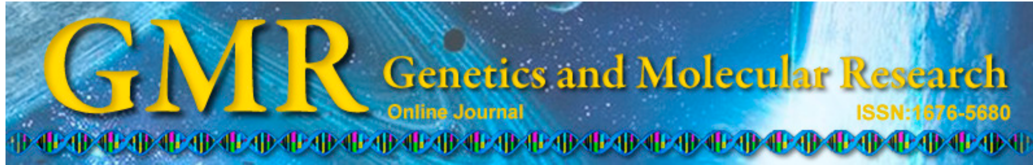

Identification and association of single-nucleotide polymorphisms in gonadotropin-inhibitory hormone (*GnIH*) gene with egg production traits in Erlang mountainous chickens

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ABSTRACT. Gonadotropin-inhibitory hormone (*GnIH*) gene is an important gene in reproduction. In this study, we screened single-nucleotide polymorphisms (SNPs) in the chicken *GnIH* gene among 204 individuals in Erlang mountainous chickens. We then analyzed the associations between polymorphisms of the *GnIH* gene and 5 egg production traits in chickens. Five SNPs (T3305C, T3310C, G3403C, G3411A, and T3591C) were detected. Associations between polymorphic loci and age at first egg, body weight at first egg, weight at first egg, egg weight in 300 days, and egg production in 300 days were analyzed using analysis of covariance. The results showed that SNP1, SNP3, and SNP4 had large effects on age at first egg, while SNP5 had a large effect on body weight at first egg; of the effect of the TT genotype was significantly higher than that of CT (P < 0.01). Further

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analysis show that the highest frequency (0.2353) haplotype H1H1 was associated with the latest age at first egg. The H4H5 haplotype had a positive effect on egg production in 300 days and a negative effect on weight at first egg. We observed no association between the H3H3 haplotype and body weight at first egg.

Key words: Egg production; Erlang mountainous chicken; *GnIH*

INTRODUCTION

The gonadotropin-inhibitory hormone (*GnIH*) gene is an important gene that is involved in the development of reproductive traits (Tsutsui et al., 2010a,b) and is a glycoprotein hormone (Tsutsui, 2009). *GnIH* as a novel Arge-Phe (RF) amide was first identified in the quail hypothalamus (Tsutsui et al., 2000). *GnIH* in the brain acts directly on the pituitary via the *GnIH* receptor to inhibit gonadotropin release, indirectly inhibiting circulating luteinizing hormone (Shimizu and Bédécarrats, 2010) and stimulating the release of follicle-stimulating hormone and prolactin in birds (Tsutsui et al., 2000; Mateos et al., 2002; Osugi et al., 2004; Bentley et al., 2006a; Tsutsui et al., 2006; Ubuka et al., 2006). Further, similar suppressive effect of *GnIH* on gonadotropin mRNA was associated with inhibition of both luteinizing hormone and follicle-stimulating hormone release in the chicken (Ciccone et al. 2004) and quail (Ubuka et al., 2006). *GnIH* affects reproductive traits by regulating these hormones during hatching (Ubuka et al., 2003). It may participate not only in neuroendocrine functions but also in behavioral (Tachibana et al., 2005; Bentley et al., 2006b) and autonomic mechanisms.

The suppressive effect of *GnIH* on gonadotropin has not been thoroughly examined. There have been no investigations on the relationship between polymorphisms in the *GnIH* gene and reproduction in chickens. Thus, we identified single-nucleotide polymorphisms (SNPs) in the chicken *GnIH* gene and analyzed whether an association exists between polymorphisms in the *GnIH* gene and egg production.

MATERIAL AND METHODS

Chicken populations

A total of 359 Erlang mountainous female chickens were included in this study. All chickens had free access to feed and water. Commercial corn-soybean diets that met all National Research Council requirements were provided in the study. At 300 days, approximately 2 mL blood was collected from the brachial vein of each individual. The next day, genomic DNA was isolated by phenolic extraction and used to genotype the *GnIH* gene.

