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Research article

Genetic variants of INHA/*Pst*I and VIPR1/*Hha*I and their relationship with reproductive traits in Silkie chicken (*Gallus gallus domesticus* Brisson)

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Abstract

The Ac chicken (Gallus gallus domesticus Brisson) is commonly raised alongside other domestic chicken breeds in the Mekong Delta region. This study was conducted to analyse the impact of INHA/PstI and VIPR1/HhaI polymorphisms on the reproductive characteristics of this breed. A total of 380 Silkie hens from 16-40 weeks of age were used, each placed in a separate cage for data collection. DNA isolation was performed using feather samples, and genotypes were detected by applying the PCR-RFLP technique. Two polymorphisms were identified, namely C829T (INHA/PstI) in exon 1 of the INHA gene and C42913T (VIPR1/HhaI) in intron 6 of the VIPR1 gene. At both sites, two polymorphisms did not follow the Hardy-Weinberg equilibrium. The INHA/PstI and VIPR1/HhaI polymorphisms demonstrated a statistically significant correlation with the total number of eggs produced and the laying rate ($p<0.05$). Specifically, hens with TT genotype (INHA/PstI) had the highest egg production (72.4 eggs/hen/24 laying weeks). In contrast, those with the CC genotype produced approximately 9 fewer eggs (63.2 eggs/hen), resulting in laying rates of 45.2% and 40.3%, respectively. Additionally, the INHA/PstI polymorphisms showed a notable and significant correlation with the average age of the first egg ($p<0.05$). In conclusion, to enhance egg production in Silkie chickens through selective breeding, it is recommended to prioritise the use of birds with the TT genotype at the INHA/PstI locus.

Keywords: Egg number, Polymorphisms, Reproductive traits, Silkie chicken, SNP.

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INTRODUCTION

The productive value of animals is determined by their ability to meet specific production demands, and this is typically measured by the quality and quantity of the product obtained during a given period. In poultry, egg production involves various synchronised metabolic and physiological processes that determine the number of eggs produced, the laying rate and egg fertility (Mench et al., 2011). Typically, the average egg production of most indigenous or native chicken breeds is low, primarily due to the existing indigenous germplasm's limited production potential, as Haunshi et al. (2009) noted. The inhibin subunit alpha (INHA) is a gonadal glycoprotein hormone that plays an essential performance in reproductive activities (Welt et al., 2002). INHA is produced by the granulosa cells of ovarian follicles hens and by the Sertoli cells of the testes roosters (Ying, 1987). Decreasing expression of the INHA gene affected an antagonistic on granulosa cell apoptosis in poultry (Chen et al., 2007). Therefore, INHA is a potential candidate gene to increase the ovulation rate in poultry. In birds, the vasoactive intestinal peptide receptor 1 (VIPR1) gene also joined the behavioural regulation mechanism associated with broodiness. VIPR1 gene was mainly expressed in the pituitary, and the differential mRNA expressed in the pituitary was connected with changes in reproductive traits (Kansaku et al., 2001; You et al., 2001; Chaiseha et al., 2004).

The black-boned chicken with white feathers, black bone and skin, also referred to as “Ac” or Silkie chicken, is famous among indigenous chickens in Vietnam. Consumers prefer their egg because it has no fishy smell, is fatty and fragrant, has high white protein, a high percentage of yolks, and is very attractive in dark colours. Silkie chicken's eggs were small (31.3-36.2 g/egg), and the hen-day egg production rate was 52.3-58.1% at 23-37 weeks old (Thuy and Ha, 2022). Because of its small size, low growth and egg production rate, Silkie chickens are mainly kept in small-scale units that are easy to access with low investment. They are a major source of income and nutritionally rich food for households. Recently, Silkie chickens have been raised industrially for egg production in Tien Giang and Long An provinces (Vietnam) on a large scale. In addition, breeding stock is still a significant obstacle due to poor breeder quality, unstable yield, and egg production has not reached its potential. Recent studies in Vietnam have concentrated on enhancing the reproductive capabilities of Silkie chickens through nutritional interventions (Phuoc et al., 2019; Thuy and Ha, 2022); however, no previous research has explored the genetic aspects related to egg production improvement. Thus, this study aimed to examine the links between two specific genetic variations in the INHA and VIPR1 genes and reproductive characteristics in Silkie hens, aiming to contribute to the future breeding program.

