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The Differential Expression and Snp Analysis of the Ovoidinhibitor Gene in the Ovaries of Laying Duck Breeds (*Anas Platyrhynchos*)

ABSTRACT

Ovoidinhibitor (OIH) is the main proteinase inhibitor in the egg white. In the present study, real-time quantitative PCR and Western-Blot were used to analyze different expression pattern of OIH in ovaries as a candidate gene of reproductive traits in Jingjiang ducks (JJ ducks) and Shaoxing ducks (SX ducks) during three laying stages. To study the polymorphism of the OIH gene in those two duck populations, we designed five pairs of primers to detect SNPs of exon 3-5, 5-6, 14-16 and intron 7, 9 of the OIH gene by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA pool sequencing methods. The results showed that OIH expression increased during the laying stage in the ovaries of both duck breeds. The relative expression levels of OIH in the egg at hatch and 180days of age were lower in JJ ducks than in SX ducks, but higher in JJ ducks than SX ducks at 500 days of age. Only exon 5-6 locus had a novel SNP. One variation (389G>A) was detected in the two tested duck populations and it was associated with some laying traits, such as body weight of hatch, age at first egg, weight at first egg, egg number at 72weeks of age. The AG genotype was associated with inferior body weight of hatch and superior weight at first egg, age at first egg and egg number at 72weeks of age. Therefore, these results suggest that OIH may be a strong candidate gene related to some laying traits in ducks.

INTRODUCTION

Ovoidinhibitor (OIH) is the main proteinase inhibitor in the albumen (Kinoshita *et al.*, 2004). It is produced in the tubular gland cell of the oviduct, which is regulated by estrogen and progesterone (Liu *et al.*, 1971). It has a broad inhibitory activity of other proteinases present in the blood plasma and in the egg white of chickens (Laskowski & Kato, 1980). Słowińska *et al.* (2014) reported that the ovoidinhibitor in the turkey reproductive tract maintains a microenvironment for the sperm in the epididymis and ductus deferens. Gao *et al.* (2016) reported that protease inhibitors, such as ovoidinhibitor, may play a key role in the degradation of egg yolk proteins. Other authors suggested that ovoidinhibitors may be related to the reproductive performance of poultry (Zhu *et al.*, 2011).

There are literature reports on the impact of OIH in poultry; however, studies on the function of OIH in the ovaries of different duck breeds are rare. Therefore, as a candidate gene for reproductive traits, the objective of this study was to determine the expression patterns of OIH in the ovaries of Jingjiang ducks (JJ duck) and Shaoxing ducks (SX ducks) during the three different laying stages, and to identify the SNP of the OIH gene associated with laying traits.



MATERIALS AND METHODS

Animal source and samples preparation

The Jingjiang (JJ) and Shaoxing (SX) are two local varieties of ducks in China. The SX duck has a small body size, early maturity (about D102) and high laying performance (over 300 eggs per year) (Zhao *et al.*, 2005). The JJ duck is an indigenous species of the Hubei Province of China, and has small body size, early maturity (about D100) and low laying performance (about 200 eggs per year) (Ding *et al.*, 2004).

In this study, 100 JJ, 100 SX, 250 JJ line I, and 226 JJ line II female ducklings (JJ lines I and II were two lines derived from the JJ breed) were reared from 0 to 500 days of age. All ducks were housed in individual cages in the same room with the controlled temperature (25±3°C), and fed a commercial diet during the experiments. At age at first egg, and at 180 days (egg production peak) and 500 days of age (egg production rapidly decreases at this age), six JJ ducks and six SX ducks were anesthetized with a mixture of 79% CO₂ and 21% O₂ for 1 min and then decapitated. The ovaries were collected after dissection, immediately frozen in liquid nitrogen, and stored at -80°C until further analyses.

Blood samples and performance measurements (body weight at hatch, and at 4 weeks, 8 weeks, 12 weeks, and 72 weeks of age, and at first egg, age at first egg, egg weight and number of eggs laid at 72 weeks of age) were collected from the 476 ducks (JJ lines I and II). Genomic DNA was obtained according to standard procedures and stored at -20 °C.

RNA isolation and cDNA synthesis

Total RNA was extracted using TRIZOL reagent, according to manufacturer's protocol (TaKaRa, Dalian,

China). Total RNA samples were separately pooled per duck and ovary. cDNA was synthesized from 1 µg total RNA, derived from the RNA pools, using PrimeScript RT-reagent kit (TaKaRa, Dalian, China) with random hexamers.

