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Research Article

Molecular Characterization of the *Ghrelin* and *Ghrelin Receptor* Genes and Effects on Fat Deposition in Chicken and Duck

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Ghrelin (GHRL) and its receptor (GHSR) are involved in various bioactivities. In this study, the complete cDNA and 5′ flanking region of the duck *GHRL* (*dGHRL*) gene and a 3717 bp fragment of the duck *GHSR* (*dGHSR*) gene were obtained. A total of 19, 8, 43, and 48 SNPs identified in 2751, 1358, 3671, and 3567 bp of the chicken *GHRL* (*cGHRL*), chicken *GHSR* (*cGHSR*), *dGHRL*, and *dGHSR* genes, respectively. Both *cGHRL* and *dGHRL* were expressed predominantly in the proventriculus, whereas the highest mRNA levels of *cGHSR* and *dGHSR* were detected in the breast muscle and pituitary. Association analysis showed that C-2047G, A-2355C, and A-2220C of the *cGHRL* gene were significantly associated with abdominal fat weight (AFW; $P = .01$), crude protein content of leg muscle (CPCLM; $P = .02$), and CPCLM ($P = .0009$), respectively. C-1459T of the cGHSR gene was also significantly associated with CPCLM ($P = .0004$). C-729T of dGHRL and A3427T of dGHSR were both significantly associated with subcutaneous fat thickness (SFT; $P = .04$). It was indicated by this study that the *GHRL* and *GHSR* genes were related to fat deposition in both chicken and duck.

1. Introduction

Small synthetic molecules termed Growth Hormone Secretagogues (GHSs) act on the pituitary gland and the hypothalamus to regulate growth hormone (GH) release. The Growth Hormone Secretagogue Receptor (GHSR or GHS-R), a G protein-coupled receptor, has been cloned from human and pig and is mainly expressed in the pituitary, hypothalamus, and hippocampus [1]. In 1999, a novel 28-amino acid gut-brain peptide named Ghrelin (GHRL) was first isolated from the mammalian stomach [2]. Similar to the synthetic molecules of GHSs, GHRL proved to be an endogenous ligand for GHSR and involved in GH secretion, food intake, and energy homeostasis [2, 3]. Even though the mature GHRL varies in structure among mammals, birds, and fish, the n-octanoylation of the Ser-3 residue is highly conserved and essential for biological activity [2, 4]. It is now well known that GHRL interacts with GHSR to mediate a variety of bioactivities through the neuroendocrine pathway.

The *GHRL* and *GHSR* genes are reportedly related to obesity in human and mouse. Body mass index and body fat were shown to be negatively correlated with plasma GHRL levels [5–8]. Two polymorphisms, Arg51Gln and Leu72Met, in the human *GHRL* gene were identified in obese subjects, and Leu72Met was significantly associated with body mass index and fat mass in some human populations [9, 10]. In addition to these two nonsynonymous SNPs, two other variations of Gln90Leu and a frameshift mutation (2 bp deletion at codon 34) were also reported in the human *GHRL* gene, and the 90Leu allele frequency was significantly higher in extremely obese children and adolescents (0.063) than in normal-weight students [11]. A novel SNP, T3056C in intron 2 of the *GHRL* gene, was significantly associated with the acylation of GHRL and some fatness traits, including high body mass index, body weight, fat mass, and skinfold thickness [12]. Among 11 SNPs identified in the 5′ flanking region of the *GHRL* gene, SNP-501A > C was significantly associated with body mass index in human [13]. In mice, the peripheral administration of GHRL was found to regulate body weight, adiposity, and UCP mRNA expression [14]. It was also interesting that central administration of GHRL enhanced rat fat ingestion [15].

Until now, the research progress on the *GHRL* and *GHSR* genes was achieved in chicken. The chicken *GHRL* (*cGHRL*) gene cDNA was first cloned by Kaiya et al. [16], and it encoded a 116-amino acid GHRL precursor and a 26-amino acid mature GHRL. The complete *cGHRL* gene comprised five exons and four introns. The first exon did not encode any amino acids, a characteristic that was conserved in mammals [17, 18]. The *cGHSR* gene was composed of two exons separated by an intron and encoded a normal *GHSR* with seven transmembrane domains (TM1 to TM7); however, a variant *GHSR* without TM6 due to a 48-bp deletion was found [19]. A total of 19 and 37 SNPs were identified in the *cGHRL* and *cGHSR* genes, respectively [20], and an 8-bp indel in the 5′ untranslated region (UTR) and some SNPs of the *cGHRL* gene were related to chicken growth and muscle fiber traits [21–23]. A 6-bp indel of the *cGHSR* gene was significantly linked with fat traits [21]. The duck *GHRL* (*dGHRL*) gene cDNA was also cloned, and the encoded dGHRL was highly similar to cGHRL [24]. Nevertheless, no further studies on the *dGHRL* and duck *GHSR* (*dGHSR*) genes have been reported.

Fat deposition is significant in both chicken and duck. Most fat accumulates as abdominal fat in chicken but as subcutaneous fat in duck; these characteristics make the chicken and duck ideal for use in this study. RACE, genome walking, real time PCR, and association analysis were performed to characterize the *GHRL* and *GHSR* genes and reveal their effects on fat deposition in chicken and duck.

2. Materials and Methods

2.1. Animals and Sample Preparation. A total of 411 birds (219 males and 193 females) from 8 chicken populations (POP1 to POP8) and 139 birds from 9 duck populations (POP9 to POP17) were used in this study (Table 1). These samples consisted of six chicken breeds, including Xinghua (XH), White Recessive Rock (WRR), Taihe Silkies (TS), Leghorn (LH), Lingnan Yellow (LY), Huiyang Beard (HB), and an F2 full-sib hybrid population (XH&WRR) described by Fang et al. [23], as well as four duck breeds, Sanshui White (SW), Peking (PK), Partridge (PT), and Lake (LK). Either blood or live tissues were collected from these birds at 9 weeks of age for the purpose of sequence cloning, variation, mRNA quantitative expression, cell culture, and marker trait association analyses (Table 1).

