Research Article

A 24 BP Indel Prolactin Gene Polymorphism and Its Association with Some Reproductive Traits in Color Dual-Purpose VLV Hens in Southern Vietnam

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Abstract | Polymorphism of the chicken prolactin gene at the 24 bp indel locus in the promoter region was studied, together with its association with the reproductive traits of color dual-purpose VLV hens. Egg production parameters were recorded as age at first egg (AFE; days), body weight at first egg (BWFE; gram/bird), mean egg weight (MEW; gram), and accumulated number of eggs (ANE) up to 38 weeks of age. DNA collected from the wing vein at 38 weeks of age was extracted from whole blood samples of 393 VLV chickens. The *24 bp* indel*/PRL* target gene was amplified by PCR and the product was separated by electrophoresis on 2% agarose gel for genotyping. Polymorphism was observed at the 24 bp PRL/indel locus, showing two alleles I and D with frequencies 0.57 and 0.43, respectively and three genotypes (II, ID, and DD) with frequencies 18.3, 49.4, and 32.3%, respectively. Hens with the DD genotype had a significantly ($P<0.05$) higher ANE than hens with the ID or II genotypes. The regression equation, $Y = 1.5437X + 58.386$ ($R^2=0.0185$) showed a positive correlation with the correlation coefficient (R) at 0.136. Polymorphism at the 24 bp *PRL/indel* with DD genotype and the correlation coefficient can be used for molecular-aided selection to improve egg production in native chickens, requiring more in-depth study.

Keywords | Dual-purpose chicken, Egg production, Polymorphism, Prolactin gene, Promoter regression

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INTRODUCTION

Population expansion, is expected to grow from 7.9 billion in 2022 to 8.6 billion in 2032 (OECD/FAO, 2023), has increased global public demand for eggs, meat, and milk. The poultry industry has two principal economic traits as egg and meat production which contribute to both local and world markets. Commercial layer breeds play a pivotal role in egg production, however, native layer hens under intensive or semi-intensive farming systems also provide efficient and profitable egg production in many countries, with high adaptation of breeds, quality of eggs, reduced diseases, and investment cost, especially in rural areas. Traditional techniques for genetic improvement followed quantitative selection but with recent advances in technology, genetic improvement of an animal population can be supported through a molecular biological approach (Cui et al., 2005; Wang et al., 2011; Lumatauw and Mu'in,

2016). Reproductive traits with low to moderate (0.13 to 0.20) estimated heritability make conventional breeding methods ineffective (Poivey, 2004; Lin et al., 2016), marker assisted (molecular-aided) selection is considerable as a capable tool for improving egg yield-related traits and enhancing economic benefits. In poultry, several candidate genes have been identified showing polymorphism associated with egg production. The prolactin (*PRL*) gene has important functions in birds, specifically in egg production in chicken (Li et al., 2009; Rowshan et al., 2012; Wilkanowska et al., 2014; Brijendra et al., 2018; Roy et al., 2020; Manoharan et al., 2021; Rohmah et al., 2022), duck (Wang et al., 2011; Chuekwon and Boonlum, 2017; Ghanem et al., 2017; Astuti, 2019; Sabry et al., 2020; Nguyen et al., 2023), goose (Ma et al., 2015; Tang et al., 2021), and quail (Yousefi et al., 2012; Lotfi et al., 2013; Eichie et al., 2016). In chicken, the size of the prolactin gene is 9.536 bp and comprises three parts as (i) three promoters (Kansaku, 2000), (ii) five exons (Ohkubo et al., 1998; Miao et al., 1999; Cui et al., 2004), and (iii) four introns (Dhara and Soller, 1999; Au and Leung, 2002). Several studies have reported that the presence of a 24 bp insertion in the promoter region of the avian prolactin gene corresponded with egg-laying activity and broody behavior in birds (Reddy et al., 2002; Jiang et al., 2009; Kulibaba and Podstreshnyi, 2012). In chicken, the 24-bp indel (insertion/deletion) polymorphism in the promoter region of the prolactin gene can be identified by the PCR technique; however, the association between the 24 bp indel polymorphism and egg production is still not clearly understood. Several studies have reported a significantly positive association between the 24 bp indel polymorphism and egg production (Cui et al., 2006; Begli et al., 2010; Rashidi et al., 2012; Sarvestani et al., 2013; Manoharan et al., 2021; Tu et al., 2023), while other studies showed no positive association (Lotfi et al., 2013; Chaovapasee et al., 2020). In Vietnam, several reports have been done on the PRL/24 indel polymorphism in Lien Minh, Ri, Mia and Silke chickens (Nguyen et al., 2018; Vinh et al., 2021; Tu et al., 2023) but have no data on VLV chicken breed, upto date. Therefore, this research aimed to investigate the polymorphism of the 24 bp in/del of the *PRL* gene in the promoter region, as well as to determine the effect of polymorphism on some reproductive traits of VLV dual-purpose color chickens.