MATERIALS AND METHODS

Experimental chickens

A total of 380 Silkie hens, ranging from 16 to 40 weeks of age, were individually housed in cages and fed a diet containing 17% CP and 2,850 Kcal/

kg ME for the entire duration of the experiment (Thuy and Ha, 2022) 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

The study recorded the age at which laying hens produced their first egg and corresponding weights. Daily egg collection took place in the afternoon, and each egg was numbered to track individual yield. To evaluate the reproductive performance of the chickens, the study focused on measuring and analysing two specific parameters: the total number of eggs produced per bird and the laying rate. Egg weight and shape index, the ratio of the egg's short diameter to its long diameter, were measured weekly for each hen throughout the entire experiment, with one egg being measured per week.

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), 8 pM each primer, and 100 ng genomic DNA. The reaction was carried out with the following conditions: denaturation (95°C for 5 min), 35 cycles of 95°C for 30 seconds, annealing for 45 seconds, extension (72°C for 45 seconds) and final extension (72°C for 10 minutes). The PCR products of each gene were split on 3.5% agarose gel for 45 minutes at 80V to identify genotypes. The polymorphic site was also confirmed by sequencing with Sanger's method. Information regarding primers and polymorphisms is provided in Table 1. The PCR products of each gene were incubated by their corresponding restriction enzymes overnight at 37°C (Table 1). The final products were split on 3.5% agarose gel for 45 minutes.

Table 1 Primers and polymorphisms of two genes used in this study

Locus	Primer sequence (5'-3')	GenBank Acc. No.	PCR size (bp)	Tm (°C)	RE	Reference
INHA/*Pst*I (T829C)	F: ATCCCACAGCCCCAAGACCGT R: TGCGGTGAGAGGGTCAGCACAG	MT892939.1	409/297/112	59	*Pst*I	This study
VIPR1/*Hha*I (C42913T)	F: CCCCGTTAAACTCAGCAGAC R: CCCAAAGTCCCACAAGGTAA	XM_418492	434/253/181	59	*Hha*I	Zhou et al. (2008)

F: Forward primer; R: Reverse primer; Tm: Annealing temperature, RE: Restriction enzyme.

Statistical analysis

Allele frequencies were determined using allele counting in accordance with Hardy-Weinberg equilibrium, and potential deviations from expected genotype frequencies were assessed with a Chi-square test. Moreover, the General Linear Model of Minitab software version 16.2.1 was used to analyse the relationship 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.

RESULTS

Allele and genotype frequencies in the Silkie chicken population

The results of the PCR-RFLP analysis of the INHA gene are depicted in Figure 1. The visible bands in the gel illustrated the distinct genotypes observed in the polymorphism. Specifically, three genotypes were identified at the INHA/*Pst*I sites, namely CC, CT, and TT. The product electrode on 3.5% agarose gel for two types of cutting with size: 297/112 bp and 409 bp, corresponding to C and T alleles. Figure 3 shows SNP analysis of INHA/*Pst*I and indicates that nucleotide T was replaced by C at site 829 (T829C) in exon 1 of the INHA gene.

The genotypes of VIPR1 gene were detected after digesting PCR products with *Hha*I restriction enzyme. The restriction fragment lengths for the C and T alleles were 253/181 bp and 434 bp (Figure 2). Similarly, Figure 4 shows SNP analysis of VIPR1/*Hha*I and indicates how nucleotide C was replaced by T at site 42913 (C42913T) in intron 6 of VIPR1 gene.

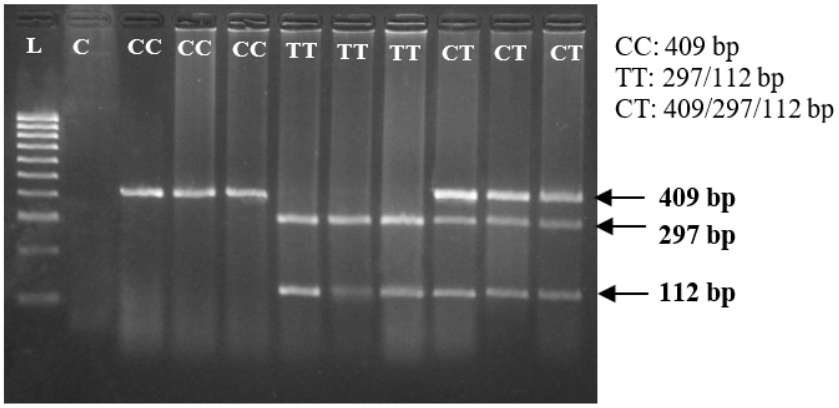

Figure 1 Presentation of PCR products of INHA/*Pst*I on 3.5% agarose gel electrophoresis
Lane L: DNA marker (100-1,000 bp); lane C: negative control; lane 3, 4, 5: CC genotype; lane 6, 7, 8: TT genotype; lane 9, 10, 11: CT genotype