Egg production trait measurements

Age at first egg was recorded when each hen began to lay. On the same day, the hen's body weights at first egg and weight at first egg were measured. Each hen's egg weight and egg numbers were recorded from the age at first egg to the age of 300 days.

DNA pool

The DNA from each chicken was isolated and 5 μL DNA from 30 birds was mixed together. This mixed DNA pool was used for amplification.

Amplification and population genotyping

Three polymerase chain reaction (PCR) primers were designed according to the sequence of *Gallus gallus GnIH* (GenBank accession No. AB193126) using the primer design program Oligo 6.0 and Primer 5.0. All primer sequences are shown in Table 1.

Table 1. PCR forward (F) and (R) primers for the *GnIH* gene.

Primer	Sequences (5'-3')	Annealing temperature	Length (bp)
P1	F: GATGACAGCTTGCTTTTC	56	264
	R: TTCAGTGCTCAGGGTTTG		
P2	F: AGTAGCTGGAATGGCACA	56	524
	R: GAGAATCCCTGAGGAAGG		
P3	F1: AGGGATGTGAATTCTAACGATCACATTC	60	546
	R1: TCCCACAGTGCAGATGTAGGATGTTAG		
	F2: AACTAAGGATCTTGAGAAGGGAATGT		
	R2: TACAGCATGACTTTGGTTAGATGATAT		

EX3 used nested PCR.

PCR was performed in a final volume of 25 μL containing 0.5 μL 2.5 ng/μL genomic DNA, 0.5 μL 10 μM of each primer, 12.5 μL 2X Master Mix (including Mg^{2+}, dNTPs, *Taq* DNA polymerase). Amplification was carried out using the following procedure: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 45 s, 56°-60°C for 35 s, and 72°C for 40 s; final elongation at 72°C for 2 min.

The PCR products were sequenced by Beijing Liuhe Genomics Biological Technology Co., Ltd. (Beijing, China). The new SNPs were identified based on sequencing results.

Statistical analysis

Data were analyzed using the GLM procedures of SAS (SAS Institute, Inc., Cary NC, USA). Genetic effects were analyzed using a general linear model procedure in the SAS package, and the following model was used:

$$Y = \mu + Bi + Sj + Gk + eijk$$

where Y = dependent variable, μ = population mean, Bi = fixed effects of breed, Sj = fixed effects of sex, Gk = genotype value, and $eijk$ = random error. The interaction G x S was not significant for any trait and therefore was not included in the model. Significant differences ($P < 0.05$) were found among different genotypes by least square means using the Duncan multiple-range test.

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Hardy-Weinberg test, haplotype construction, and linkage disequilibrium analysis

Hardy-Weinberg equilibrium was analyzed using the Hardy-Weinberg test 3.0 software. Based on the 5 SNPs present in all 204 experimental birds, haplotypes were constructed using the PHASE 2.0 program to reconstruct haplotypes from the population data. Analysis of linkage disequilibrium was carried out using the online software SHEsis (http://analysis.bio-x.cn/SHEsisMain.htm).

RESULTS

SNP genotypes of the chicken *GnIH* gene

PCR products were developed for screening individuals in the population. Three target gene fragments were sequenced and found to contain SNPs (Figure 1). Both the homozygous and heterozygous individuals of different genotypes were sequenced for further analysis. We identified 5 mutations, including a G/C mutation at position 3403 nucleotides (nt), T/C mutation at position 3305, 3310, and 3591 nt, respectively, and a G/A mutation at 3411 nt in the DNA sequence of the chicken (accession No. SB193126) (Figure 1).