The SW commercial population (POP14), including 100 ducks (52 males and 48 females), were slaughtered at 90 days old, and body weights at hatch (g) and 90 days old (g), carcass weight (g), fat thickness under skin (mm), fat width (mm), eviscerated weight (g), abdominal fat pad weight (g), and abdominal fat pad ratio (%) were recorded. All birds were raised in separate pens and fed with commercial corn-soybean-based diets that met NRC requirements. All chickens were collected from Guangdong Wens Foodstuff Corporation Ltd. (Guangdong, China), and all ducks were provided by Guangdong Sanshui Lianke Duck Farm (Guangdong, China).

Genomic DNA was extracted from EDTA-anticoagulated blood. Total RNA was extracted from the isolated tissues using an improved Trizol isolation method (Invitrogen, California, USA) following the manufacturer's instructions. One microgram of total RNA was used to obtain cDNA by reverse transcription with use of the Rever Tra Ace Transcriptase Kit (Toyobo, Japan) and random hexamers.

2.2. Primers. Thirty-three primer pairs (PM1 to PM33) were designed and consisted of PM1 to PM6 (6) for the *cGHRL* gene, PM7 to PM10 (4) for the *cGHSR* gene, PM11 for the chicken *β*-actin gene, PM12 to PM24 (13) for the *dGHRL* gene, PM25 to PM32 (8) for the *dGHSR* gene, and PM33 for the duck *β-actin* gene (Table 2). PM12 and PM26 were both genomic primers, each of which consisting of three reverse primers and a random forward primer supplied by the Genomic Walking Kit. PM14 was a primer pair for 3′ RACE, and the remaining 30 primer pairs were gene-specific primers. The GENETOOL software (http://www.biologysoft.com/) was used to design the primers based on reported sequences of the chicken *GHRL* (GenBank AB075215), *GHSR* (GenBank AB095994), and *β-actin* genes (GenBank NM_205518), as well as duck *GHRL* (GenBank AY338466), *GHSR* (GenBank EU005225), *β-actin* (GenBank EF667345), and goose *GHRL* (GenBank AY338465). All primers were commercially synthesized by Shanghai Biosune Biotechnology Co. Ltd. (Shanghai, China).

2.3. PCR, PCR-RFLP, and Sequencing. PCR was performed in 25 μL reactions containing 50 ng chicken genomic DNA, 1 × PCR buffer, 12.5 pmol primers, 100 μM of each dNTP, 1.5 mM $MgCl_2$, and 1.0 U *Taq* DNA polymerase (Sangon Biological Engineering Technology Company, Shanghai, China). PCR was run in a Mastercycler gradient (M. J. Research Co. Ltd., USA) with the following procedure: 3 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 45 seconds at the annealing temperature (58–62°C) and 1 minute at 72°C, and a final extension of 5 minutes at 72°C. PCR products were checked for size and quality on 1% agarose gels.

The PCR product of PM5 was sequenced for genotyping of the A-2220C, A-2246G, A-2355C, C-2399del, C-2030T, and C-2047G polymorphisms in the *cGHRL* gene. The PCR product of the PM7 reaction was digested with *Tat I* with the PCR-RFLP method for genotyping C-1896A in the *cGHSR* gene. The PCR product of the PM8 reaction was digested by *Hin1 II* with the PCR-RFLP method for genotyping the C-1459T SNP in the *cGHSR* gene. The PCR product of the PM9 reaction was digested with *Tail I* with the PCR-RFLP method for genotyping the A-1022C SNP in the *cGHSR* gene. The PCR product of the PM20 reaction was digested with *Csp6 I* with the PCR-RFLP method for genotyping the C-729T SNP in the *dGHRL* gene. The PCR product of the PM22 reaction was digested with *Tail I* with the PCR-RFLP method for genotyping the T985C SNP in the *dGHRL* gene. The PCR product of the PM30 reaction was digested with *Csp6 I* with the PCR-RFLP method for genotyping

Table 1: Chicken and duck samples used in this study.

No.	Species	Population[(1)]	Samples	Blood/live tissues	Purpose
POP1	Chicken	XH	6 (3 ♂; 3 ♀)	Blood for DNA	*GHRL* and *GHSR* gene variation
POP2	Chicken	WRR	6 (3 ♂; 3 ♀)		
POP3	Chicken	TS	6 (3 ♂; 3 ♀)		
POP4	Chicken	LH	6 (3 ♂; 3 ♀)		
POP5	Chicken	XH&WRR	373 (200 ♂; 173 ♀)	Blood for DNA	Association analysis
POP6	Chicken	LY	6 (3 ♂; 3 ♀)	18 tissues [(2)]	Real time PCR
POP7	Chicken	HY	6 (3 ♂; 3 ♀)		
POP8	Chicken	LY	2 ♀	Subcutaneous and abdominal adipose tissues	Cell culture
POP9	Duck	SW	1 ♂	Blood for DNA and proventriculus tissue	*GHRL* gene sequence cloning
POP10	Duck	SW	6 (3 ♂; 3 ♀)	Blood for DNA	*GHRL* and *GHSR* gene variation
POP11	Duck	PK	6 (3 ♂; 3 ♀)		
POP12	Duck	PT	6 (3 ♂; 3 ♀)		
POP13	Duck	LK	6 (3 ♂; 3 ♀)		
POP14	Duck	SW	100 (52 ♂; 48 ♀)	Blood for DNA	Association analysis
POP15	Duck	SW	6 (3 ♂; 3 ♀)	18 tissues	Real time PCR
POP16	Duck	PT	6 (3 ♂; 3 ♀)		
POP17	Duck	SW	2 ♀	Subcutaneous and abdominal adipose tissues	Cell culture

[(1)]XH = Xinghua chicken, WRR = White Recessive Rock, TS = Taihe Silkies, LH = Leghorn, XH&WRR = an F2 population crossed by XH and WRR, LY = Lingnan Yellow, HY = Huiyang Beard, SW = Sanshui White duck, PK = Peking duck, PT = Partridge duck, and LK = Lake duck.
[(2)] The 18 tissues used were pituitary, cerebrum, lung, abdominal fat, liver, testis, proventriculus, ovary, subcutaneous fat, spleen, kidney, oviduct, leg muscle, uropygial gland, hypothalamus, glandularis, small intestine, cerebellum, heart and breast muscle.

the T404C SNP in the *dGHSR* gene. The PCR product of the PM31 reaction was digested with *Csp6 I* with the PCR-RFLP method for genotyping the A3427G SNP in the d*GHSR* gene. Digestion products were detected by agarose gel (1.5%) electrophoresis, and genotypes were determined by specific profiles (Supplementary Figure 1 in Supplementary Material available on-line at doi: 10.1155/2009/567120).