Real-time quantitative PCR

All PCR primers for real-time quantitative RT-PCR are shown in Table 1. Primers were chosen to generate specific products, of around 200 bp of β -actin and OIH. The β -actin (EF667345) and OIH (NM_001030612) sequences for the designed primers were retrieved from GenBank. Real-time quantitative PCRs were performed in a 20-µL mixture containing 10 µL 2×SYBR Green/Fluorescein qPCR Master Mix (TaKaRa, Dalian, China), 1 µL template cDNA (1 µg/µL), 0.4 µM forward and reverse primers, and 8.2 µL dd H₂O. Real-time quantitative PCR was performed on an ABI 7500 real-time quantitative PCR thermal cycling instrument (Applied Biosystems, USA). The real-time quantitative PCR amplification was performed in triplicate. The cycling conditions were as follows: 1 cycle of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s.

Western-Blotting Analysis

The OIH protein from each tissue was extracted and separated in 12% gradient SDS polyacrylamide gels. The separated OIH protein was then transferred onto a polyvinylidene difluoride (PVDF) membrane. The specific antibody for OIH was used at 1:1000 dilution. All incubations were implemented in TBSE solution (containing 5% dry milk). Immunodetection was executed using Pierce chemiluminescence (Thermo, USA). The signal intensity was determined by the density measurement method.

Table 1 – Primers used for real time quantitative PCR and SNP detecting of duck OIH gene

Primer name	Product size (bp)	Primer sequence (5'-3')	Amplified region	Annealing Temperature X (°C)
β -actin	150	F:5'-ACGGTGCTGTCTGGTGGTA-3 R:5'-TGCTGACATGGGAGAGCAG-3	-	60
OIH	243	R:5'- TGCGTTGCAGAAGAAACATC-3' R:5'- TGCGTTGCAGAAGAAACATC-3'	-	60
P1	668	F: CAGATGGCTCCACATACAG R: GCAAATCCCACATTCGTT	exon3-5	53
P2	579	F: GTAGCCTGCCCAAGGATTC R: CCGATCTCCAGCTTGCAAT	intron 5	55
P3	569	F: CGGCACCGATGGTTTCAC R: ATCTCCTGCCTGCATTT	intron 7	54
P4	718	F: AAAATGCAGGCAGGAGAT R: TTTCTTGTTGGGTATTGA	intron 9	51
P5	974	F: AAGAACGGAAGATGTGAAG R: TAACACGCTGCCATACTCA	exon 14-16	52



PCR amplification, SNP identification, and genotyping

According to duck OIH gene sequences (NC_006100), five pairs of primers (shown in Table 1) were designed to amplify the OIH gene exon3 to16 regions and to detect their SNP.

The PCR assays were performed in an Eppendorf Mastercycler gradient (Eppendorf, Germany). The final volume was 15 μ L, and contained 7.5 μ L 2 \times Taq PCR mix (Dongsheng, Guangdong, China), 10 pM of each primer, and 50 ng genomic DNA. The cycling condition consisted of an initial denaturation step at 95 $^{\circ}$ C for 4 min followed by 35 cycles of 95 $^{\circ}$ C for 30 s, annealing at X $^{\circ}$ C (Table 1) for 35 s, 72 $^{\circ}$ C for 30 s, and a final extension at 72 $^{\circ}$ C 10 min.

Genomic DNA of JJ lines I and II was used as template and the sequenced sequences from different samples were aligned to search for base variations.

The PCR-RFLP was used to analyze the polymorphisms in duck OIH. It was performed by mixing 8 μ L of the PCR products (with primer exon 5-6), 10 U Taq I restriction enzyme (New England Biolabs, USA) and 1 μ L 10 \times reaction buffer. The mixtures were incubated at 37 $^{\circ}$ C for 12 h and separated at 150 v in 3% agarose gel.

Statistical analyses

The real-time quantitative PCR data were calculated using the $2^{-\Delta\Delta Ct}$ comparative CT method. The results are expressed as mean \pm standard deviation. Ct values were tested for normality (Kolmogorov-Smirnov test) prior to analysis. Statistical analysis of data was performed by one-way analysis of variance followed by LSD post-hoc test.