MATERIALS AND METHODS

Chickens

A total of 393 VLV chickens (64 males and 329 females, Figure 1), reared at the VIGOVA Poultry Research and Development Center were used in this study. The VLV is an improved dual-purpose chicken line, with body weight at 38 wks of age was 3.2 kg/male and 2.8 kg/female and egg yield was 170 egg/hen at 68 wks of age (Hoang et al., 2017) developed from chicken breed imported into Vietnam more than 30 years ago.

Figure 1: Representative VLV chicken breed. *(A and B) adult VLV rooster and hen at the time of sample collection (38 weeks of age); (C) VLV in a confined ground area at the growing stage, and (D) caged hens at the laying stage.*

The birds were individually identified by a wing band, housed in a confined large cage area from one day chick to 04 weeks of age and in the confined ground area from 5 to 20 weeks of age, and then reared in individual cages from 21 weeks. Trade feeds were supplied according to age as 0 to 8 weeks (20% crude protein - CP and 2,900 kcal of metabolizable energy - ME) *ad libitum*, 9 to 19 weeks (15% CP and 2,700 kcal of ME) under a restricted feeding regime, and for the laying stage from 20 weeks (17.5% CP and 2,750 kcal of ME) *ad libitum*. Clean fresh water was supplied daily.

The chickens were weighed (BW) at the end of 8 and 20 weeks of age before they were fed in the morning to monitor the feeding regime and control BW before maturity (data not shown). Reproductive traits related to egg production were recorded as age at first egg (AFE; in days), body weight at first egg (BWFE, g/bird), accumulated number of eggs (ANE; egg) calculated as the number of eggs laid until 38 weeks of age, and mean egg weight (MEW; in grams) as the average daily egg weight collected during 37 and 38th week of age.

Collection of samples and DNA extraction

Blood samples were collected at 38 weeks of age from

393 individual healthy and free diseases chickens. A fresh blood sample (1 mL per bird) was taken via the wing vein, following the method of Nguyen et al. (2023). Genomic DNA was extracted using a TopPURE® kit for total blood DNA extraction (ABT-Vietnam) to obtain the OD value using a Bio-drop Machine (UK), qualified by electrophoresis on 1% agarose gel, and then stored at -80 °C until used (Nguyen et al., 2023).

Primer information, amplification and electrophoresis

One set of primers (5'-3') was used as Forward-TTTAATATTGGTGGGTGAAGAGACA and Reverse-ATGCCACTGATCCTCGAAAACTC with 130/154 bp fragment length (Cui et al., 2006).

A polymerase chain reaction (PCR) was performed for genotypic analysis of the extracted DNA (12.5 µL) from 393 individual samples using a BIOER Machine (China), according to Nguyen et al. (2023), comprising 6.25 µL (2X) My Taq™ Mix (Bioline, UK), 1 µL primers (10 pM each; Phu Sa-VIE), 1 µL DNA template (50 ng/µL), and 4.25 µL ddH_2O. The PCR procedure involved cycling conditions as initial denaturation at 95 °C for 4 min followed by repeated 35 cycles at (1) 95 °C for 30 s for denaturation, (2) 59 °C for 30 s for annealing, (3) 72 °C for 30 s for extension, and (4) 72 °C for 5 min for the final extension. The genotypic was identified as 154 bp (Insertion/Insertion; II), 154/130 bp (Insertion/Deletion; ID), and 130 bp (Deletion/Deletion; DD) by 2% agarose gel electrophoresis (60 min, 50V) with a 50 bp DNA ladder under UV light (GelDoc It2-UVP, USA).