2.4. RT-PCR and 3′ RACE. Total RNA was extracted from the proventriculus from an SW duck by the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. A proventriculus cDNA library was prepared using the SMART RACE cDNA amplification kit (Clontech Laboratories, Mountain View, CA). Primer-directed RT-PCR and 3′ rapid amplification of cDNA ends (RACE) were used to generate duck GHRL cDNAs, amplified using primers P1 to P3 (Table 2). A cDNA library was prepared using the SMART RACE cDNA amplification kit (Clontech Laboratories, Mountain View, CA). The 3′ ends of the cDNAs were amplified with a specific forward primer and reverse random primers from the SMART RACE cDNA amplification kit following the manufacturer's instructions.

2.5. Genome Walking. To obtain the 5′ flanking region of the duck *GHRL* and *GHSR* genes, genome walking was performed using the Genome Walking Kit (TakaRa, Japan). The duck genomic DNA was purified using the Genomic DNA Purification Kit (U-gene, China) and was then used for genome walking. The genome walking procedure included three reactions: the first PCR reaction used the outer adaptor primer AP1 from the kit and an outer gene-specific primer PM12R1 (or PM26R1); the second reaction used primers AP2 and PM26R2; the third reaction used primers AP3 and PM26R3. Each reaction was performed step-by-step following the kit instructions. PCR products of the third reaction were purified using the Gel Extraction Kit (U-gene, China), cloned into the pGEM-T easy vector (Promega, USA), and sequenced by Shanghai Biosune Biotechnology Co. Ltd. (Shanghai, China).

2.6. Quantitative Real Time PCR. The mRNA levels of *GHRL* and *GHSR* in 18 tissues of chickens and ducks were determined by quantitative real time PCR with SYBR green dye using *β-actin* as an internal positive control (IPC). The 15 μL PCR mixture contained 1 μL of cDNA, 7.5 μM Super Mix (Toyobo, Japan), 10 μM of each primer (PM6, PM10, PM11, PM24, PM32, and PM33), and 6.0 μL DEPC H_2O. Real time PCR was run on an ABI 7500 (Applied Biosystems, Foster City, CA, USA) using the following program: initial denaturation at 95°C for 4 minutes, then 40 cycles of 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 45 seconds. Each sample was repeated three times and the average mRNA level was obtained for further analysis. Two random PCR products of PM6, PM10, PM11, PM24, PM32, and PM33 were sequenced by Shanghai Biosune Biotechnology Co. Ltd. (Shanghai, China) to confirm that specific fragments of the *cGHRL*, *cGHSR*, chicken *β-actin*, *dGHRL*, *dGHSR*, and duck *β-actin* genes were obtained. Quantitative values were obtained from the Ct values, which were the inverse ratio relative to the starting PCR product. The relative quantification was obtained by $2^{-\Delta Ct}$ in which

Table 2: Descriptions of the 33 primers used in this study.

Gene	Primer	Forward/reverse	Sequence (5′ to 3′)	Annealing temp (°C)	Purpose
cGHRL	PM1	PM1F	tgctgaaggaccgaaaacaaa	56	SNP identification
		PM1R	gccttgacagatgccttagtg		
	PM2	PM2F	aagaaagctggtaactgcactag	55	SNP identification
		PM2R	ggtgggctggtggagtta		
	PM3	PM3F	tgcgttctgctactctttttcat	63	SNP identification
		PM3R	gggccaaggaggagtgtct		
	PM4	PM4F	cggcagacactcctcctt	57	SNP identification
		PM4R	gccatccttccaactgtgtatat		
	PM5	PM5F	tatgcgttctgctactcttt	58	Genotyping by sequencing
		PM5R	tggaagcgatcactatacc		
	PM6	PM6F	catacagcaacaaaaggatac	63	Real time PCR
		PM6R	tgtggttgtccttcagct		
cGHSR	PM7	PM7F	gtgggtcagggcatcaaactc	58	SNP identification and PCR-RFLP
		PM7R	tcgagggctgctgaattttatg		
	PM8	PM8F	ggctcttccttttggtttgtct	60	SNP identification and PCR-RFLP
		PM8R	tcgccctctctctgattcacct		
	PM9	PM9F	tgatgccactggtctgagaat	58	Genotyping by PCR-RFLP
		PM9R	tatccagctgcccatgtaaat		
	PM10	PM10F	tgggcgtcgagcatgagaat	63	Real time PCR
		PM10R	ccacgactagcatcttcacag		
Chicken *β-actin*	PM11	PM11F	ccccaaagccaacagagaga	63	Real time PCR
		PM11R	ggtggtgaagctgtagcctctc		
dGHRL	PM12	PM12F	Genomic Walking Kit	According to the kit	Genomic Walking
		PM12R1	ttccatcagttctagacgagt		
		PM12R2	tgtttgtccatactcttgatact		
		PM12R3	gcccctgctttatctgtatc		
	PM13	PM13F	ccgcctggtgaagaaaac	56	RT-PCR
		PM13R	aagcctacacatccacctgcaat		
	PM14	PM14F	gaggcaagctgaaggacaaccaca	54	3′ RACE
		PM14R	3′ RACE Kit		
	PM15	PM15F	gctggttttcccgtgtaattc	56	RT-PCR
		PM15R	tgtttgtccatactcttgatact		
	PM16	PM16F	gctggttttcccgtgtaattc	54	Intron 1 amplification
		PM16R	tgtttgtccatactcttgatact		
	PM17	PM17F	tggtttggctggctctagt	56	Intron 2 amplification
		PM7R	PM12R3		
	PM18	PM18F	aaagcaggggcagaagat	57	Intron 3 amplification
		PM18R	PM12R2		
	PM19	PM19F	agggtcctggtccaaaaat	54	Intron 4 amplification
		PM19R	PM12R1		
	PM20	PM20F	cgcatggtagccttcacacac	56	SNP identification and PCR-RFLP
		PM20R	agccgatgggttagcagagag		
	PM21	PM21F	tcggctccatcagttgcagttat	56	SNP identification
		PM21R	cggcgatgtatttttgctgttg		
	PM22	PM22F	tccccacgacagagtagtttgag	56	SNP identification and PCR-RFLP
		PM22R	gccttcccctgcttcctaa		
	PM23	PM23F	atagcagcattttagaagtga	54	SNP identification
		PM23R	ttgcctcagcttttcact		
	PM24	PM24F	cgtgtaattcctctctgctaa	63	Real time PCR
		PM24R	cgatgtatttttgctgttgtt		

