-old chicks resulting in high costs for chick production (Mookprom et al., 2017). Selection in the closed flock of Betong chickens in the Sunwanvajokkasikit Poultry Farm of Kasetsart University, Thailand (KU line) has emphasized growth performance (Sopannarath and Jeanmas, 2016; Bungsrisawat et al., 2018). Thus, the low egg production of the Betong chickens in general, and of the KU line in particular, makes it necessary to select for reproduction traits to optimize the overall production of KU line of Betong chickens.

Prolactin (PRL), a peptide hormone secreted by the anterior pituitary gland has shown a diverse spectrum of biological activities and functions in all vertebrates (Bhattacharya et al., 2011). It is involved in many functions in chickens, such as growth, development, reproductive and bloodiness behavior (Bole-Feysot et al., 1998). Cui et al. (2006), Kunhareang et al. (2012), Sarvestani et al. (2013) and Mitrofanova et al. (2017) have studied the association between polymorphism in the 5'-flanking region (5FA) of the *PRL* gene and growth and egg production traits in chicken. The expression of the

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PRL gene depends on the 5FA sequence (Rashidi et al., 2012). A 24 bp insertion (I)/deletion (D) at site -358 in 5FA of the *PRL* gene, called ecotropic viral integration site-1 encoded factor (*Evi-1*) binding site, represses the expression of the *PRL* gene in White Leghorn chickens (Cui et al., 2006). The frequency of the I allele was high in laying hens. Moreover, the effects of single nucleotide polymorphisms (SNPs) at sites -2161 and -2402 in 5FA of the *PRL* gene were significantly associated with broodiness behavior and egg production in chickens (Jiang et al., 2005; Cui et al., 2006; Sarvestani et al., 2013). Thus, the aims of this study were to determine the allele, genotype and haplotype frequencies of SNPs in 5FA of the *PRL* gene and to estimate the effect of the haplotypes of the *PRL* gene on body weight and egg production in the KU line Betong chicken.

Materials and Methods

Animal welfare

Animal care protocols were approved by the Kasetsart University Institutional Animal Care and Use Committee (Approval no. ACKU59-AGR-011) and were in accordance with the guidelines on animal care and use under the Ethical Review Board of the Office of National Research Council of Thailand (NRCT) for the conduct of scientific research.

Data and blood sample

A sample of 297 pullets randomly chosen from four hatching batches was used in this study. The body weight of pullets at age 16 wk (BWT) was recorded before placing them in individual cages. They were fed a commercial layer feed ration of approximately 100 g/bird/d and were provided with a photoperiod of 16 hours light/d. Hen weight at first egg (WFE), age at first egg (AFE), and first egg weight (FEW) were recorded when hens laid their first egg. Egg number (ENO) was counted from their first egg to their last egg at age 42 wk. A blood sample (1,000 µL) from each individual hen was placed into a microcentrifuge tube containing EDTA anticoagulant (50 µL 0.5M pH 8.0) and stored at -20°C before the DNA extraction process.

DNA extraction and polymerase chain reaction amplification

Blood samples were pretreated with 0.9% NaCl before DNA extraction. The DNA was extracted from a whole blood sample using the phenol-chloroform method according to Sambrook et al. (1989). DNase/RNase free water (Bio Basic; Canada) was used for DNA dissolution and the result was stored at -20°C until use. The DNA concentration and degree of purity were determined using a Biodrop Duo Micro Volume Spectrophotometer (Innovative Solutions; UK).

Polymorphisms in 5FA of the *PRL* gene were identified using two techniques of polymerase chain reaction (PCR) base, namely an allele-specific technique (an insertion/deletion) and PCR-restriction fragment length polymorphism (RFLP). Following Cui et al. (2006) and Sarvestani et al. (2013), the 24 bp insertion/deletion at site -358 in 5FA of the *PRL* gene was amplified using *PRL-1* primers (Table 1) and the SNP at sites -2161 and -2402 in 5FA of the *PRL* gene were amplified using primers *PRL-2* (Table 1) to identify genotypes at these sites using gel electrophoresis analysis. The PCR was conducted in a 25 µL reaction mixture containing 100 ng genomic DNA (50 ng/µL), 1.0× buffer for KOD-Plus-Neo, 2.5 mM $MgSO_4$, 0.2 mM each of dNTPs 0.2 µM forward and reverse primers, and 0.5 units Taq-KOD-Plus-Neo (Toyobo, Japan). The process started from an initial denaturation and continued for 5 min at 95°C and followed by 35 cycles of the following stages: denaturation for 30 s at 95°C, primer annealing for 30 s at 62°C (for *PRL-1* primers) or 64°C (for *PRL-2* primers), and DNA chain synthesis for 30 s at 72°C with a final extension for 5 min at 72°C. The PCR was carried out on a Mastercycler® Nexus Gradient (Eppendorf, Germany). For RFLP analysis, the PCR products from *PRL-2* primers were digested with *CviQI* (New England BioLabs; USA) and *AluI* (New England BioLabs; USA) for genotyping of SNP at sites in 5FA of the *PRL* gene, respectively. The digestion reaction was carried out in a 20 µL mixture containing 1×NE buffer, 250 ng PCR product and restriction enzyme (New England BioLabs; USA). The PCR products from *PRL-1* primers and the digestion products from *PRL-2* primers were electrophoresed directly on 3% agarose with 0.5×TAE buffer. They were stained using ethidium bromide and the genotype bands were visualized under ultraviolet light. These results were confirmed using sequencing analysis and the sequences were aligned using the Clustal X software (Larkin et al., 2007).