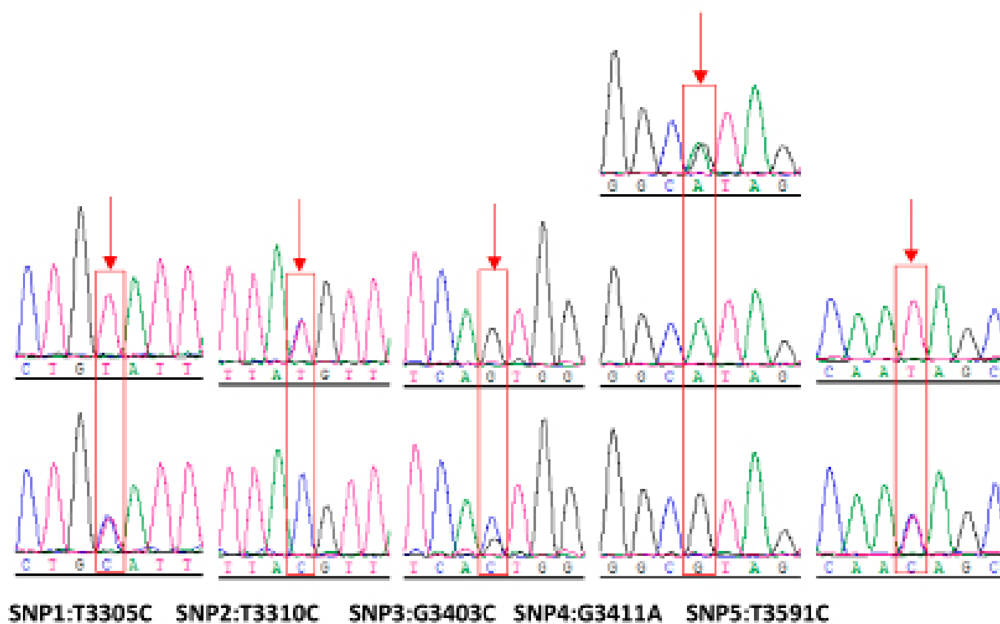

Figure 1. Sequencing results of the SNP1-5; arrow indicates the mutation site. SNP1: sequencing result of T/C heterozygote and T/T homozygote at the 3305 site. SNP2: sequencing result of T/C heterozygote and C/C homozygote at the 3310 site. SNP3: sequencing result of G/C heterozygote and G/G homozygote at the 3403 site. SNP4: sequencing result of A/G heterozygote and G/G homozygote at the 3411 site. SNP5: sequencing result of T/C heterozygote and T/T homozygote at the 3591 site.

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Frequencies of genotypes and alleles

There was no CC homozygous genotype in SNP1, SNP3, and SNP5. The frequencies of the genotypes and alleles are shown in Table 2. In SNP1 and SNP3, the frequency of the TT homozygous genotype (0.7892) was higher than that of the TC heterozygous genotype (0.2108). The frequency of allele T (0.8946) was significantly higher than the C alleles (0.1054). In SNP2, we observed no TT homozygous genotype. The frequency of the TC heterozygous genotype (0.1471) was lower than the CC homozygous genotype (0.8529). The frequency of allele T (0.0735) was significantly lower than of the C alleles (0.9265). In SNP4, the frequency of the AG heterozygous genotype (0.6275) was significantly higher than the AA (0.2402) and GG (0.1323) homozygous genotypes. In SNP5, the frequency of the TT heterozygous genotype (0.8578) was higher than the TC homozygous genotype (0.1422). The frequency of allele T (0.9289) was significantly higher than the C alleles (0.0711). All SNPs conformed to Hardy-Weinberg equilibrium ($P > 0.05$), except SNP4. The polymorphic information content (PIC) of SNP4 was moderate ($0.25 < PIC < 0.5$), while the SNP PIC values were low ($PIC < 0.25$).

Table 2. Genotypic and allelic frequencies of SNPs of *GnIH* gene.

SNPs	Number	Gene frequency		Genotype frequency			χ^2	PIC
	204	T	C	TT	TC	CC		
SNP1		0.8946	0.1054	0.7892	0.2108	-	2.831 (P = 0.092)	0.1708
		T	C	CC	TC	TT		
SNP2		0.0735	0.9265	0.8529	0.1471	-	1.285 (P = 0.257)	0.1269
		G	C	GG	CG	CC		
SNP3		0.8946	0.1054	0.7892	0.2108	-	2.831 (P = 0.092)	0.1708
		A	G	AA	AG	GG		
SNP4		0.5539	0.4461	0.2402	0.6275	0.1323	14.48 (P < 0.01)	0.3721
		T	C	TT	TC	CC		
SNP5		0.9289	0.0711	0.8578	0.1422	-	1.914 (P = 0.274)	0.1233

Linkage disequilibrium analysis

The results of linkage disequilibrium analysis are shown in Table 3 and Figure 2. SNP1 and SNP3 were in complete linkage disequilibrium ($D' = 1.000$, $r^2 = 1.000$). There was no difference between these 2 SNPs, and thus we analyzed only 1 SNP. Others SNPs showed weak linkage disequilibrium ($r^2 < 0.33$).