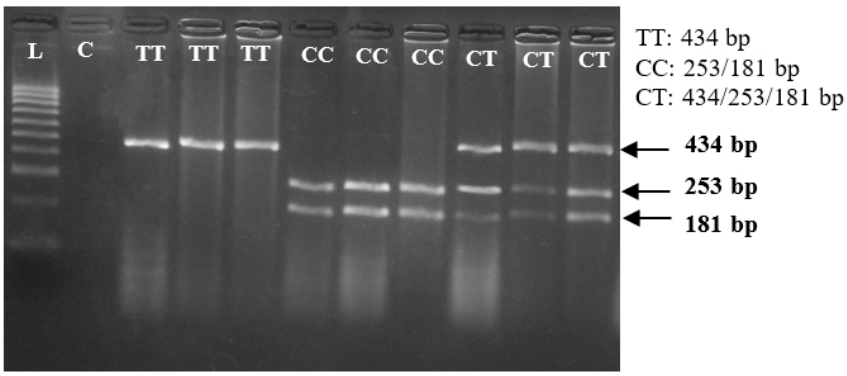

Figure 2 Presentation of PCR products of VIPR1/HhaI on 3.5% agarose gel electrophoresis
Lane L: DNA marker (100-1,000 bp); lane C: negative control; lane 3, 4, 5: CC genotype; lane 6, 7, 8: TT genotype; lane 9, 10, 11: CT genotype

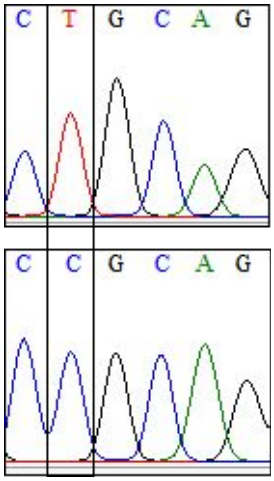

Figure 3 SNP analysis of INHA/*Pst*I

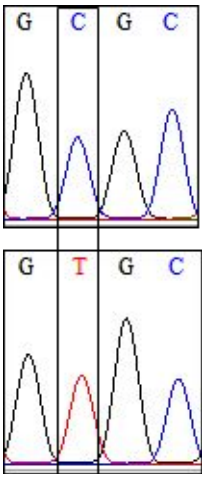

Figure 4 SNP analysis of VIPR1/*Hha*I

The INHA/*Pst*I locus had a lower C allele frequency (0.23) compared to the T allele frequency (0.77) in the population. However, the frequency ratio of the C allele (0.88) was higher than that of the T allele frequency (0.12) in the VIPR1/*Hha*I locus. Additionally, the results indicated that the genotypic frequencies of the INHA/*Pst*I and VIPR1/*Hha*I loci in the Silkie chicken population did not adhere to the Hardy-Weinberg principle ($p<0.001$).

Table 2 Distribution of allele and genotype frequencies of two genes in the Silkie hen population (n = 380)

Polymorphisms	Genotypes			Alleles		HWE χ^2
INHA/*Pst*I (T829C)	CC 0.09 (n = 33)	CT 0.29 (n = 110)	TT 0.62 (n = 237)	C 0.23	T 0.77	13.2**
VIPR1 (C42913T)	CC 0.81 (n = 306)	CT 0.15 (n = 57)	TT 0.04 (n = 17)	C 0.88	T 0.12	31.6**

HWE: Hardy-Weinberg equilibrium, n: hen number, **: $p<0.001$

Association of two polymorphisms with reproductive traits in Silkie chicken

The association of two polymorphisms on observed traits is shown in Table 3. The INHA/*Pst*I and VIPR1/*Hha*I polymorphisms had a highly significant association with the egg productivity of Silkie hens in 24 weeks of laying ($p<0.005$). Amongst genotypes of INHA/*Pst*I, the number of eggs produced by those with TT genotype in 24 weeks was 72.4 eggs, which was higher than that of CT (65.3 eggs) and CC genotype (63.2 eggs). A similar trend was found in VIPR1/*Hha*I locus as the total number of eggs of hens with CC, CT and TT genotypes were significantly different ($p<0.005$). Silkie hens bearing the CC genotype produced more eggs (69.7) in 24 weeks than those with the CT genotype (62.4 eggs).