Data analysis

Observations of the amplicons and genetic constitutions were made following the procedures of Darabi et al. (2010) and Nguyen et al. (2023). Allele and genotype frequency, and expected heterozygosity (He) were performed according to Nei and Kumar's Formula (2000), with the value of observed heterozygosity (Ho) based on Weir's Formula (1996). The polymorphism information content (PIC) was appraised according to Botstein et al. (1980). ANOVA analysis was applied to test the effect of the 24 bp *PRL/indel* polymorphism in the promoter region of the prolactin gene on reproductive traits together with Tukey's test, and significant difference was set at $P<0.05$. A regression analysis was conducted to predict the association of reproductive traits with genotypes, with the values coded as 1 for II, 2 for ID, and 3 for DD genotypes before analysis. The obtained coefficient correlation (R) was then compared to the standard explanation by Schober et al. (2018) based on the value of R as negligible (0.00-0.10), weak (0.10-0.39), moderate (0.40-0.59), strong (0.60-0.79), and very strong (0.80-1.0).

RESULTS AND DISCUSSION

Target gene amplification by PCR assay and identification of polymorphism

A fragment of the 24 bp (130/154 bp) *PRL* gene in the promoter region was amplified in all the chicken samples, with the representative electrophoresis images 154/130 bp in length, as shown in Figure 2. The mutation form of insertion/deletion (I/D) was identified in the promoter region of the prolactin gene of the sampled chicken population. Two alleles (D and I) and three genotypes were observed as II (insertion of segment of 24 bp), DD (deletion of segment of 24 bp), and ID (insertion/deletion of segment of 24 bp). Several previous studies also successfully amplified the target gene in the promoter region of the prolactin gene in chicken (Jiang et al., 2005; Cui et al., 2006; Alipanah et al., 2011; Dementieva et al., 2020; Yadav et al., 2018; Ahmadi et al., 2019) and in quail or geese (Yousefi et al., 2012; Eichie et al., 2016; Tang et al., 2021) concurring with our results.

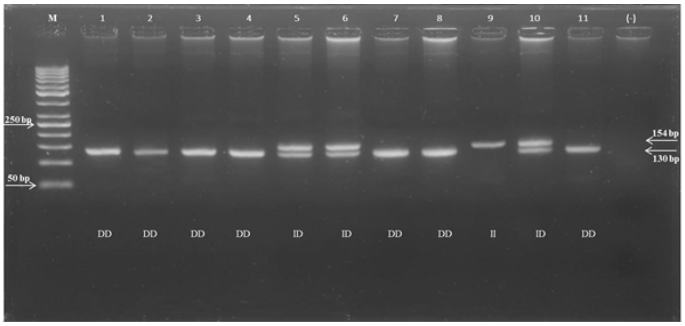

Figure 2: Representative electrophoresis of the PCR products of 24 bp indel in the promoter region of the prolactin gene on 2% agarose gel. *M: DNA ladder (50 bp). No. 1-4; 7-8 and 11: individual samples with DD genotype; Nos. 5-6 and 10: ID genotype; No. 9: II genotype; (-): Negative control.*