Table 2: Continued.

Gene	Primer	Forward/reverse	Sequence (5′ to 3′)	Annealing temp (°C)	Purpose
dGHSR	PM25	PM25F	cccctgagcaccaacgagt	56	Intron 1 amplification
		PM25R	ggcaaccagcagagtatga		
	PM26	PM26F	Genomic Walking Kit	According to the kit	Genomic Walking
		PM26R1	cggaccgatgttctttctctttt		
		PM26R2	gacgggcaggaaaaagaagac		
		PM26R3	ggcccagaggatgaggatga		
	PM27	PM27F	tggcgttctccgacctgctcat	57	SNP identification
		PM27R	acacagaccctcagaaacac		
	PM28	PM28F	cgggtggcttaaataacaacag	56	SNP identification
		PM28R	tgccctttttcctcagtttctc		
	PM29	PM29F	cggaccataattaaacacctgaa	56	SNP identification
		PM29R	caggtagaagaggacaaaggaca		
	PM30	PM30F	tggcgttctccgacctgctcat	56	Genotyping by PCR-RFLP
		PM30R	acacagaccctcagaaacac		
	PM31	PM31F	gcaggttgtttagatatggct	56	Genotyping by PCR-RFLP
		PM31R	cgtgaaaaggcaaccagcagag		
	PM32	PM32F	cccctgagcaccaacgagt	63	Real time PCR
		PM32R	ccaccacaactaacattttcaca		
Duck *β-actin*	PM33	PM33F	acgccaacacggtgctg	63	Real time PCR
		PM33R	gggtccggattcatcatactc		

$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{IPC}}$. The data are expressed as the mean ± S.E. Statistics were analyzed by SAS Students *t*-test that measures analysis of variance with a significance level of 0.05.

2.7. Statistics Methods

2.7.1. Sequence BLAST, Prediction of Transcription Factors, and Haplotypes. The homology of GHRL among human, mouse, chicken, turkey, emu, goose, and duck was studied. Identity percentages were obtained from amino acid BLAST with the DNASTAR software (http://www.dnastar.com/). The obtained sequences of *GHRL* and *GHSR* were aligned with the Mega 3.1 software (http://www.megasoftware.net/). The 798-bp 5′ flanking region of the *dGHRL* gene obtained in this study was used to predict the potential binding sites for transcription factors by an online service (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl). In the chicken F2 population (XH&WRR) and the duck commercial population (SW), haplotypes were inferred based on the genotype data with the PHASE 2.1 software (http://en.wikipedia.org/wiki/Phase) [25].

2.7.2. Nucleotide Diversity (θ). To evaluate the nucleotide diversity, the normalized numbers of SNPs (θ) were calculated by dividing K, the number of observed nucleotide changes, by the total sequence length in base pairs (L) and correcting for the sample size (n) as described in [26], using the following formula:

$$\theta = \frac{K}{\sum_{i=1}^{n-1} i^{-1} L}. \quad (1)$$

2.7.3. Marker-Trait Association Analysis. The effects of 13 SNPs identified in this study on fatty traits were estimated by marker-trait association analysis. These SNPs included six in the *cGHRL* gene (C-2030T, C-2047G, A-2220C, A-2246G, A-2355C, and C-2399del), three in the *cGHSR* gene (C-1896A, C-1459T, and A-1022C), two in the *dGHRL* gene (C-729T and T985C), and two in the *dGHSR* gene (T404C and A3427G). Marker-trait association analysis was performed using the SAS GLM procedure (*SAS Institute, 1996*) and the genetic effects were analyzed by a mixed procedure according to the following model:

$$Y = \mu + G + D + H + S + e, \quad (2)$$

where Y is an observation on the trait, μ is the overall population mean, G is the fixed effect of the genotype, D is the random effect of dam, H is the fixed effect of hatch, S is the fixed effect of sex (male or female), and e is the residual random error.

3. Results

3.1. Cloning the Full-Length cDNA of the dGHRL Gene. The full-length cDNA of the *dGHRL* gene (835 bp) was amplified using primers PM13 to PM15. It comprised 115 bp of the 5′ untranslated region (5′ UTR), the 351 bp open reading frame (ORF) encoding a 116-amino acid (aa) peptide, and 369 bp of the 3′ UTR (Figure 1) (GenBank EF613551). The SW duck GHRL precursor (116 aa) included a signal peptide (23 aa), the mature GHRL (26 aa), and a long C-terminal peptide (67 aa). Compared with the other reported mallard dGHRL precursor sequence (NCBI accession number: AY338466), there were some different amino acids (W16R, G21A, A53T,