Table 3. Linkage disequilibrium analysis of SNP loci of *GnIH*.

Locus	SNP1	SNP2	SNP3	SNP4	SNP5
SNP1		0.009	1.000	0.146	0.009
SNP2	1.000		0.009	0.098	0.006
SNP3	1.000	1.000		0.146	0.009
SNP4	0.999	0.999	0.999		0.077
SNP5	1.000	0.076	1.000	0.898	

r^2 is above the diagonal for SNPs and D' is below the diagonal.

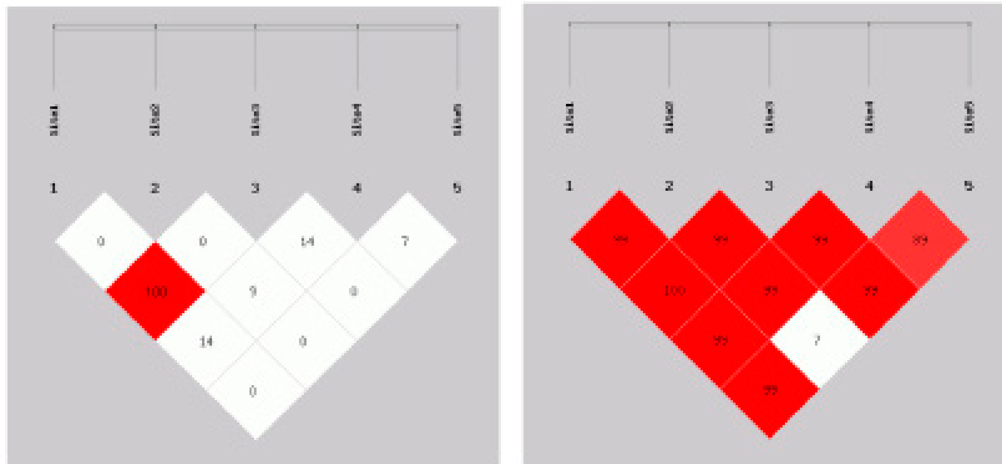

Figure 2. Map of D' and r^2. Left map indicate r^2, right map indicate D'.

Associations between SNPs in the *GnIH* gene and egg production traits

The associations between the *GnIH* genotypes and egg production traits in Erlang mountainous chickens were analyzed, and the least square means of 3 genotypes are listed in Table 4. The SNP1/SNP3 was significantly associated with age at first egg ($P < 0.01$) and egg production in 300 days ($P < 0.05$). Age at first egg in chickens with the TC/CG genotypes was significantly lower than that in those with TT/GG ($P < 0.01$). Egg production in 300 days in chickens with the TC/CG genotypes was significantly higher than that in those with TT/GG ($P < 0.05$). SNP2 was only significantly associated with egg production in 300 days, with TC significantly higher than CC ($P < 0.05$). SNP4 was significantly associated with 2 traits, age at first egg ($P < 0.01$) and body weight at first egg ($P < 0.05$). Both traits of AA were significantly higher than GG ($P < 0.05$). SNP5 was significantly associated with body weight at first egg, with TT significantly higher than CT ($P < 0.01$).

Haplotypes, construction of haplotypes, and their frequencies

Haplotypes were constructed with 5 SNPs in all 204 experimental chicken, by employing the PHASE program to reconstruct haplotypes from population data. Table 5 shows that 6 haplotypes with minor allelic frequencies greater than 2% were identified based on these 5 SNPs. Three main haplotypes, H1, H3, and H6, accounted for 85.54% of the observations. Table 6 shows that 9 diplotypes were successfully constructed. Only the frequency of H1H2 was lower than 0.01. Other diplotypes, H1H1, H1H6, H1H3, H1H4, H3H4, H1H5, and H4H5, were subjected to association analysis in this experiment.