The gene frequencies were determined for each population by direct counting. Hardy-Weinberg equilibrium (HWE) was tested using χ^2 -test of PopGene32 (version 3.2) in each population. The association analyses between OIH genotypes and laying traits (including body weight at hatch, and 4, 8, 12, 72 weeks of age, weight at first egg, age at first egg, egg weight and egg number at 72 weeks of age) were analyzed according to the general linear model (GLM) procedure of the software SPSS (version 18.0). All analyses were performed first using a full animal model, and next using a reduced animal model. The full animal model included fixed effects of SNP genotypes, populations, and random effects (including permanent environment, animal and residual). The effect associated with populations was not matched in the linear model, and the preliminary analyses

indicated that this effect had no significant influence on variability of the traits in the analyzed populations.

Therefore, the following reduced linear model was used for final analysis:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where Y_{ij} is the trait measured on each of ij th animal; μ was the overall population means; G_i is the fixed effect associated with the i th genotype; and e_{ij} is the random error. The least square analysis was used to determine the difference between genotypes.

RESULTS

Expression of duck OIH

The mRNA expression of OIH in ovaries of JJ and SX ducks collected at the three different ages was determined by real-time quantitative PCR. As Figure 1 shows, the mRNA of OIH was detected in all examined ovary samples. The relative expression of the OIH mRNA transcript in the ovaries continuously increased during the laying period (from the first egg to 500 days of age). The relative expression level of OIH mRNA in the ovaries ($p < 0.05$) was significantly different between the two breeds from at age at first egg and 180 days of age, but not at 500 days of age ($p > 0.05$). Furthermore, in the ovaries, the relative mRNA expression of OIH between age at 1st egg and 180 days of age was lower in JJ ducks compared with SX ducks, but was higher in JJ ducks than in SX ducks at 500 days of age.

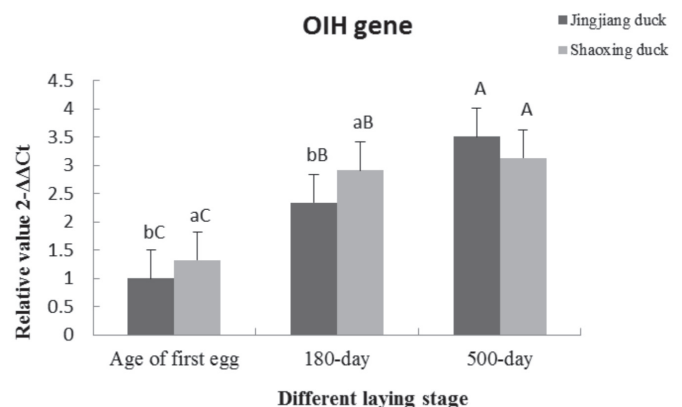


Figure 1 – Development changes of the relative expression levels of OIH mRNA in the ovaries of Jingjiang and Shaoxing ducks. Note: Different lowercase letters (a and b) indicate significant difference ($p < 0.05$) between Jingjiang and Shaoxing ducks; different uppercase letters (A, B and C) indicate significant age of first egg, 180-day and 500-day differences ($p < 0.05$) in the same duck population.

The method of Western-Blot was used to examine whether the OIH protein could be detected in ovary samples. The predicted duck OIH protein weight was 49kDa. The results are shown in Figure 2. The OIH protein was detected in all ovaries of both SX and JJ



ducks, and the results did not show any significant differences ($p>0.05$) in their RNA expression of OIH between the two breeds at any of the evaluated periods. This may be probably due to the fact that part of the mRNA of OIH was not translated into the protein in the ovaries at the different stages.