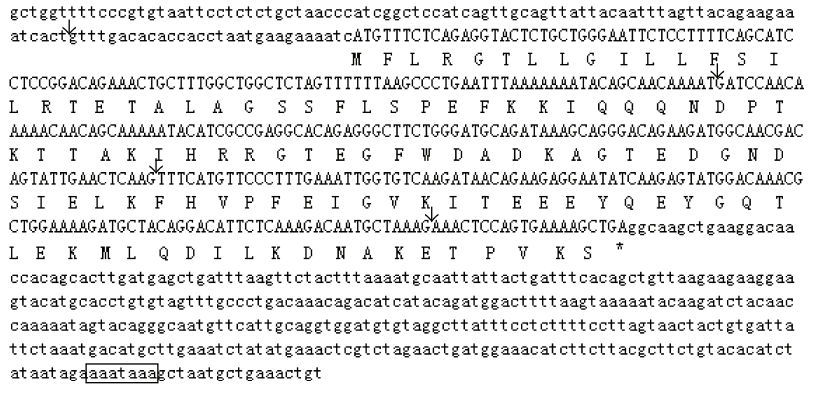

Figure 1: cDNA and deduced amino acid sequence of the *dGHRL* gene. The arrowheads indicate the locations of introns. Polyadenylation signal is boxed. The asterisk (*) represents the putative stop codon (TGA). The nucleotide sequence is deposited in GenBank (EF613551).

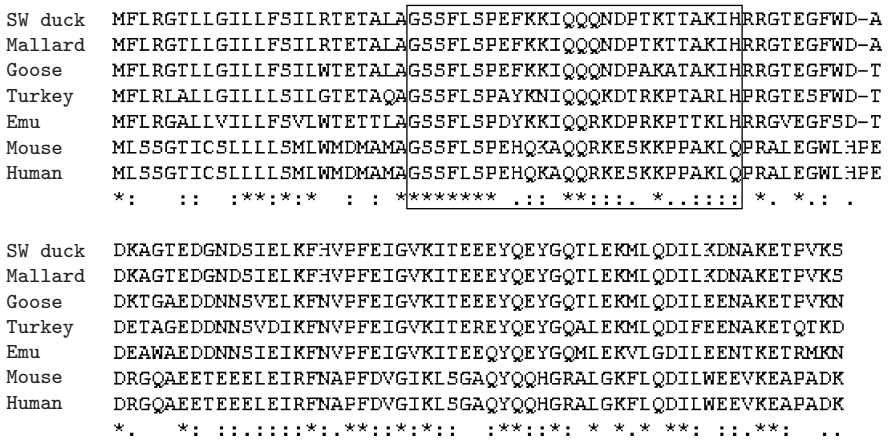

Figure 2: Alignment of GHRL sequences among 7 species. The mature peptides were boxed.

T59A, A64T, and G71S). The putative SW dGHRL precursor showed identities of 88.8%, 72.4%, 69.8%, 71.6%, 37.9%, and 37.1% with its counterparts of goose, chicken, turkey, emu, mouse, and human, respectively (Figure 2). As far as the mature peptides are concerned, however, dGHRL had much higher homology with the other six species (92.3%, 65.4%, 65.4%, 69.2%, 53.8%, and 50.0% with goose, chicken, turkey, emu, mouse, and human, resp.) (Figure 2).

3.2. Genomic Organization of the dGHRL and dGHSR Genes. The sequence ensemble generated with the PM12 to PM19 products gave rise to a 3670 bp genomic fragment of the *dGHRL* gene, which included 798 bp of the 5′ flanking region, 883 bp of five exons (134, 137, 114, 109, and 389 bp for exon 1 to exon 5) and 1989 bp of four introns (168, 469, 458, and 894 bp for intron 1 to intron 4). Like other species, the four introns of the *dGHRL* gene all followed the "GT-AG" rule, and moreover, exon 1 did not encode any amino acids (Supplementary Figure 2). The obtained *dGHRL* genomic sequences were submitted to the NCBI database (GenBank EF613552).

The 3717 bp sequence of the *dGHSR* gene was deduced by genomic walking (PM26), PCR amplification (PM25) and sequence ensemble with reported sequences (Genbank EU005225). It comprised two incomplete exons (591 and 148 bp for exon 1 and exon 2) and one intron (2978 bp) that followed the "GT-AG" intron rule (Supplementary Figure 2). The obtained *dGHSR* gene sequences were also submitted to the NCBI database (GenBank FJ194548).

3.3. Online Prediction of Transcription Factor Binding Sites in the dGHRL Gene. Online prediction of transcription factor binding sites in the 5′ flanking region of the *dGHRL* gene showed that a typical TATA box was present in 23 bp upstream of the transcription start site, and potential binding sites for some transcription factors were found, including one AML-1a site (runt-factor AML-1), two cap 1 sites (cap signal for transcription initiation), two CdxA 1 sites, three GATA-1 sites (GATA-binding factor 1), as well as one Oct-1 site (octamer factor 1) (Figure 3).

3.4. SNPs and Nucleotide Diversity. A total of 19 SNPs (10 transitions, 8 transversions, and 1 indel) and 1 polyA polymorphism were identified in 2751 bp of the 5′ UTR region of the *cGHRL* gene, while 8 SNPs (5 transitions and 3 transversions) were identified in 1358 bp of the 5′ UTR of the *cGHSR* gene (Table 3). Forty-three SNPs (27 transitions, 15 transversions, and 1 triallelic SNP), 1 SSR, and 1 9-bp

Figure 3: Online prediction of transcription factor binding sites and the TATA box in the 5′ flanking region of the *dGHRL* gene.

indel were identified in 3671 bp of the *dGHRL* gene, while 48 SNPs (35 transitions and 13 transversions) and 5 indels were found in 3567 bp of the *dGHSR* gene (Table 3). Eleven SNPs were located in the coding regions of the *dGHRL* gene, but only four SNPs were nonsynonymous and led to amino acid changes in the C-terminal extension peptide. For the *dGHSR* gene, however, no nonsynonymous SNPs were found within five SNPs in the coding regions (Table 3). A triallelic SNP (GA/C at nt 1774) was observed in the coding region of the *dGHRL* gene, which was related to three different amino acids (A/P/T at aa 59) (Table 3).

The average bps per SNP were 145, 170, 86, and 75 for the *cGHRL*, *cGHSR*, *dGHRL,* and *dGHSR* genes, respectively. The nucleotide diversities (θ) corrected for sample size were 1.83×10^{-3}, 1.56×10^{-3}, 3.10×10^{-3}, and 3.56×10^{-3} for the *cGHRL*, *cGHSR*, *dGHRL,* and *dGHSR* genes, respectively.