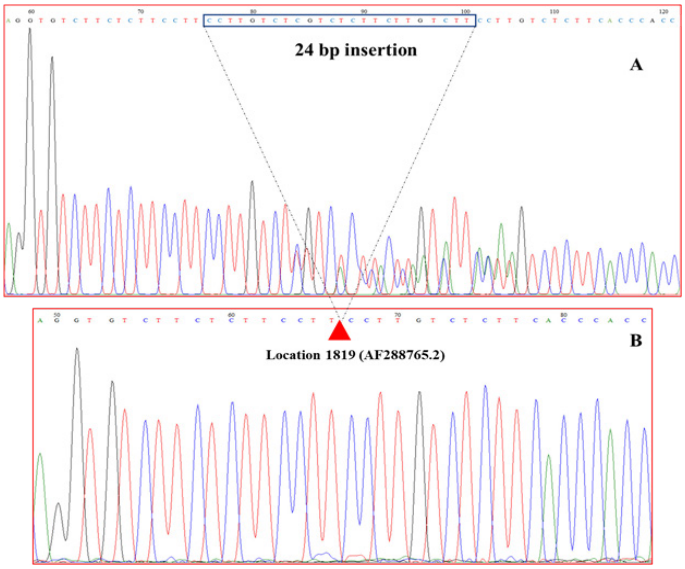

Figure 3: Representative two sequences of the 24 bp *PRL/indel* in the promoter region. *A: insertion of 24 bp (I allele) and B: deletion of 24 bp (D allele) at site 1819 (AF288765.2).*

Table 1: Genotypic and allelic frequency in VLV chicken population at the *24 bp PRL/indel* polymorphic site.

Population	Parameter	Genotypic frequency			Allelic frequency		He	PIC
		II	ID	DD	I	D		
Female	N	63	146	120	0.413	0.587	0.485	0.367
	Observed frequency	0.191	0.444	0.365				
	Expected frequency	0.170	0.485	0.345				
Male	N	9	48	7	0.516	0.484	0.499	0.374
	Observed frequency	0.141	0.750	0.109				
	Expected frequency	0.266	0.499	0.234				
Total	N	72	194	127	0.43	0.57	0.49	0.37
	Observed frequency	0.183	0.494	0.323				
	Expected frequency	0.185	0.490	0.325				

He: expected heterozygosity; PIC: Polymorphism Information Content.

The sequence analysis results are shown in Figure 3, indicating that mutation at this locus formed two alleles (D and I). The I allele (insertion of 24 bp; Figure 3A) and D allele (deletion of 24 bp at site 1819 of AF288765.2; Figure 3B) were identified and three genotypes were observed as illustrated in Figure 2. A similar pattern was also reported by Tu et al. (2023).

The allele and genotype frequencies of the 24 bp *indel/PRL* were analyzed, with results presented in Table 1. According to sex, the frequencies of the D and I alleles in a group of hens were 0.587 and 0.413, while frequencies in roosters were 0.484 and 0.516, respectively. Frequencies of the II, ID, and DD genotypes were 19.1, 44.4, and 36.5% in hens and 14.1, 75.0, and 10.9% in roosters, respectively. In the total population, the frequency of the I allele (0.43) was lower than the D allele (0.57), while the genotypic frequencies of the three genotypes (DD, ID, and II) were 0.323, 0.494, and 0.183, respectively (Table 1).

The frequencies of the I and D alleles were different and varied among the chicken populations and breeds. The frequency of the I allele varied from low to medium in non-commercial breeds and was commonly dominant in layer commercial breeds. Cui et al. (2006) noted that the frequency of the I allele varied from 0.02 to 0.20 in Taihe Silkies breed for the F0 and F1 generations, 0.05 in Yangshan breed, 0.17 in Nongdahe breed, 0.22 in White Rock, and 1.0 in White Leghorn chickens. The frequency of the I allele also varied among different populations and breeds of chicken such as 0.72 in local Iranian (Begli et al., 2010), 0.59 in Mazandaran (Rashidi et al., 2012), "0" in Poltava Clay (Kulibaba, 2015), 0.31 in Papua chicken (Lumatawn and Mu'in, 2016), while for Vietnamese native chicken breeds the I allele frequency was 0.13 in Lien Minh (Nguyen et al., 2018), 0.12 and 0.19 in Ri and Mia, respectively (Vinh et al., 2021), and 0.19 in Silkie (Tu et al., 2023). In this study, the I allele frequency was 0.43, considered as medium level.

As shown in Table 1, the value of He (expected heterozygosity) was 0.49, indicating that the investigated population was divergent. Wang et al. (2011) suggested that the heterozygosity parameter could be used to estimate the level of genetic diversity within or among populations, with a value of heterozygosity greater than 0.5 considered high genetic diversity (Karabag et al., 2016).