Statistical analysis

Descriptive statistics were computed with the UNIVARIATE procedure of SAS software (SAS, 2017). The genotypic and allelic frequencies of each site were calculated following Falconer and Mackay (1996). The Hardy-Weinberg equilibrium of genotypes at each site was tested by comparing the observed and expected genotypes with a χ^2 test. The effect of *PRL* haplotype on body weight and egg production traits was analyzed using the GLM procedure and least square means were estimated. The model is shown in Equation 1:

$$y_{ijk} = \mu + H_i + B_j + HB_{ij} + e_{ijk} \quad (1)$$

Table 1 Primer characteristics of prolactin gene in Betong chicken (KU line)

Primer name	Length (bp)	Primer	Sequence
PRL-1	130–154	Forward	TTTAATATTGGTGGGTGAAGAGACA
		Reverse	ATGCCACTGATCCTCGAAAACTC
PRL-2	439	Forward	AGAGGCAGCCCAGGCATTTTAC
		Reverse	CCTGGGTCTGGTTTGGAAATTG

where y_{ijk} is the observation of BWT, WFE, AFE, FEW and ENO. μ is the overall mean of the population, H_i is the fixed effect of the *PRL* haplotype (i = DDGGTT, DDGGCT, DDGGCC, IDCCCC, and IICCCC), B_j is the fixed effect of hatching batch (j = 1, 2, 3, 4) , HB_{ij} is the fixed effect of interaction between *PRL* haplotype and hatching batch, and e_{ijk} is the random residual error ($e \sim N(0, \sigma^2)$).

Results and Discussion

The descriptive statistics of body weight and egg production traits in Betong chicken (KU line) are shown in Table 2. The means (± SD) of BWT, WFE, AFE, FEW and ENO were 1,463.07 ± 165.43 g, 2,078.11 ± 256.85 g, 195.05 ± 39.27 d, 37.74 ± 5.47 g and 52.51 ± 31.79 eggs, respectively. The means of BWT and FEW in this study agreed with other reports for Betong chicken (Chanjula et al., 2004; Putsakul et al., 2010; Wangtaweesukkamol et al., 2013; Thepparat et al., 2016). Furthermore, the means of WFE, AFE, FEW and ENO also agreed with those of other Thai native chickens (Chotesangasa et al., 1994; Kunhareang et al., 2012; Boonkum et al., 2014; Mookprom et al., 2017). However, the mean of AFE was higher than that in a Betong chicken population in southern Thailand (161 d; Chanjula et al., 2004) and the mean of BWT was higher than in a population of native chicken in northeast Thailand (Leotargul et al., 1996). These differences may have been due to the years of selection for BWT in the KU line of Betong chicken.

The presence of a single fragment of the PCR product from primer *PRL-1* was found in genotype II (154 bp) and genotype DD (130 bp) while genotype ID was characterized by the presence of two fragments (154 and 130 bp; Fig. 1). The 24 bp insertion at site -358 is shown in Fig. 2. For SNPs at site -2161 in 5FA of the *PRL* gene, genotypes CC and GG were characterized by the presence of a fragment of 439 and 405 bp, respectively, while the genotype CG was found in two fragments (439 and 405 bp; Fig. 3). The 34 bp fragment of genotype GG and genotype CG migrated to the base of the gel. The mutation point from G to C at site -2161 in 5FA of the *PRL* gene is shown in Fig. 4. The digestion products from primer *PRL-2* with *AluI* for genotype TT were found in three fragments (304, 81 and 54 bp), while the genotype CC was characterized by the presence of four fragments (160, 144, 81 and 54 bp). The genotype CT was found in five fragments (304, 160, 144, 81 and 54 bp; Fig. 5). The 160 and 144 bp fragments were not clearly visible. However, the results were confirmed by the sequencing technique and the mutation point from T to C at site -2402 is shown in Fig. 6.