3.5. Tissue-Specific Expression of the cGHRL, cGHSR, dGHRL, and dGHSR Genes. The highest mRNA level of the *cGHRL* gene was found in the proventriculus and then the pituitary, cerebrum, abdominal fat, subcutaneous fat, hypothalamus, small intestine, heart, and breast muscle. The highest mRNA level of the *dGHRL* gene was also found in the proventriculus and then the abdominal fat, testicles, subcutaneous fat, uropygial gland, and breast muscle (Figure 4; Supplementary Table 1).

The highest mRNA level of the *cGHSR* gene was detected in the breast muscle and then the subcutaneous fat, leg muscle, abdominal fat, heart, spleen, liver, uropygial gland, cerebrum, proventriculus, pituitary, and testicles. The highest mRNA level of the *dGHSR* gene was detected in the pituitary and then the subcutaneous fat, hypothalamus, small intestine, testis, cerebellum, and cerebrum, whereas little mRNA was found in the other tissues (Figure 4; Supplementary Table 1).

When the HB and LY chicken breeds were compared, significant differences in the *GHRL* mRNA levels were found in the proventriculus, subcutaneous fat, lung, and kidney ($P < .05$), whereas significant differences in the *GHSR* mRNA levels were found in the hypothalamus and breast muscle ($P < .05$). When the PT duck and SW duck breeds were compared, significant differences in the *GHRL* mRNA levels were found in the proventriculus and subcutaneous fat ($P < .05$), whereas no significant differences in the *GHSR* mRNA levels were found in any tissues ($P > .05$) (Figure 4; Supplementary Table 1).

3.6. Associations of SNPs with Fatty Traits in Chicken and Duck. For the *cGHRL* gene, C-2047G and A-2355C were found to be significantly associated with abdominal fat weight (AFW; $P = .01 < .05$) and crude protein content of leg muscle (CPCLM; $P = .02 < .05$), and A-2220C was significantly associated with CPCLM ($P = .0009 < .01$). In addition, haplotypes across six SNPs (C-2030T, C-2047G, A-2220C, A-2246G, A-2355C, and C-2399del) were significantly associated with AFW ($P = .04 < .05$), abdominal fat ratio (AFR; $P = .04 < .05$), and CPCLM ($P < .0001$). As far as the three genotypes of each locus were concerned, CC of C-2047G, CC of A-2220C, and AA of A-2355C had the highest values of association with AFW and CPCLM, indicating that the advantageous alleles for fat deposition were C-2047, A-2220, and A-2355 (Table 4). For the *cGHSR* gene, C-1459T was shown to be significantly associated with CPCLM ($P = .0004 < .01$). Individuals with the CC genotype had the highest CPCLM association, indicating that C rather than T was the dominant allele for CPCLM (Table 4).

In duck, C-729T and haplotypes of C-729T and T985C of the *dGHRL* gene were both significantly associated with subcutaneous fat thickness (SFT; $P = .04$ and .05, resp.). Individuals with the CT genotype of C-729T had the highest SFT. For the *dGHSR* gene, A3427G and haplotypes of T404C and A3427G were both significantly associated with SFT ($P = .04$ and .05, resp.). Individuals with the TT genotype of C3427T had the highest SFT, indicating that T rather than C was the dominant allele for fat deposition (Table 4).

4. Discussion

4.1. Molecular Characterization of the GHRL and GHSR Genes. The genomic organization of the *GHRL* and *GHSR* genes was conserved in chicken and duck (Supplementary Figure 2). The cDNAs of the *cGHRL* and *cGHSR* genes were reported by Kaiya et al. [16] and Tanaka et al. [19],

Table 3: SNPs identified in the *cGHRL*, *cGHSR*, *dGHRL,* and *dGHSR* genes.

Gene	No.	SNP[1]	Region	Note [2,3,4]	No.	SNP	Region	Note [2,3,4]
cGHRL	1	C-495T	5′ flanking	19369777[(2)] *Dde I* [(3)]	11	A-2220C	5′ flanking	19371502
	2	C-517T	5′ flanking	19369799	12	A-2246G	5′ flanking	19371528 *Tas I*
	3	C-873G	5′ flanking	19370155	13	A-2355C	5′ flanking	19371637
	4	T-1038C	5′ flanking	19370320	14	C-2399del	5′ flanking	19371681
	5	C-1253T	5′ flanking	19370535 *Hin1 II*	15	G-2861T	5′ flanking	19372143
	6	T-1562G	5′ flanking	19370844	16	G-3264C	5′ flanking	19372546
	7	A-1589G	5′ flanking	19370871	17	C-3290T	5′ flanking	19372572 *Tai I*
	8	G-1950A	5′ flanking	19371232	18	G-3321T	5′ flanking	19372603
	9	C-2030T	5′ flanking	19371312	19	T-3324C	5′ flanking	19372606
	10	C-2046G	5′ flanking	19371329				
cGHSR	1	A-1907G	5′ flanking	18787994	5	T-1288C	5′ flanking	18789055
	2	C-1896A	5′ flanking	18788005 *Tat I*	6	A-1022C	5′ flanking	18789321 *Tail I*
	3	C-1608T	5′ flanking	18788293	7	T-892C	5′ flanking	18789762 *Bsa JI*
	4	C-1459T	5′ flanking	18788442 *Hin1 II*	8	G-834C	5′ flanking	18789820 *Hin6 I*
dGHRL	1	G221A	5′ flanking		23	T1128C	Exon 2	
	2	T256C	5′ flanking		24	T1145C	Exon 2	G5 Syn[(4)], *Csp6 I*
	3	T263C	5′ flanking		25	C1179G	Exon 2	R17G, *Msp I*
	4	G273T	5′ flanking		26	A1244T	Intron 2	
	5	A276C	5′ flanking		27	T1278C	Intron 2	
	6	A303G	5′ flanking		28	G1390A	Intron 2	
	7	A342C	5′ flanking		29	A1756G	Exon 3	T53A, *Hin 6 I*
	8	T401C	5′ flanking	*Csp6 I*	30	G1761A	Exon 3	E54 Syn
	9	C405T	5′ flanking		31	G1774A/C	Exon 3	A59P/T
	10	A532C	5′ flanking		32	A1789G	Exon 3	T64A
	11	A628G	5′ flanking		33	C1803T	Exon 3	N68 Syn
	12	C638T	5′ flanking		34	A1807G	Exon 3	G70S
	13	A645G	5′ flanking		35	A1943C	Intron 3	*HpyCH4IV*
	14	C686G	5′ flanking		36	C2070T	Intron 3	
	15	C691A	5′ flanking		37	T2115C	Intron 3	*TailI*
	16	A736T	5′ flanking		38	C2179T	Intron 3	
	17	C778A	5′ flanking		39	A2324G	Exon 4	E89 Syn
	18	C1028A	Intron 1		40	G2345A	Exon 4	T96 Syn
	19	A1070G	Intron 1		41	A2351G	Exon 4	E98 Syn
	20	A1108G	Exon 2		42	G2409C	Intron 4	
	21	A1118G	Exon 2		43	T2509C	Intron 4	
	22	A1117G	Exon 2					
dGHSR	1	C320T	Exon 1	A Syn, *Msp I*[(4)]	25	C2815A	Intron	
	2	T359C	Exon 1	N Syn	26	C2843T	Intron	
	3	G398C	Exon 1	T Syn, *Bsa JI*	27	C2861T	Intron	
	4	T404C	Exon 1	Y Syn, *Csp6 I*	28	G2863C	Intron	
	5	G548A	Exon 1	T Syn	29	C2883T	Intron	
	6	C779T	Intron		30	T2906C	Intron	
	7	T1364A	Intron		31	C2939A	Intron	
	8	C1437T	Intron		32	T2958A	Intron	
	9	T1456C	Intron		33	G2993A	Intron	
	10	T1469C	Intron		34	G3001A	Intron	