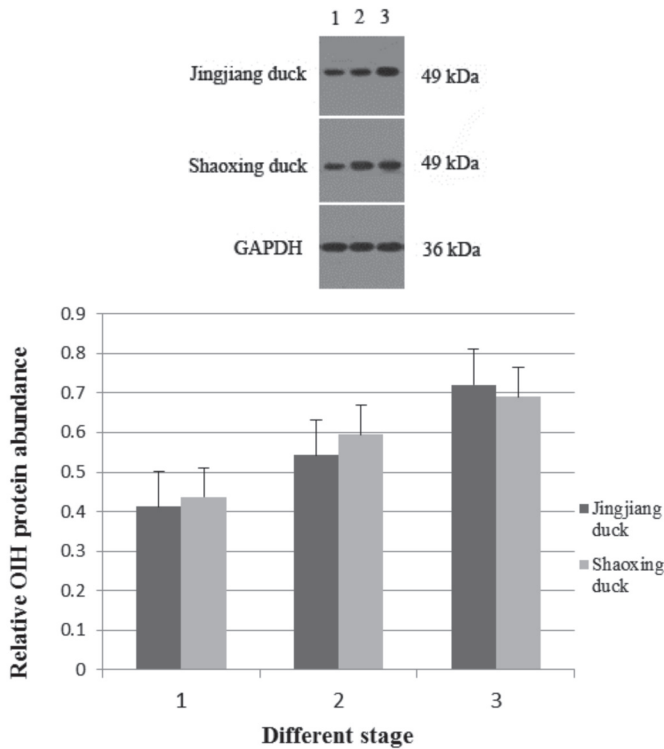


Figure 2 – Western-blot results of OIH in the ovaries of Jingjiang and Shaoxing ducks. Note: 1 is stage of age at first egg; 2 is stage of 180 days of age; 3 is stage of 500 days of age; (a) Western blot results of OIH; (b) relative expression of OIH in the ovaries of JJ and SX ducks

Identified SNP

The sequences amplified with all primers (P1, P2, P3, P4 and P5 of exon 3-5, exon 5-6, intron 7, intron 9 and exon 14-16) were aligned between JJ duck lines 1 and 2. No mutations were detected in exon 3-5, intron 7, intron 9, or exon 14-16. There was one variation locus in exon 5-6, G→A at the 389-nucleotide position (389G>A), which was detected by restriction endonucleases *TaqI*. This variation was a synonymous mutation and did not cause any amino acid changes. This SNP was detected by *PCR-RFLP* using the amplification product of exon 5-6. We submitted the sequences of two homozygotes of exon 5-6 to GenBank, and gain accession numbers *KC977993* (for the GG genotype) and *KC977994* (for the AA genotype).

As Figure 3 shows, the G to A mutation at locus 389 expressed three genotypes: GG, GA and AA, and the partial sequences of three genotypes are shown in Figure 4.

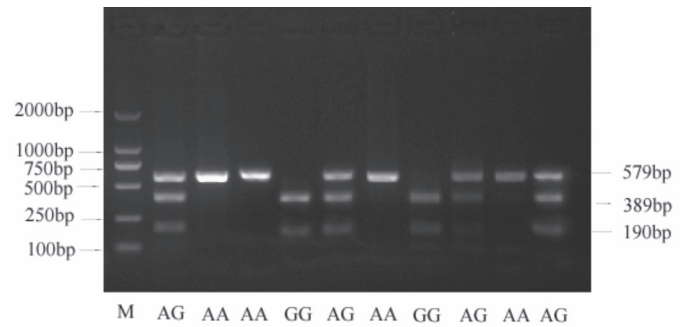


Figure 3 – PCR-RFLP band pattern on a 3% agarose gel. Genotype AA: 579 bp; genotype AG: 579bp + 389 bp + 190 bp; genotype GG: 389 bp + 190 bp