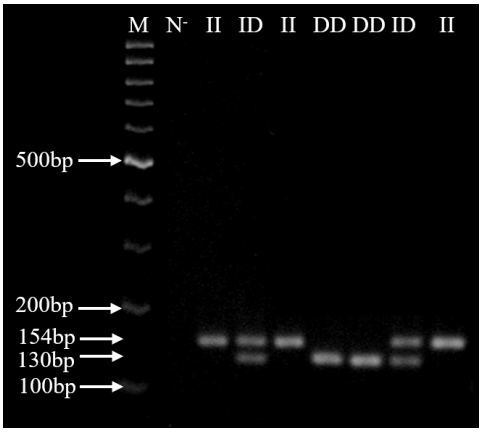

Fig. 1 Genotyping of the 24 bp insertion/deletion at site -358 in the 5'-flanking region of prolactin gene using polymerase chain reaction with 3% agarose gel electrophoresis analysis, where M=100 bp ladder

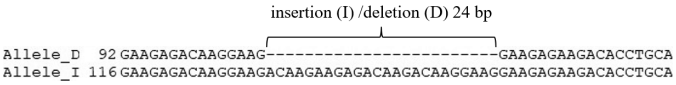

Fig. 2 Insertion (I)/deletion (D) 24 bp at site -358 in 5'-flanking region of prolactin gene

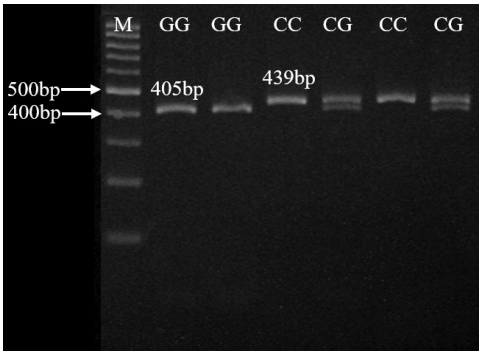

Fig. 3 Restriction analysis of the polymerase chain reaction products from primer *PRL-2* (439 bp) digested with *CviQI* using 3% agarose gel electrophoresis, where GG genotype = restriction fragment of 405 bp, CG genotype = restriction fragments of 439 and 405 bp and CC genotype = restriction fragment of 439 bp and M=100 bp ladder

Table 2 Descriptive statistics for body weight and egg production traits in Betong chicken (KU line)

Trait	Number	Mean	SD	Minimum	Maximum
BWT (g)	294	1,464.69	165.26	1,023.00	2,131.00
WFE (g)	289	2,080.24	257.27	1,541.00	2,778.00
AFE (d)	297	195.09	39.39	128.00	299.00
FEW (g)	278	37.76	5.49	22.00	50.00
ENO (egg)	297	52.45	31.84	1.00	120.00

BWT = body weight at age 16 wk; WFE = hen weight at first egg; AFE = age at first egg; FEW = first egg weight; ENO = egg number at age 42 wk.

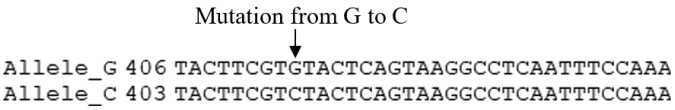

Fig. 4 Single nucleotide polymorphisms at site -2161 in 5'-flanking region (5FA) of prolactin gene.

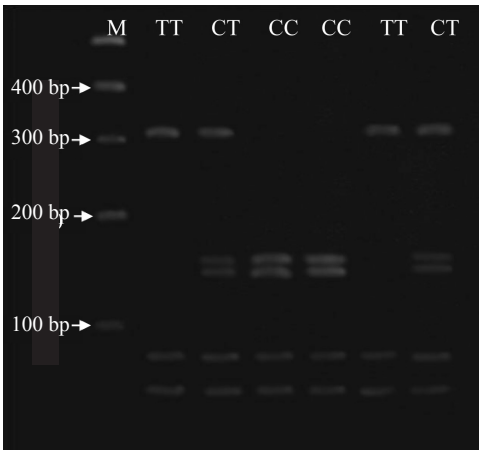

Fig. 5 Restriction analysis of the polymerase chain reaction products from primer *PRL-2* (439 bp) digested with *AluI* using 3% agarose gel electrophoresis, where TT genotype = restriction fragments of 304, 81 and 54 bp, CT genotype = restriction fragments of 304, 160, 144, 81 and 54 bp and CC genotype = restriction fragments of 160, 144, 81 and 54 bp, M=100 bp ladder

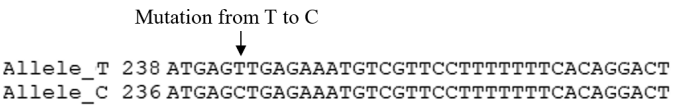

Fig. 6 Single nucleotide polymorphisms at site -2402 in 5'-flanking region (5FA) of prolactin gene.