Table 3: Continued.

Gene	No.	SNP[1]	Region	Note [2,3,4]	No.	SNP	Region	Note [2,3,4]
dGHSR	11	C1478T	Intron		35	A3304G	Intron	
	12	A2393T	Intron		36	A3037G	Intron	
	13	C2430T	Intron		37	T3050G	Intron	
	14	A2431G	Intron		38	G3080A	Intron	
	15	T2578C	Intron		39	T3208A	Intron	*Taa I*
	16	T2591A	Intron	*Tail I*	40	C3329T	Intron	*Taa I*
	17	T2619A	Intron		41	T3340A	Intron	
	18	C2657T	Intron		42	G3362A	Intron	
	19	C2681T	Intron		43	G3363A	Intron	
	20	A2682G	Intron		44	T3380C	Intron	
	21	G2746A	Intron		45	C3424T	Intron	*Csp6 I*
	22	T2792C	Intron		46	A3427G	Intron	
	23	G2794T	Intron		47	A3451G	Intron	
	24	G2803A	Intron		48	C3543T	Intron	

[1]The first nucleotide of start codon was marked as +1, and the next upstream nucleotide was −1. SNP position was determined based on reported sequences of GenBank EF613552 (*dGHRL* gene) and FJ194548 (*dGHSR* gene), respectively. [2]SNP position was determined according to the released chicken genomic sequence (http://genome.ucsc.edu/). [3]Restriction enzyme was used for PCR-RFLP. [4]SNP caused a codon and amino acid change.

