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ASSOCIATIONS OF POLYMORPHISMS IN PROLACTIN AND DOPAMINE RECEPTOR D2 GENES WITH REPRODUCTIVE TRAITS ON SILKIE CHICKEN

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ABSTRACT: The Silkie chicken (*Gallus gallus domesticus* Brisson) is one of domestic chicken breeds with commercial rearing and breeding potentials for egg production. Prolactin (PRL) and dopamine D2 receptor (DRD2) are potential genes associated with reproductive traits in chickens. This study was conducted to analyze the association of PRL and DRD2 insertion/deletion (Indel) polymorphisms with chicken reproductive traits in Silkie chickens. A total of 380 hens from 16-40 weeks of age were used, with each one being placed in a separate cage. DNA isolation was performed using feather samples, and genotypes were detected using the Indel technique. Two polymorphisms consisting of 24 base pair (bp) Indel in the promoter region of the PRL gene and 22 bp Indel in the promoter region of the DRD2 gene were identified. At both sites, the Indel polymorphisms did not follow the Hardy-Weinberg equilibrium. In addition, with the exception of total eggs over 23 weeks of laying in the PRL gene, the analysis revealed no association between these polymorphic loci and any traits collected. In conclusion, birds with the DD genotype produced the maximum egg yield (73.6 eggs/hen), whereas those with the II genotype produced approximately 9 fewer eggs (64.1 eggs/hen), resulting in laying rates of 45.7% and 40.1%, respectively. For enhancing the egg-laying capacity of Silkie chickens via selective breeding, opting for DD birds with DD genotype of PRL Indel is highly recommended.

Keywords: Egg production, Indel technique, Polymorphism, Reproductive traits, Silkie chicken.

INTRODUCTION

The productive value of animals is determined by their ability to meet certain production demands and this is typically measured by the quality and quantity of the product obtained during a given period (Rodina et al., 2019; Martinelli et al., 2020). In poultry, the process of egg production involves various synchronized metabolic and physiological processes that determine the number of produced eggs, rate of laying, and egg hatchability (Mench et al., 2011). Typically, the average egg production of most indigenous or native breeds/fowls is low (Nguyen et al., 2020), and this low productivity in backyard farming is primarily due to the limited production potential of the existing indigenous germ plasm, as noted by Haunshi et al. (2009). Many factors, particularly environmental and genetic ones, have a significant impact on these traits (Niknafs et al., 2012; Tongsiri et al., 2019; Loengbudnark et al., 2023). When considering the genetic effects on egg production in chickens, prolactin (PRL) (Mohamed et al., 2016) and dopamine D2 receptor (DRD2) (Xu et al., 2010) have been identified as potential candidate genes. Prolactin, a hormone generated by the pituitary gland, is important in the regulation of egg formation since it increases broodiness, which temporarily suppresses ovarian activity and prevents egg laying (Sharp et al., 1984). In the promotor region of PRL gene, the mutation at -358 position with 24 base pair (bp) insertion/deletion (Indel) influenced egg productivity in different chicken breeds (Jiang et al., 2005; Cui et al., 2006; Begli et al., 2010; Yousefi et al., 2012; Lotfi et al., 2013). Additionally, in birds, dopamine was found to have a crucial function in the secretion of PRL as it binds to its specific receptors, namely, dopamine D1 receptor and DRD2 (Youngren et al., 1998). It has been reported that the DRD2 gene governs the reproductive traits of poultry and is associated with the number of chicken eggs produced after 300 days (Xu et al., 2011b) as well as the age at which the first egg is laid (Xu et al., 2011a).