During the 24 weeks of laying, among the INHA/*Pst*I genotypes, hens having the TT genotype had a higher laying rate than those with the CT and CC genotype ($p<0.005$). As a result, the laying rate was also the highest in TT birds (45.2%). Moreover, chickens with the CC and TT genotype had the highest laying rate (43.5%) in the polymorphic site of the VIPR1 gene.

Table 3 Association of two polymorphisms with reproductive traits in Silkie chicken

Parameters	Genotypes (INHA/*Pst*I)			Genotypes (VIPR1/*Hha*I)		
	CC (n=33)	CT (n=110)	TT (n=237)	CC (n=306)	CT (n=57)	TT (n=17)
AFE (day)	123±1.02^{a}	119±0.68^{b}	119±0.54^{b}	120±0.40	120±0.74	122±1.33
BWFE (g)	731±7.22	735±4.79	729±3.78	734±2.80	735±5.24	726±9.42
Laying days	157±1.02^{b}	161±0.68^{a}	161±0.54^{a}	160±0.40	160±0.74	158±1.33
Total eggs	63.2±2.63^{b}	65.3±1.74^{b}	72.4±1.38^{a}	69.7±1.02^{a}	62.4±1.90^{b}	68.7±3.43^{ab}
Laying rate (%)	40.3±1.70^{b}	40.6±.12^{b}	45.2±0.89^{a}	43.5±0.66^{a}	39.0±1.23^{b}	43.5±2.21^{ab}
Egg weight (g)	35.1±0.19	35.5±0.13	35.3±0.10	35.2±0.07	35.4±0.14	35.2±0.25
Egg shape (%)	76.7±0.09	76.7±0.06	76.6±0.04	76.6±0.03	76.7±0.06	76.8±0.11

AFE: Age at first egg; BWFE: Body weight at first egg.
^{a,b}: Means with different letters in the same row differ significantly ($p<0.005$)

Hens with genotype CT (INHA/*Pst*I polymorphism) had the greatest body weight (735 g/hen), the largest egg weight (35.5 g/egg) and the highest egg shape index (76.7%). Besides, a relation between INHA/*Pst*I polymorphism and the first egg's average age was also determined ($p<0.005$). Hens with the CC genotype had a later average age at the first egg (123 days) than those with the CT and TT genotypes (119 days). Moreover, chickens carrying TT genotype (INHA/*Pst*I) had the longest laying days (161 days/hens). This result also indicated that the TT genotype had early sexual maturity that was economic benefits in egg production.

DISCUSSION

In this study, T allele frequency of the VIPR 1/Hha I variant differed from Chinese chicken breeds. Zhou et al. (2008) reported T allele frequencies of the VIPR1/ HhaI as 0.15 in Red Jungle Fowls, 0.08 in Xinghua chickens, 0.03 in Ningdu Sanhuang chickens, 0.01 in Baier Huang chickens, 0.00 in Leghorn layers. Furthermore, the C+42913T and C+53327T polymorphisms in the VIPR 1 gene are associated with broodiness in Chinese chicken breeds ($p<0.05$). The C allele frequency of the VIPR1/HhaI locus in this study differed from indigenous Noi chicken (0.78) (Ngu et al., 2015) and Lien Minh chicken (0.62) (Nguyen et al., 2018). The observed genotypic frequencies did not follow the expectations of the Hardy-Weinberg law due to factors such as genetic drift, mutation, migration, non-random mating, or natural selection. These factors can disrupt the equilibrium of genotypic frequencies and lead to deviations from the expected proportions predicted by the Hardy-Weinberg law (Masel, 2012).