Construction of haplotypes and their associations with chicken egg production traits

Mixed model analysis revealed significant associations between haplotypes with 4 traits, age at first egg, body weight at first egg, weight at first egg, and egg production in 300

Table 4. Association of SNPs and egg production traits.

SNPs	Genotype	N	Age at first egg	Body weight at first egg	Weight at first egg	Egg weight in 300 days	Egg production in 300 days
SNP1/SNP3	CC/CC	0	-	-	-	-	-
	TC/CG	43	138.91 ± 0.93^{B}	2096.51 ± 30.70	39.74 ± 0.41	60.02 ± 1.17	125.47 ± 1.47^{a}
	TT/GG	161	142.90 ± 0.75^{A}	2151.77 ± 15.29	39.60 ± 0.29	59.31 ± 0.24	118.16 ± 1.62^{b}
SNP2	TT	0	-	-	-	-	-
	TC	30	143.20 ± 1.52	2160.83 ± 18.64	39.53 ± 1.06	59.40 ± 0.22	127.73 ± 3.04^{a}
	CC	174	141.86 ± 0.70	2136.55 ± 15.80	39.64 ± 0.22	59.51 ± 0.42	118.31 ± 1.44^{b}
SNP4	AA	49	144.63 ± 1.67^{A}	2215.19 ± 38.23^{a}	39.61 ± 0.43	59.92 ± 0.36	114.47 ± 2.91
	AG	128	141.74 ± 0.73^{AB}	2130.90 ± 17.47^{a}	39.63 ± 0.32	59.47 ± 0.51	121.76 ± 1.43
	GG	27	138.89 ± 1.02^{B}	2122.86 ± 26.61^{b}	39.67 ± 0.65	58.82 ± 0.46	119.41 ± 5.03
SNP5	CC	0	-	-	-	-	-
	CT	29	139.45 ± 1.47	2019.31 ± 48.49^{B}	39.41 ± 0.65	58.88 ± 0.49	125.38 ± 3.00
	TT	175	142.49 ± 0.69	2160.14 ± 13.35^{A}	39.66 ± 0.26	59.59 ± 0.39	118.75 ± 1.46

Least square means within a row under the same SNP locus lacking a common lowercase superscript differ significantly ($P < 0.05$), within a row lacking a common uppercase superscript differ great significantly ($P < 0.01$).

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days (Table 7). The H3H4 haplotype had a negative effect on body weight at first egg, with the lowest body weight at first egg associated with earliest age at first egg. However, H1H1 showed the highest frequency (0.2353) associated with latest age at first egg. The H4H5 haplotype had a positive effect on egg production in 300 days, negative effect on weight at first egg, highest egg production in 300 days, and lowest trait of weight at first egg. The results revealed that the H3H3 haplotype had a positive effect on body weight at first egg.

Table 5. Haplotypes inferred based on the 5 single-nucleotide polymorphisms.

Haplotype	SNP1	SNP2	SNP3	SNP4	SNP5	Frequency
H1	T	C	G	A	T	0.5515
H2	T	C	G	A	C	0.0025
H3	T	C	G	G	T	0.1985
H4	T	C	G	G	C	0.0686
H5	T	T	G	G	T	0.0735
H6	C	C	C	G	T	0.1054

Table 6. Hapoltype frequencies in *GnIH*.

Diplotypes	H1H1	H1H6	H1H3	H3H3	H1H2	H1H4	H3H4	H1H5	H4H5
Frequency	0.2353	0.2108	0.2206	0.0735	0.0049	0.0784	0.0294	0.1176	0.0294

Table 7. Associations between diplotypes of the *GnIH* gene and egg production traits.