Among indigenous chickens in Vietnam, black-boned chicken, named “Ac” or Silkie chicken with white feathers, black bone and skin, is a famous breed. Silkie chicken brings many benefits to Vietnamese because of its therapeutic potential from meat and is used as a medical food for improving human health. In terms of nutritional value, chicken meat contains essential amino acids and iron. The Silkie eggs were also chosen by consumers because they have no fishy smell, are fatty, fragrant, high in white protein, have a high percentage of yolks, and a very attractive dark color (Phuong

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et al., 2023). The eggs of Silkie chicken were of small size (31.3-36.2 g/egg) and hen-day-egg production rate was 52.3-58.1% at 23-37 weeks old (Thuy and Ha, 2022). Because of their small size and low growth and egg production rate, Silkie chickens were mainly kept on a small scale and easy to access with a low investment. They were a major source of income and nutritionally rich food for households. Recently, the Silkie chickens have been raised industrially for egg production in Tien Giang and Long An provinces (Vietnam) on a large scale. However, breeding stock is still a major obstacle due to poor breeder quality and unstable yield; besides, egg production has not reached its potential. In Vietnam, some recent studies focused on nutrition to improve the reproductive performance of Silkie chickens (Phuoc et al., 2019; Thuy and Ha, 2022), but there have been no publications investigating and selecting this breed to improve egg production.

Therefore, this study aimed to investigate the associations of two polymorphisms of PRL and DRD2 genes with reproduction traits in Silkie hens.

MATERIALS AND METHODS

Experimental chickens

A total of 380 Silkie chicken hens, ranging in age from 16 to 40 weeks, were individually housed in cages and fed a diet containing 17% crude protein and 2,850 Kcal/kg metabolized energy for the entire duration of the experiment. Clean water was available to the chickens at all times. All chickens were vaccinated against common diseases before the 40-week experimental period of laying.

Phenotypic data

For laying hens, age at first egg (AFE) and body weight at first egg (BWFE) were recorded. In addition, eggs were collected daily at 5 P.M. and numbered to monitor individual yield. To evaluate the chickens' reproductive performance, the following parameters were recorded and calculated: total egg yield (per bird basis) and laying rate. The weight of the eggs and their shape index (the ratio of the short diameter of the egg to the long diameter) were measured every week (one egg/hen) throughout the entire experiment.

DNA extraction and genotyping

Genomic DNA isolated from chicken feathers (Bello et al., 2001) was subjected to amplification in a thermal cycler. The polymerase chain reaction (PCR) was performed in a 25 µl reaction containing 12.5 µM PCR Master Mix 2X (Phu Sa Genomics Joint Stock Company, Vietnam), 20 pM each primer, and 100 ng genomic DNA. The reaction was carried out with the following conditions: denaturation (95^{o}C for 5 minutes), 35 cycles of 95^{o}C for 30 seconds, annealing for 45 seconds, extension (72^{o}C for 45 seconds), and final extension (72^{o}C for 10 minutes). The PCR products of each gene were split on 3.5% agarose gel for 45 minutes at 80V for the identification of genotypes. The polymorphic site was also confirmed by sequencing with Sanger's method (Sanger et al., 1977). Information regarding primers and polymorphisms is provided in Table 1.

Table 1 - Information for primers and polymorphisms

Locus	Sequence (5'-3')	GenBank Acc. No.	PCR size (bp)	Tm (°C)	References
PRL Indel	F: TTTAATATTGGTGGGTGAAGAGACA R: ATGCCACTGATCCTCGAAAACTC	AF288765	154/130	54	Cui et al. (2006)
DRD2 Indel	F: TGCACTTCAATCCTTCCCAGCTT R: TTGCGCTGCCCATTGACCA	EU313425.1	187/165	62	Xu et al. (2010)

F: Forward primer; R: Reverse primer; Tm: Annealing temperature

Data analysis

The frequencies of alleles were calculated through allele counting in accordance with the Hardy-Weinberg equilibrium and the potential deviations from the expected genotype frequencies were tested using a Chi-square test. Moreover, the General Linear Model of Minitab software version 16.2.1 was used to analyze the association between genotype and egg yield and egg traits using the model of $Yij = \mu + Gi + \xi ij$ (where Yij: traits observed; µ: general mean, Gi: influence of genotype; ξij: random error). Data are presented as Least square mean ± Standard error.

Ethical considerations

The study was performed by authorized, qualified, and trained veterinarians, scientists, and technicians, in compliance with the guidelines of the Institutional Animal Ethics Committee (IAEC).