At the VIPR 1/HhaI polymorphic site, the dominant C allele frequency was also reported in native Ningdu Sanhuang chickens (Xu et al., 2011 a) and local Noi chickens (Ngu et al., 2015; Vu and Ngu, 2016). The selection pressure to improve reproductive performance may be responsible for the increased CC genotype frequency with higher egg production and laying rate than other individuals. By contrast, hens with TT genotype of INHA /PstI locus had higher genotype frequency and egg production than other individuals, leading to a higher frequency of the T allele than the C allele in the population. The present study indicated that the CC genotype (VIPR 1/HhaI polymorphism) had positive effects on increasing egg production. Previous reports showed that the CC genotype (VIPR1 gene) corresponded to the highest egg yield in 20 weeks of laying in Noi hens (Ngu et al., 2015; Vu and Ngu, 2016) and Lien Minh chicken (Nguyen et al., 2018). In the Ningdu Sanhuang breed, VIPR 1/HhaI polymorphism significantly affected total egg numbers at 300 days of age (Xu et al., 2011 b). The egg production of Ac hens in the present study was high compared to Bang Troi chickens at 37-40 weeks old (34.1%) (Thinh et al., 2020). According to Hoa et al. (2021), the egg yield of the black Noi hens was 74.3 eggs /hen, and the production was 77.9 eggs /hen for dark brown Noi hens at 25- 50 weeks old. The total number of eggs in Ho and Dong Tao chickens was 88.5 eggs/hen and 94.9 eggs/hen, respectively (Duy et al., 2020). Besides, the Noi chickens with II genotype (NPY/DraI) had 38.9 eggs /hen /20 laying weeks (Ngu et al., 2015). The present results showed that the selection significantly improved the reproductive performance of the experimental flocks. In this study, the age of sexual maturity of Ac chickens was earlier than some other native chicken breeds in Vietnam, such as Lien Minh chickens (186-187 days old in VIPR1/HhaI polymorphism and 178-189 days old in VIPR1/ TaqI polymorphism) (Nguyen et al., 2018) and Noi chickens (161-168 days

old) (Hoa et al., 2021). This result also indicated that the TT genotype (INHA/PstI locus) had early sexual maturity, which was economically beneficial in egg production. Furthermore, this finding suggests that the TT genotype at the INHA/PstI locus exhibited early sexual maturity, which carries economic advantages regarding egg production.

The egg weight in the present study was much smaller than that of other local chickens in Vietnam, such as Ri chickens (41.7 g/egg) and Mia chickens (44.7 g/egg) (Moula et al., 2012), H'mong chickens (51.4 g/egg) (Phuong et al., 2017), Lien Minh hens (40.2-40.6 g/egg in VIPR1/HhaI polymorphism and 39.9-41.5 g/egg in VIPR1/TaqI polymorphism) (Nguyen et al., 2018), 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, Ac chickens had lower egg weight than indigenous chickens in Southern Ethiopia (46.6 g/egg in the lowland, 48.6 g/egg in the midland and 45.4 g/egg in the 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 plateau) (Legesse and Kefyalew, 2023).

Functional factors were observed in both introns, which have the potential to regulate gene expression (Haddrill et al., 2005; Wang and Shashikant, 2007), and exons, which can cause changes in the polypeptide sequence (Roos and Boer, 2021). Mutations in introns can affect RNA synthesis and processing, consequently influencing the translation products. The mutation in the 5′ flanking region might be responsible for regulating the transcription level (Xu et al., 2005). It means that polymorphisms in the 5′ regulatory region played a major role in regulating the transcription of the VIPR1 gene, and the results showed that the C42913T in intron 6 of VIPR1/HhaI was associated with egg production. The VIPR1 is located in the gastrointestinal tract, genitourinary system, smooth muscle and exocrine glands. It activated adenylyl cyclase and increased cAMP by binding to the G protein (Ishihara et al., 1992).

Consequently, hens experienced enhanced feed intake, increased egg production, and improved enzymes in the digestive system due to the activation of the VIPR1 gene.

CONCLUSION

The polymorphisms of INHA /*Pst*I and VIPR 1/*Hha*I demonstrated a significant relationship with the total number of eggs produced by Ac chickens between 16 and 40 weeks of age. Utilizing birds with the TT genotype at the INHA /*Pst*I locus is recommended for increasing egg production in Ac chicken breed.

AUTHOR CONTRIBUTIONS

Le Thanh Phuong; Investigation, methodology, formal analysis, manuscript preparation, editing, and finalisation

Tran Trung Tu; Conceptualization and design of the experiment, investigation, editing, and finalisation.

Nguyen Trong Ngu; Conceptualization and design of the experiment, investigation, supervision, editing, and finalisation.

CONFLICT OF INTEREST

We have no conflict of interest.

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