Genotypic and allelic frequencies

All three possible genotypes were found at sites -358, -2161 and -2402 in 5FA of the *PRL* gene. At site -358, the genotypic frequency of homozygous II (0.047) was low while the genotypic frequency of homozygous DD was high (0.667; Table 3). Kunhareang et al. (2012) reported that the pattern of the homozygous DD genotype in 5FA of the *PRL* gene was commonly found in Thai native chicken. In Mazandaran native chicken (Rashidi et al., 2012), Far native fowl (Sarvestani et al., 2013) and Pushkin breed chicken (Mitrofanova et al., 2017), the frequencies of homozygous II were higher than those of homozygous DD. In addition, Kulibaba and Podstreshnyi (2012) confirmed that the genotypic frequency of homozygous II in 5FA of the *PRL* gene in the dual-purpose line of Ukrainian chicken was much lower (0.03) than in the layer line of Ukrainian chicken (0.50). At site -2161, the genotypic frequencies of GG, CG and CC were 0.667, 0.094 and 0.239, respectively and at site -2402, the genotypic frequencies of TT, CT and CC were 0.667, 0.222 and 0.111, respectively (Table 3).

High allelic frequencies of D (0.81), G (0.714) and T (0.778) were obtained at sites -358, -2161 and -2402, respectively (Table 3). These results agreed with the report of Cui et al. (2006) in Chinese chicken (Taihe Sikies 1). Kulibaba and Podstreshnyi (2012) indicated that the frequency of the T allele at site -2402 in the *dual-purpose* Ukrainian line of chicken (0.845) was substantially higher than in the layer line of Ukranian chicken (0.255). Further, the homozygous GG genotype at site -2161 and the homozygous TT genotype at site -2402 were commonly found in both broiler and dual-purpose chickens. In contrast, homozygous CC at sites -2161 and -2402 were common in layer chicken only (Cui et al., 2006; Kulibaba and Podstreshnyi, 2012). Therefore, the results from the current study suggested that Betong chicken (KU line) could be classified as a broiler or a dual-purpose chicken.

The genotypes of the 24 bp insertion/deletion at site -358 in 5FA of the *PRL* gene did not differ from the expected Hardy-Weinberg equilibrium (χ^2 =1.500). In contrast, the frequencies of the genotypes at sites at -2161 and -2402 in 5FA of the *PRL* gene were different from their corresponding values under Hardy-Weinberg equilibrium (χ^2 = 175.751 and χ^2 = 37.883, respectively). Even though the chickens in the current study were randomly sampled in the population, the three positions were linked on chromosome 2 (Kulibaba and Podstreshnyi, 2012; Sarvestani et al., 2013). Unlinked loci reach independence after one generation of random mating, whereas linked loci with a recombination rate reduce linkage disequilibrium after generations.

Only 5 *PRL* haplotypes were found in the current study (Table 4) and the frequency of haplotype DDTTGG was the highest (0.667), whereas the frequency of haplotype IICCCC was the lowest (0.047). These results confirmed the dependency of the *PRL* gene at sites -358, -2161 and -2402 in 5FA and the D, T and G alleles were transported together.

Table 3 Genotypic and allelic frequencies of prolactin gene and χ^2 test to examine Hardy-Weinberg equilibrium in Betong chicken (KU line) (n=297)

Position	Genotypic frequency			Allelic frequency		χ^2
-358	DD	ID	II	D	I	1.500
	0.667	0.287	0.046	0.810	0.190	
-2161	GG	CG	CC	G	C	175.751
	0.667	0.094	0.239	0.714	0.286	
-2402	TT	CT	CC	T	C	37.883
	0.667	0.222	0.111	0.778	0.222	

$\chi^2_{0.05,\ df=2}$ = 5.99

Table 4 Haplotype frequencies of the prolactin gene in Betong chicken (KU line) (n=297)

Haplotype	Haplotype frequency
DDGGTT	0.667
IDCGCT	0.094
IDCCCT	0.128
IDCCCC	0.064
IICCCC	0.047

Prolactin haplotype and hatching batch effects on body weight and egg production

The interaction between the *PRL* haplotype and hatching batch as well as the *PRL* haplotype were not significant for BWT, WFE, AFE, FEW and ENO (Table 5). However, hatching batch was significant for BWT, WFE, AFE, and ENO. The least squares mean for hatching batch 1 was non-estimable (Table 5), and the least squares means for hatching batches 2, 3 and 4 for WFE were not significantly different.