RESULTS AND DISCUSSION

Allele and genotype frequencies in the Silkie chicken population

Figures 1 and 2 display the genotype analysis of two genes. The bands depicted in the agarose gel electrophoresis allowed for the differentiation of genotypes for each polymorphism. The study detected three genotypes, which also

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resulted from a mutation in the promoter region of the PRL gene. The 24 bp Indel polymorphism presented a DNA fragment size of 154 bp for the I allele and 130 bp for the D allele (Figure 1a, b). Moreover, the promoter region of the PRL gene from Silkie chicken had 24 inserted nucleotides (TGTCTCTTCTTGTCTTCCTTGTCT) (Figure 3a,b).

A 22-base pair insertion/deletion (Indel) was found in the DRD2 gene of Silkie chickens. Figure 2a illustrates two alleles, the I allele (187 bp) and the D allele (165 bp). The promoter region of the DRD2 gene in Silkie chickens had a 22 bp (GTTGCTACCCTTAGCAAAGGCT) insertion, as shown in Figure 2b. Figure 4 presents a partial DNA sequence with 22 nucleotides inserted in the I allele of the DRD2 gene.

Table 2 present the allele and genotype frequencies of two genes. The PRL Indel locus had a lower I allele frequency (0.30) compared to the D allele frequency (0.70) in the population. However, the frequency ratio of the I allele (0.54) was higher than the D allele frequency (0.46) in the DRD2 Indel locus. Additionally, the results indicate that the genotypic frequencies of the DRD2 and PRL Indel loci in the Silkie chicken population did not adhere to the Hardy-Weinberg principle ($p<0.001$).

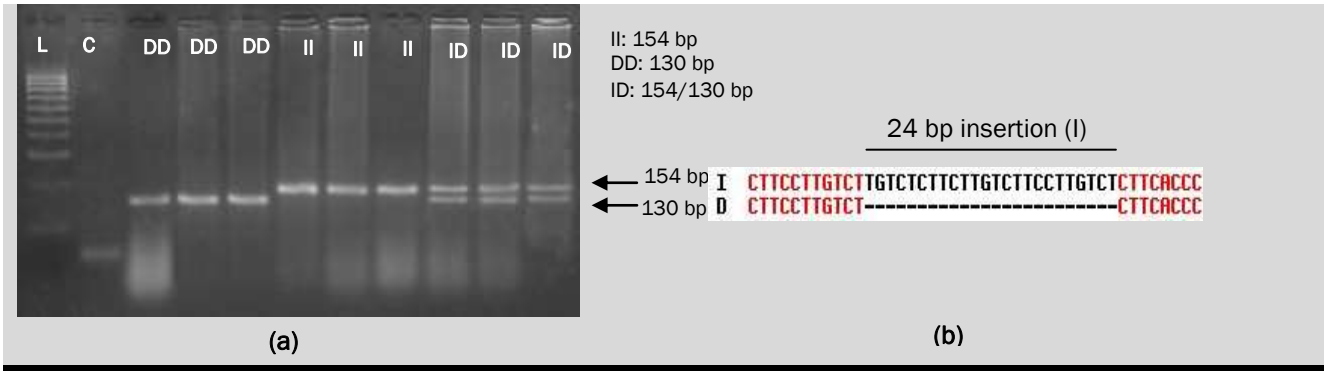

Figure 1 - Genotypes of PRL Indel. a) PCR products displayed on 3.5% agarose gel electrophoresis; b) 24 bp insertion sequences; Lane L: DNA marker (100-1,000 bp); lane C: negative control; lane 3, 4, 5: DD genotype; lane 6, 7, 8: II genotype; lane 9, 10, 11: ID genotype