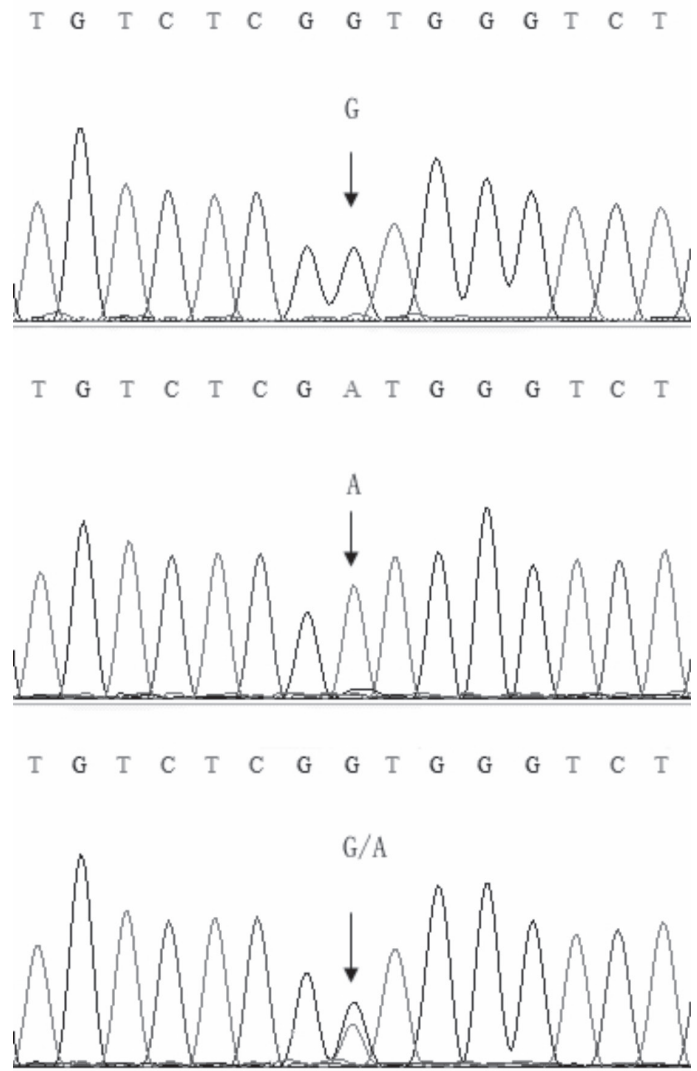


Figure 4 – Sequencing shows that the sequence amplified by primer intron 5 contains the 389G>A mutation. AA genotype contains allele A at 389th position; GG genotype contains allele G at 389th position; AG genotype contains both A allele and G allele at 389th position.

Allele and genotype of OIH gene exon5-6

The frequencies of allele and genotypes of OIH gene exon 5-6 in the two duck populations are shown Table 2. In the 389 locus of exon 5-6, the G allele was



Table 2 – Gene and genotype distribution in exon 5-6 locus of OIH gene in two duck populations

Population	Number	Genotype frequencies			Gene frequencies		χ^2 (HWE)
		AA	GG	AG	A	G	
JJ duck Line I	250	0.1280	0.5200	0.3520	0.3040	0.6960	3.7174
JJ duck Line II	226	0.1770	0.3186	0.5044	0.3363	0.6637	9.5740**

Note: χ^2 (HWE): Hardy-Weinberg equilibrium χ^2 value; ** showed that $p < 0.01$.

the preponderant allele in the evaluated populations, and the JJ duck line II population deviated from Hardy-Weinberg equilibrium (HWE) ($p < 0.01$).

Association analysis of OIH gene exon 5-6

We analyzed the association of the SNP with laying traits of two duck populations (JJ lines I and II). The results are presented in Table 3. In JJ duck line I, heterozygotes (AG) were significantly younger

at first egg than those with genotypes AA and GG ($p < 0.05$), and egg number at 72 weeks of age was significantly different among the AG, GG and AA genotypes ($p < 0.05$). In JJ duck line II, the genotype AA was significantly heavier at hatch, and produced a higher number of eggs at 72 weeks of age ($p < 0.05$) than genotypes GG and AG. Moreover, body weight at first egg of the genotype GG was significantly lower compared with genotype AG ($p < 0.05$).

Table 3 – Least square means and standard errors of the laying traits in two duck populations

Traits	JJ duck line I (Mean \pm S.E.)			JJ duck line II (Mean \pm S.E.)		
	AA (n=34)	GG (n=130)	AG (n=86)	AA (n=34)	GG (n=112)	AG (n=80)
Body weight of hatch (g)	44.01 \pm 0.79	44.18 \pm 0.58	43.31 \pm 0.67	47.09 \pm 0.53a	41.42 \pm 0.71b	40.47 \pm 0.68b
Weight at 4 weeks (g)	508.34 \pm 15.35	505.71 \pm 9.59	491.56 \pm 12.16	546.26 \pm 13.03	535.74 \pm 13.42	530.70 \pm 13.87
Weight at 8 weeks (g)	886.22 \pm 22.04	858.30 \pm 14.23	860.23 \pm 20.57	912.86 \pm 34.17	865.99 \pm 20.56	904.77 \pm 23.41
Weight at 12 weeks (g)	1263.31 \pm 47.66	1218.16 \pm 24.69	1234.97 \pm 32.01	1316.46 \pm 41.31	1252.14 \pm 26.84	1327.73 \pm 31.85
Weight at first egg (g)	1408.85 \pm 43.99	1364.9 \pm 24.02	1427.88 \pm 23.30	1327.73 \pm 18.94ab	1332.22 \pm 24.72b	1459.04 \pm 23.43a
Age at first egg (d)	149.00 \pm 3.88a	146.38 \pm 2.09a	136.00 \pm 1.22b	137.27 \pm 2.24	140.81 \pm 2.09	137.15 \pm 1.09
Egg weight (g)	67.29 \pm 0.97	66.26 \pm 0.85	64.26 \pm 0.73	65.74 \pm 1.16	65.00 \pm 0.63	66.02 \pm 0.88
Weight 72 weeks (g)	1404.58 \pm 34.31	1402.55 \pm 27.03	1358.33 \pm 28.75	1398.89 \pm 57.88	1697.08 \pm 30.24	1410.96 \pm 31.03
Egg number at 72 weeks	199.69 \pm 8.13c	268.22 \pm 7.68b	315.79 \pm 7.28a	268.81 \pm 7.02b	306.14 \pm 5.61a	307.92 \pm 9.14a