Diplotypes	N	Age at first egg (days)	Body weight at first (g)	Weight at first egg (g)	Egg weight in 300 days (g)	Egg production in 300 days (days)
H1H1	48	145.15 ± 1.63^{A}	2128.13 ± 26.63^{A}	39.63 ± 0.44^{A}	59.95 ± 0.37	114.02 ± 2.93^{BC}
H1H6	43	138.91 ± 0.93^{AB}	2096.51 ± 30.70^{A}	39.74 ± 0.41^{A}	60.02 ± 1.17	125.47 ± 1.47^{ABC}
H1H3	45	142.71 ± 1.39^{AB}	2223.33 ± 22.56^{A}	38.89 ± 0.40^{A}	59.30 ± 0.68	118.04 ± 3.04^{BC}
H3H3	15	140.80 ± 1.42^{AB}	2249.33 ± 40.18^{A}	40.47 ± 0.76^{A}	58.10 ± 0.91	107.20 ± 6.97^{C}
H1H4	16	142.88 ± 1.94^{AB}	2196.67 ± 40.70^{A}	40.00 ± 0.84^{A}	58.27 ± 1.02	117.75 ± 3.36^{BC}
H3H4	6	134.00 ± 1.95^{B}	1913.75 ± 56.99^{B}	41.83 ± 1.01^{A}	58.83 ± 0.54	129.67 ± 8.24^{AB}
H1H5	24	144.25 ± 1.84^{A}	2163.96 ± 21.48^{A}	40.54 ± 1.24^{A}	59.25 ± 0.27	124.75 ± 3.50^{ABC}
H4H5	6	139.00 ± 1.47^{AB}	2148.33 ± 39.36^{A}	35.50 ± 0.50^{B}	60.00 ± 0.43	139.67 ± 2.67^{A}

Least squares means within a row under the same SNP locus lacking a common uppercase superscript differ great significantly ($P < 0.01$).

DISCUSSION

GnIH interacts with some hormones relate to reproduction (Maddineni et al., 2008a; Sari et al., 2009). Thus, variations within genes have important effects on biological traits. Previous studies on the chicken genome have shown higher *GnIH* expression at sexual maturity in chicken ovaries, diencephalon (Maddineni et al., 2008b), and follicular maturation (Maddineni et al., 2008a). In the chicken hypothalamus, pituitary, ovary, and oviduct, *GnIH* mRNA expression of broody and after brooding chickens was higher than that of laying hens (He, 2009). An increasing number of studies have focused on chicken *GnIH* because of its role in central and peripheral reproductive function. Most of these studies demonstrated that *GnIH* is related to hormone regulation and reproduction, and lack of the SNPs is related to egg production. However, in the present study, 5 SNPs (T3305C, T3310C, G3403C, G3411A,

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and T3591C) were identified in Erlang mountainous chickens. Comparison of these SNPs revealed distinct mutation loci. SNP1, SNP3, and SNP4 had large effects on age at first egg, while SNP5 had large effects on body weight at first egg.

There are some limitations to single-marker analysis, such as noise, which obscures the results. Significant associations between individual SNPs and phenotypic traits were analyzed by single-factor analysis, not mixed model analysis. Therefore, it was unclear whether an association between the SNPs and the examined traits exists. Haplotype or haplotype blocking provided a practical solution for overcoming these limitations. Haplotypes were constructed for 5 SNPs and used to analyze the associations between haplotype combinations and 5 egg production traits.

Based on our results, chickens with the H1H1 and H3H3 haplotypes began laying later and the total of 300-days eggs were lower. The frequency of H1 was 55.15% and H3 was 19.85%; these haplotypes may be disadvantageous for improving egg production traits.

However, the H4H5 haplotype combination should be advantageous for improving egg production traits. This result implies that an interaction exists between different SNPs. Our data showed that associations between haplotypes and egg production were more accurate than those for single SNPs, and that the haplotypes generally provided more information than did 1 SNP.

In summary, commercial breeding programs for layer chicken have become increasingly complex, making the use of molecular marker-assisted selection methods for improving reproduction important. Our results indicated that 5 SNP markers were associated with egg production, and thus the *GnIH* gene plays an important role in regulating egg production in chickens. The *GnIH* gene shows great potential for use in molecular marker-assisted selection programs for controlling egg production.

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