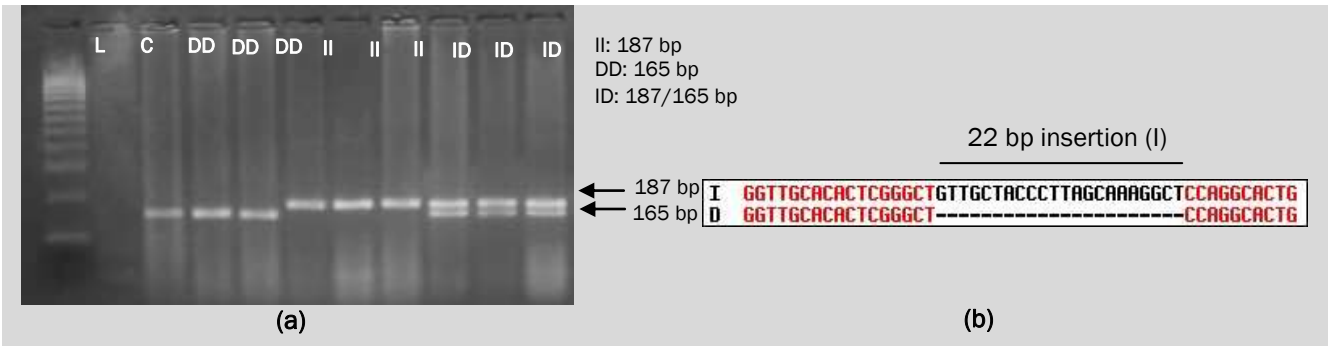

Figure 2 - Genotypes of DRD2 Indel. a) PCR products presentation on 3.5% agarose gel electrophoresis; b) 22 bp insertion sequences; Lane L: DNA marker (100-1,000 bp); lane C: negative control; lane 3, 4, 5: DD genotype; lane 6, 7, 8: II genotype; lane 9, 10, 11: ID genotype

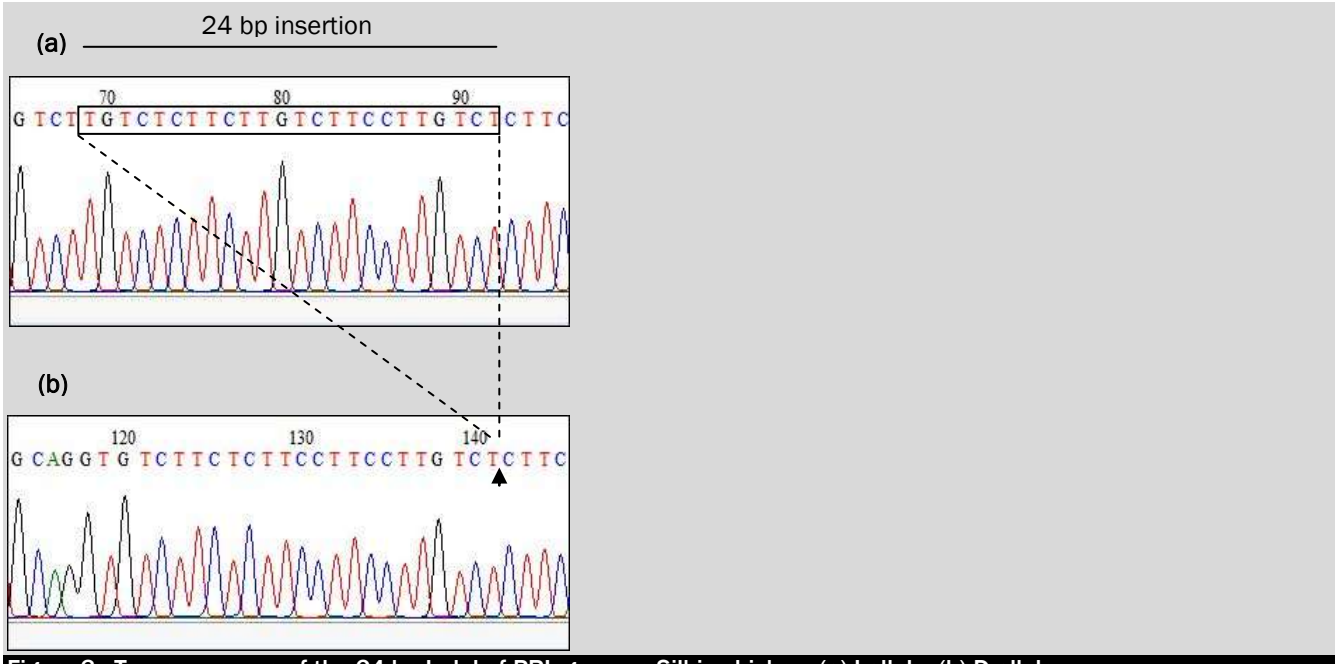

Figure 3 - Two sequences of the 24 bp Indel of PRL gene on Silkie chicken. (a) I allele; (b) D allele