The PIC value was 0.37 (Table 1) with 0.25<PIC<0.5 indicating moderate polymorphism at the 24 bp *PRL/indel* locus (Botstein et al., 1980; Chesnokov and Artemyeva, 2015).

Association of polymorphism at the 24 bp PRL/indel site and some reproductive traits in VLV hens

The associations between the 24 bp *PRL/indel* polymorphism and some reproductive traits are shown in Table 2. Results revealed that grouped hens with the DD genotype had significantly higher accumulated egg numbers up to 38 weeks of age compared to the ID and II genotypes (57.0, 55.0, and 54.1 eggs/bird, P<0.05). No effect of 24 bp *PRL/indel* genotypic polymorphism on average egg weight was found among the genotypes (DD, ID, and II: 54.3, 54.3, and 55.1 gram/egg, respectively; P>0.05). No significant difference in age at first egg was found among groups of hens with different genotypes (DD, ID, and II) (171.5, 170.9, and 170.6 days, respectively; P>0.05) or body weight at first egg among the three genotypes (2,330.5, 2,362.4, and 2,314.4 g/bird), as shown in Table 2.

Table 2: Effects of a 24 bp *PRL/indel* genotype on some reproductive traits of VLV chicken breed.

Genotype	N	Age at first egg (days)	Body weight at first egg (g/bird)	Egg yield up to 38 weeks of age (egg)	Mean egg weight (g)
DD	120	171.5±1.5	2,330.5±19.2	57.0^{a}±0.7	54.3±0.4
ID	146	170.9±1.1	2,362.4±19.7	55.0^{b}±0.7	54.3±0.4
II	63	170.6±1.7	2,314.4±44.4	54.1^{b}±1.1	55.1±0.7

Data are presented as mean ± SEM. Within the column, number with different superscripts are significantly different (P<0.05).

The prolactin hormone is a polypeptide that plays a key role in egg production, and enhancement of PRL secretion causes the onset of nesting behavior, which results in lower egg production (Sharp et al., 1998). In birds, the *PRL* gene is recognized as an applicant marker for egg productive traits because it performs a pivotal function in broodiness behavior that specifically controls egg yield variability by reducing egg biosynthesis during the nesting period (Chen et al., 2007; Perdamaian and Daryono, 2020). Previous research reported that insertion of the 24 bp *PRL/indel* in the promoter region was positively correlated with laying rate in hens and negatively correlated with incubation behavior (Reddy et al., 2007; Cui et al., 2006; Jiang et al., 2009; Wang et al., 2009, 2011; Kulibaba and Podstreshnyi, 2012). Using the 24 bp *PRL/indel* as a candidate marker gene in the selection program of Silkie chicken showed no significant associations between genetic variation and productive traits (Wada et al., 2008), while Begli et al. (2010) reported no significant association between the 24 bp PRL/indel site polymorphism and growth traits in local fowls of Yazd Province. The II and ID genotypes were significantly associated with an increase in egg number in an Iranian fowl population from Yazd Province. By contrast, several researchers reported that the deletion allele (D) was associated with an increasing number of eggs as well as egg weights (Yadav et al., 2018; Tu et al., 2023), concurring with our results and not supporting some of the earlier findings.

For maturity age, Xu et al. (2011) also noted that hens with the ID genotype had an average age at first egg slightly lower than the DD genotype, in agreement with our results (Table 2).

The onset of broodiness triggers an increase in the prolactin hormone in the plasma of tropical chicken breeds, resulting in the regression of ovarian follicles and lower egg production (Li et al., 2013; Banu et al., 2017). Jiang et al. (2005) mentioned that insertion of the 24 bp fragment in the promoter region of the *PRL* gene may suppress a transcriptional factor binding site, resulting in decreased PRL expression and contributing to broodiness behavior, especially in hens. Cui et al. (2006) reported that the heterozygous genotype for insertion and deletion (In/Del) had the highest expression level of PRL mRNA in several lines of Chinese chickens.