Despite the fact that the *PRL* haplotype could not explain BWT and egg production, the least squares means for the haplotype with the highest frequency (DDTTGG) for BWT, WFE, AFE, FEW and ENO were 1,455.23 g, 2,058.01 g, 194.16 d, 37.56 g and 52.00 eggs, respectively, and the least squares means for the haplotype with the lowest frequency (IICCCC) were 1,403.23 g, 2,027.79 g, 184.85 d, 37.90 g and 61.44 eggs, respectively. The 24 bp insertion/deletion at site -358 in 5FA of the *PRL* gene had a non-significant effect on body weight in Pushkin chicken (Mitrofanova et al., 2017) and Silkie fowl (Rahman et al., 2014). Conversely, Xu et al. (2011) reported a significant effect of the 24 bp insertion/deletion at site -358 in 5FA of the *PRL* gene on ENO. Furthermore, Sarvestani et al. (2013) found that the homozygous II genotype at site -358 in 5FA of the *PRL* gene and homozygous CC at sites of -2161 and -2402 had higher egg production than other genotypes in Far native chicken. Moreover, Cui et al. (2006) confirmed that the 24 bp insertion/deletion at site -358 in 5FA of the *PRL* gene as well as haplotyped in the *PRL* gene significantly affected ENO in White Leghorn, Yangshan chicken, Taihe Silkies1, Taihe Silkies2, White Rock and Nongdahe chicken. Cui et al. (2006) indicated that interactions between SNP at site -2402 and other SNPs in 5FA of the *PRL* gene enhanced the *PRL* transcriptional output. Nevertheless, while polymorphisms of the three positions in the *PRL* gene were found in the current study, the haplotype effect could not account for a significant fraction of the variation for body weight and egg production. Therefore, these haplotypes would not be useful for selection programs aimed at improving body weight and egg production in the KU line of Betong chicken.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Table 5 Least squares means (LSM) and standard errors (SE) of prolactin haplotypes, hatching batches and their effects on body weight and egg production traits

Item †	Trait									
	BWT (g)		WFE (g)		AFE (d)		FEW (g)		ENO (eggs)	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE
PRL haplotype										
DDGGTT	1,455.23	9.96	2,058.01	16.89	194.16	2.86	37.56	0.41	52.00	2.13
IDCGCT	1,430.78	29.11	2,061.34	48.71	203.57	8.30	38.18	1.28	50.98	6.16
IDCCCT	1,456.22	22.35	2,058.80	39.13	197.94	6.47	37.93	0.93	52.90	4.81
IDCCCC	Not est	Not est	Not est	Not est	Not est	Not est	Not est	Not est	Not est	Not est
IICCCC	1,403.23	36.30	2,027.79	61.42	184.85	10.51	37.90	1.45	61.44	7.81
Hatching batch										
1	Not est	Not est	Not est	Not est	Not est	Not est	Not est	Not est	Not est	Not est
2	1,540.41^{a}	22.26	2,132.68^{a}	37.67	196.02^{ab}	6.45	37.62	0.91	42.55^{b}	4.79
3	1,466.63^{b}	27.18	2,137.67^{a}	45.99	212.25^{a}	7.87	40.07	1.18	34.28^{b}	5.85
4	1,362.25^{c}	23.14	2,092.67^{a}	39.37	189.88^{b}	6.59	38.22	0.94	62.66^{a}	4.90
p-value										
H	0.555		0.904		0.673		0.897		0.831	
B	<0.001		<0.001		0.024		0.083		<0.001	
HB	0.096		0.121		0.620		0.778		0.520	

BWT = body weight at age 16 wk; WFE = hen weight at first egg; AFE = age at first egg; FEW = first egg weight; ENO = egg number at age 42 wk; Not est = Not estimable; H = fixed effect of *PRL* haplotype; B = fixed effect of hatching batch; HB = fixed effect of interaction between *PRL* haplotype and hatching batch. LSM with different lowercase superscripts within each column are significantly ($p < 0.05$) different.

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