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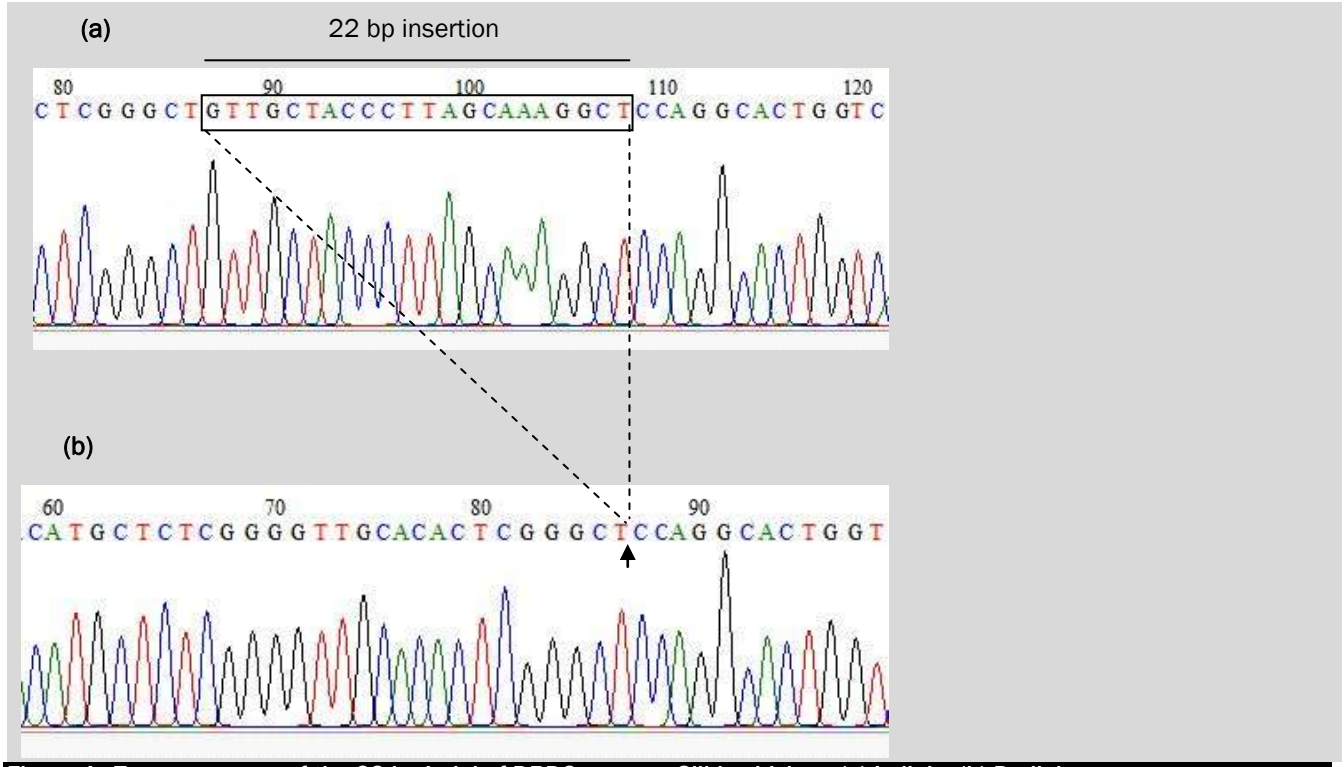

Figure 4 - Two sequences of the 22 bp Indel of DRD2 gene on Silkie chicken. (a) I allele; (b) D allele

Table 2 - Distribution of allele and genotype frequencies of two genes in the Silkie hen population

Locus	Genotype frequency			Allele frequency		HWE (χ2)
PRL Indel	DD	ID	II	D	I	
	0.59 (223)	0.22 (86)	0.19 (71)	0.70	0.30	80.8***
DRD2 Indel	DD	ID	II	D	I	
	0.26 (101)	0.38 (144)	0.36 (135)	0.46	0.54	21.1***

HWE: Hardy-Weinberg Equilibrium; (n): hen number; ***: p<0.001

Association of two polymorphisms on reproductive traits in Silkie chicken

During the 23 weeks of laying, among the PRL Indel genotypes, chickens bearing the DD genotype produced a higher number of eggs than those with the II genotype (p<0.001). As a result, the laying rate was also the highest in DD birds. For the polymorphic site of the DRD2 gene, no association was found for all the examined parameters (Table 3).