Note: Values with different superscripts within the same column differ significantly at $P < 0.05$ (a, b, c); n was the number of this genotypes in the population.

DISCUSSION

Ovinhibitor (OIH) is a Kazal-type serine inhibitor. It is the main protease inhibitor found in the chicken plasma, and known to inhibit the activity of trypsin, chymotrypsin, and elastase (Zahnley, 1975; Shechter *et al.* 1977; Vered *et al.*, 1981). OIH is a three-domain protein and shares its structure with the ovomucoid protein (Moore *et al.*, 2004). It is reported that OIH is highly expressed in the magnum and liver of chickens, followed by the uterus, egg yolk, and eggshell precursors, respectively, and that its expression increases in the liver during sexual maturation and subsequently decreases in mature hens (Bourin *et al.*, 2011). However, to date, the expression pattern of OIH in ovaries of laying ducks has not been detected. Real-time quantitative PCR has become a standard method to measure gene expression by evaluating the amount of mRNA produced (Ong *et al.*, 2002). So, in this study, the expression of OIH mRNA in the ovaries at age at first egg, and 180 days and 500 days of age were researched using real-time quantitative PCR.

In this study, OIH mRNA expression level increased from age at 1st egg until 500 days of age in the ovaries of both JJ and SX ducks, and was significantly different between age at first egg and 180 days. These results are inconsistent with the OIH expression levels reported in the liver of mature chickens (Bourin, *et al.*, 2011), and suggest that the profile of OIH expression is different among species and tissues.

SX and JJ ducks are both local Chinese breeds, with SX ducks presenting high egg production and JJ ducks low egg production. In this study, we found that the OIH mRNA expression in JJ ducks was lower than in SX ducks at age at first egg and 180 days of age, whereas at 500 days of age, the OIH mRNA expression in JJ ducks was higher than in SX ducks. In addition, the Western-Blot results have showed similar OIH mRNA expression pattern. These results indicate that OIH is closely related to the laying performance of ducks and may play an important role in the ovary during the egg-laying period.

The candidate gene method is a very powerful method to detect associations of gene polymorphisms



with economic traits in farm animals (Schwerin, 2003). In view of the function of OIH, in this research, the OIH gene was chosen as a candidate gene to study the association between gene polymorphisms and laying traits in ducks.

Goliasova & Wolf (2004) reported that in populations subjected to selection, in general, deviations of genotype frequencies from the Hardy-Weinberg equilibrium are expected in some loci that impact traits under selection. The results of this study indicate that the JJ duck II population deviated from the Hardy-Weinberg equilibrium (*HWE*) ($p < 0.05$) for the OIH gene. One of the reasons may be that the evaluated population was subjected to intensive selection during commercial breeding.

In this study, one novel mutation was found in exon 5-6 of the OIH gene using *PCR-RFLP* methods. We analyzed the associations between genotypes and laying traits, including hatching weight, weight at 4, 8, 12, and 72 weeks of age, weight at first egg, age at first egg, egg weight, egg number at 72 weeks of age. The results indicated the AG genotype presented inferior hatching weight and superior weight at first egg, age at first egg, and egg number at 72 weeks of age. This is in accordance with Wang (2015), who suggested that the SNP of OIH gene introns 10 and 11 may be related with some laying traits in ducks. Therefore, we postulate that the OIH gene may have a quantitative trait locus (*QTL*), which controls some laying traits in the exon 5-6, and that the genotype AG may be used as a SNP marker for inferior hatch weight and superior weight at first egg, age at first egg, and egg number at 72 weeks of age. Nevertheless, we concluded that, in order to prove the associations of the mutation of the OIH gene exon 5-6, more samples and further analyses should be carried out.

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