Several intrinsic and extrinsic factors affect broodiness in birds. Intrinsic factors include genetic divergences due to mutation and other mechanisms related to epigenetic conditions, for example, DNA methyl/demethylation or histone acetyl/deacetylation. Extrinsic factors include photoperiodism, THI (temperature-humidity index), and feed constitutions which change bird behavior toward broodiness which is associated with egg production capacity (Izutsu et al., 2001, 2002; Geng et al., 2014; Perdamaian and Daryono, 2020). Beside this, changing the feeding style from small-holders scale to industrial system is also considerable the factor that affecting the broody behavior and/or laying rate of hens.

A regression analysis was performed between the 24 bp PRL indel genotypes and egg yield for up to 38 weeks of age, as shown in Figure 4. Results indicated that the *PRL* gene affected 1.85% of the variation in ANE up to 38 weeks of age, with a similar result also reported by Purwantini et al. (2020). In this study, the correlation coefficient between genotypes with ANE was small (0.136), suggesting a relatively small effect of genotype on egg yield. According to Schober et al. (2018), the weak correlation coefficient (0.136) suggested that many other elements affected the ANE trait as both environmental (extrinsic) and genetic (intrinsic) factors. Reproductive traits are generally controlled by polygenes (Goraga et al., 2012) and some genetic factors affect reproduction. The *PRL* and other marker genes require more in-depth study. The correlation coefficient in this study was relatively small but could still be used as a basic database for a selection program.

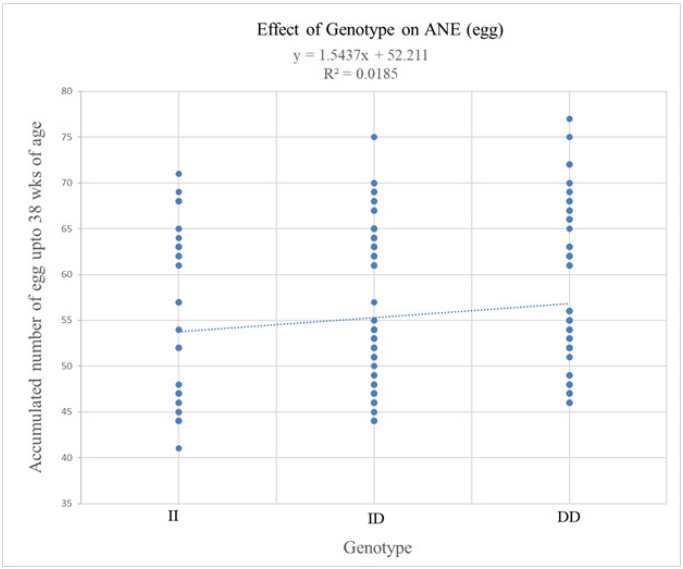

Figure 4: Genotype effects of the 24 bp indel prolactin gene on the accumulated number of eggs laid by VLV hens up to 38 weeks of age. ANE: accumulated number of eggs.

CONCLUSIONS AND RECOMMENDATIONS

We, for the first time, determined the polymorphism at the 24 bp *PRL/indel* locus in the promoter region in the VLV color dual-purpose chicken breed. The dominant of D allele and DD genotypes were found and hens bearing the DD genotype produced higher egg numbers up to 38 weeks of age than hens with the ID and II genotypes. In case of the breeding program, handling the D allele in the

population should be considered. Polymorphism at the 24 bp *PRL/indel* site can be used for molecular-aided selection to improve egg production in native chickens, requiring more in-depth study.

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NOVELTY STATEMENT

This is the first study in Southern Vietnam on the Prolactin gene polymorphism in the promoter regions of dual-purpose VLV chickens carrying the 24 bp *PRL/indel*. The D allele had a higher frequency and positive effect on the total number of eggs up to 38 weeks of age. A positive association and correlation between egg production and DD genotype implies that carefully managing breeding programs to employ the frequency of the D allele due to it is considerable marker gene to improve egg production.

AUTHOR'S CONTRIBUTION

All authors discussed the experimental design, read and approved the manuscript at each step. NTN managed all the research activities including writing the manuscript. TTVT and TTTN contributed equally to DNA extraction, amplification of the target gene and sequence analysis. TTH and DTN, contributed equally to collect the blood samples, productivity data and performed the statistical analyses.

Conflict of interest

The authors have declared no conflict of interest.

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