Table 3 - Association of INDEL polymorphisms with reproductive traits in laying chickens

Parameters	Genotypes (PRL)			Genotypes (DRD2)		
	DD (n=223)	ID (n=86)	II (n=71)	DD (n=101)	ID (n=144)	II (n=135)
AFE (day)	119±0.36	119±0.58	120±0.65	120±0.56	119±0.47	119±0.48
BWFE (g)	733±2.5	733±4.1	731±4.5	734±3.9	730±3.3	733±3.4
Laying days	161±0.4	161±0.6	160±0.6	161±0.6	162±0.5	161±0.5
Total eggs	73.6±0.94^{a}	70.6±1.51^{a}	64.1±1.67^{b}	69.1±1.45	70.6±1.22	68.5±1.25
Laying rate (%)	45.7±0.60^{a}	43.8±0.97^{a}	40.1±1.08^{b}	43.1±0.93	43.8±0.79	42.7±0.80
Egg weight (g)	35.3±0.07	35.3±0.11	35.6±0.12	35.4±0.10	35.3±0.09	35.3±0.09
Egg shape (%)	76.6±0.03	76.6±0.05	76.6±0.06	76.6±0.05	76.6±0.04	76.6±0.04

AFE: Age at first egg; BWFE: Body weight at first egg.^{a,b}: Means with different letters in the same row differ significantly (p<0.05).

The I allele frequency of the PRL Indel in this study differs from that of Chinese chicken breeds. Cui et al. (2006) reported I allele frequencies of the PRL Indel as follows: 0.02 in Taihe Silkie F0 generation, 0.2 in Taihe Silkie F1 generation, 0.05 in Yangshan, 0.17 in Nongdahe, 0.22 in White Rock, and 1.00 in White Leghorn chickens. Furthermore, the I allele was identified in native Iranian chickens with a frequency of 0.72 (Begli et al., 2010), in Mazandaran chickens with a frequency of 0.59 (Rashidi et al., 2012), and in Poltava clay chickens with a frequency of 0.00 (Kulibaba, 2015).

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DRD2 and PRL Indel polymorphisms did not conform to the Hardy-Weinberg equilibrium, possibly due to small sample sizes or the exclusive use of Silkie hens in the population. On the other hand, in the Pushkin chicken breed, Mitrofanova et al. (2017) discovered a 22 bp Indel in the DRD2 gene, with an I allele frequency of 0.41, lower than the D allele frequency of 0.59. Similarly, Datumada and Thongsaiklaing (2020) found that the I allele frequency of the DRD2 Indel in Thai native chickens was 0.23, lower than the D allele frequency of 0.77. For the PRL Indel, Lumatauw and Mu'in (2016) detected I allele frequency of 0.31 in Papua local chickens. Moreover, several previous studies reported similar results, the I allele frequency was 0.13 in Lien Minh chickens (Nguyen et al., 2018), 0.19 in Ri chickens, and 0.12 in Mia chickens (Vinh et al., 2021). In a study on Ningdu Sanhuang chickens, Xu et al. (2011b) analyzed the PRL Indel and found that hens with the ID genotype produced an average of 97.3 eggs, which was higher than the 94.0 eggs produced by chickens with the DD genotype. Additionally, hens with the ID genotype had an average first egg age of 191.38 ± 14.09 days, slightly earlier than the 195.71 ± 16.15 days for the DD genotype (Xu et al., 2011a). Furthermore, the ID genotype in PRL Indel exhibited a higher average egg weight (47.6 g/egg) compared to other genotypes in Lien Minh chickens (Nguyen et al., 2018).

The egg weights in the present study were much smaller than other local chickens in Vietnam such as Ri chickens (41.7 g/egg) and Mia chickens (44.7 g/egg) (Moula et al., 2012), Bang Troi chickens (48.4 g/egg) (Thinh et al., 2020), black Noi hens (48.3 g/egg), and dark brown Noi hens (49.7 g/egg) (Hoa et al., 2021). In addition, Silkie chickens had egg weight lower than indigenous chickens in southern Ethiopia (46.6 g/egg in lowland; 48.6 g/egg in midland and 45.4 g/egg in highland) (Berhanu et al., 2022) and Sidama region (Ethiopia) (44.9 g/egg in lowland; 49.5 g/egg in midland; and 42.9 g/egg in highland) (Legesse and Kefyalew, 2023). This study identified a 22-bp Indel in the DRD2 gene of Silkie chickens. The researchers analyzed the genetic diversity of this DRD2 Indel polymorphism in Silkie chickens and found that the frequency of the Indel allele was relatively low in the population (Xu et al., 2011b). The study provides insight into the genetic variation present in the DRD2 gene of Silkie chickens and highlights the potential implications of this variation for their dopamine signaling and related functions.

It's worth noting that genetic variation in the DRD2 gene has been studied in various species, including humans, and has been associated with a range of behavioral and physiological traits, such as addiction, impulsivity, and obesity (Switala et al., 2022). However, the specific effects of the DRD2 gene Indel polymorphism on reproductive traits in chickens are not well-established in the scientific literature, and further research would be needed to investigate any potential associations.

In the works of various authors, it was found that the presence of a 24-bp insertion in the promoter region of the avian prolactin gene is positively correlated with the intensity of egg-laying activity in birds and broody behavior (Jiang et al., 2009; Kulibaba and Podstreshnyi, 2012). The results of Jiang et al. (2005) have shown that PRL could be a genetic marker in breeding against broodiness in chickens. Prolactin gene in chicken (cPRL) specifically on the promoter region is a candidate gene for brooding behavior (Shimada et al., 1991; Dunn et al., 1998), and egg production (Cui et al., 2006). The cPRL promoter gene is an important part that responsible for the expression or the function of cPRL. The position of promoter in cPRL gene is located at the starting point (Lewin, 1997) and has its role in activating the early transcription for the gene expression. If a mutation occurs in this promoter region, the cPRL gene will not function and fail to express. Therefore, the promoter region is a crucial part of the gene as it controls the initiation of gene expression by binding to RNA polymerase and other transcription factors. Mutations in the promoter region can affect the binding of these factors and thus alter the expression of the gene. In the case of cPRL, a mutation in the promoter region can disrupt the early transcription process and prevent the gene from being expressed, leading to a loss of function. This can have significant effects on the chicken's behavior and physiology, as demonstrated by the association of cPRL promoter gene with brooding behavior and egg production.

Recently, it was found that the Tellicherry native chicken population has a high frequency of the I allele, which is associated with a 24bp Indel polymorphism in the promoter region of the prolactin gene (Manoharan et al., 2021). The study suggests that this polymorphism could be used as a potential molecular marker for selection and breeding in native chickens. Furthermore, the presence of an association between the 24bp insertion polymorphism and egg production was observed, indicating that this polymorphism could also be used for improving egg production in Tellicherry native chicken population (Manoharan et al., 2021). In prior research (Ngu et al., 2015), a point mutation (DRD2/*Bse*GI) was reported in Noi chicken, another indigenous Vietnamese breed. However, this polymorphism exhibited no impact on the birds' egg production. The DD genotype of the 24-bp Indel polymorphism in the prolactin gene has been found to be associated with a higher laying rate in chickens.

CONCLUSION

In the local Silkie chicken, a 24-bp Indel genetic variation was discovered in the promoter region of the prolactin gene. The D allele, which carries this Indel, was observed at a high frequency of 0.70. This indicates that by carefully managing breeding practices to manipulate the frequency of the D allele, it may be possible to improve egg production.

DECLARATIONS

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Authors' contribution

This work was conducted with contribution of all authors. T.T. Tu and N.T. Ngu designed the experimental procedures. T.T. Tu and L.T. Phuong performed the experiments. T.T. Tu, L.T. Phuong and N.T. Ngu interpreted the data and prepared the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors have not declared any conflict of interests.

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