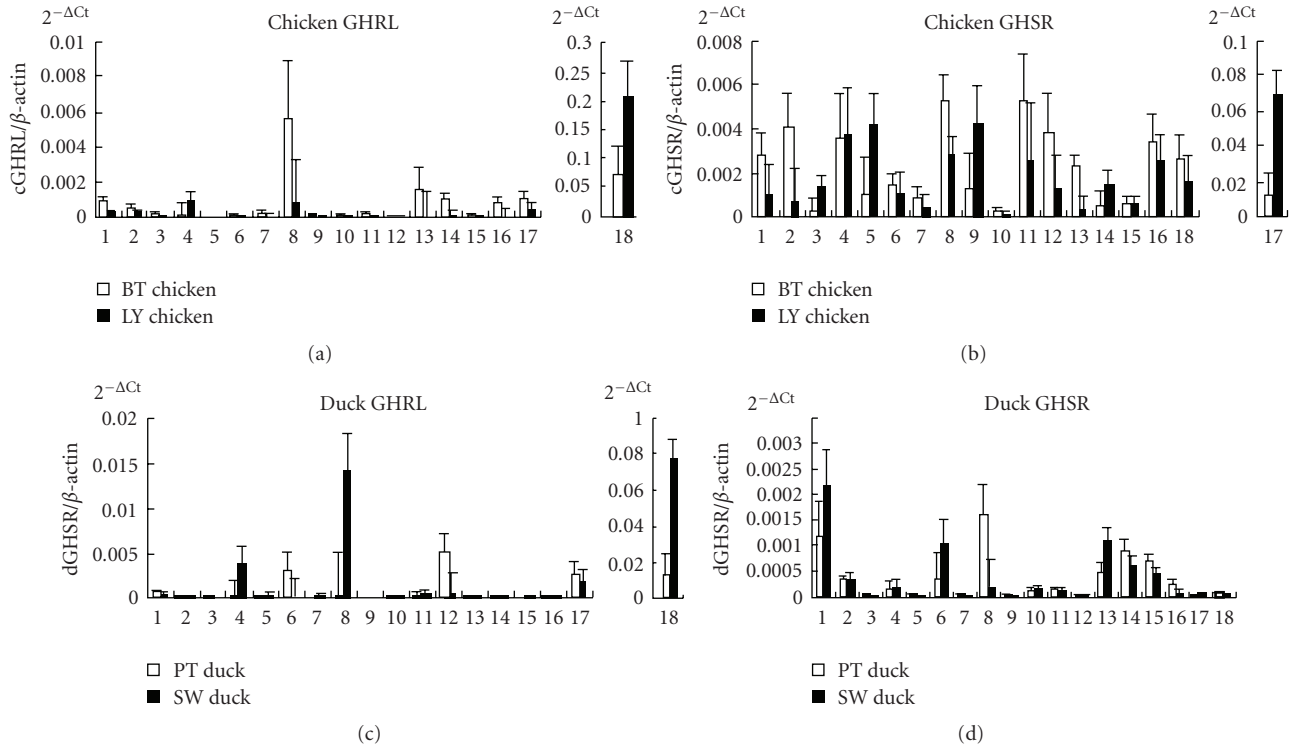

Figure 4: Relative mRNA expression levels of the *GHRL* and *GHSR* genes in adult chicken and duck tissues. The vertical axis indicates the 2^{-Ct} value (mean ± S.E.M., $n = 6$). Pituitary, Cerebrum, Lung, Abdominal fat, Liver, Testis, Gizzard, Subcutaneous fat, Spleen, Kidney, Leg muscle, Uropygial gland, Hypothalamus, Small intestine, Cerebellum, Heart, Breast muscle, and Proventriculus are considered.

respectively. The genome sequences of the *cGHRL* and *cGHSR* genes were also previously known. The reported *cGHRL* and *cGHSR* genes were 2706 and 4121 bp long, respectively, and comprised five and two exons each [17, 19, 27]. From the cloning and sequencing performed in this study, the *dGHRL* cDNA (GenBank EF613551) had a 351-bp ORF that encodes a 116-aa precursor. The predicted mature dGHRL was a 26-aa peptide, and only two amino acids were different from cGHRL. The first seven amino acids, including a serine residue at position 3 (site of *n*-octanoylation), of the mature dGHRL are identical to those of chicken, turkey, goose, and emu (Figure 2) [17, 24].

Table 4: Effects of SNPs on fatty traits in chicken and duck.

Gene	SNP	Traits	*P*-value	Genotype and traits		
cGHRL	C-2047G	AFW	.01	29.01 ± 1.089^{a} (C/C)	25.57 ± 3.46^{b} (C/G)	23.30 ± 5.676^{b} (G/G)
	A-2220C	CPCLM	.0009	20.71 ± 0.1892^{a} (A/A)	20.03 ± 0.319^{a} (A/C)	22.47 ± 0.7554^{B} (C/C)
	A-2355C	CPCLM	.0203	21.83 ± 0.4864^{a} (A/A)	20.75 ± 0.228^{b} (A/C)	20.58 ± 0.1889^{b} (C/C)
cGHSR	C-1456T	CPCLM	.0004	67.77 ± 4.598^{a} (C/C)	66.88 ± 1.097^{a} (C/T)	63.40 ± 0.7162^{B} (T/T)
dGHRL	C-729T	SFT	.0408	1.52 ± 0.22^{a} (T/T)	2.44 ± 0.32^{b} (C/T)	2.02 ± 0.48^{ab} (C/C)
dGHSR	C-3427T	SFT	.0407	0.80 ± 0.77^{a} (C/C)	1.79 ± 0.30^{b} (C/T)	1.93 ± 0.23^{b} (T/T)

Note that only significant associations are shown in this table. AFW = abdominal fat weight; CPCLM = crude protein content of leg muscle; SFT = subcutaneous fat thickness. Values within a row without a common superscript letter differ and values with superscript letters differ significantly ($P < .05$ and $P < .01$ each). Genotypes of a and b mean they differed significantly ($P < .05$) and genotypes of a and B mean they differed great significantly ($P < .01$).

The obtained *dGHRL* gene sequence (GenBank EF613552) also comprised five exons and four introns, and the first exon did not encode any amino acids. This characteristic was conserved in chicken, turkey, human, and rodent [16–18, 28, 29]. The obtained 3717 bp sequence of the *dGHSR* gene (GenBank FJ194548) consisted of two exons and one intron and encoded a 347-aa mature receptor protein. All introns of the *cGHRL, cGHSR, dGHRL,* and *dGHSR* genes followed the GT-AG rule, which is conserved in eukaryotic genes.

Online prediction indicated that a typical TATA box and a number of transcriptional factor binding sites (AML-1a, cap 1, CdxA 1, GATA-1, and Oct-1) were present in the 5′ flanking region of the *dGHRL* gene. These transcriptional factor binding sites were also found in the *GHRL* gene promoter regions of human and chicken [29, 30], and a "TATATAA" sequence was reported to be located at the c*GHRL* gene promoter [18]. GATA regulatory motifs were first found in the promoters of globulin and other erythroid-specific genes [31]. AML-1 is also a key regulator of hematopoiesis and plays an important role in development of all hematopoietic lineages [32]. It is possible that *dGHRL* gene expression in hematopoietic cells would be regulated by GATA-1 and AML-1a. The transcription factor cap-1 is a key factor of the cap-dependent translation initiation mechanism. Oct-1 is one of the transcription factors with a POU homeodomain and is expressed in most tissues including the brain [33]. Moreover, Oct-1 plays very important roles in the transcriptional regulation of gonadotropin-releasing hormone and aldolase C gene expression in the brain [34, 35]. CdxA is one of the vertebrate caudal proteins that play important roles in establishment of the body plan during early development [36]. These transcription factor binding sites in the putative promoter region might affect *dGHRL* gene expression.

The *GHRL* and *GHSR* genes were found to be more variable in poultry than in mammals. As indicated by this study, the SNP densities were 145, 170, 86, and 75 bp per SNP for the *cGHRL* (2751 bp/19 SNP), *cGHSR* (1358/8), *dGHRL* (3671/43), and *dGHSR* (3567/48) genes, respectively (Table 3). In humans, a total of 11 SNPs were found in 1657 bp of *GHRL* gene with an SNP density of 1 SNP/151 bp [13]. The reported density for the human *GHSR* gene (5 SNP of 1101 bp) was 220 bp per SNP [37]. Based on the dbSNP data (http://www.ncbi.nlm.nih.gov), a total of 8 and 32 SNPs were reported in 2922 and 6494 bp of the mouse *GHRL* and *GHSR* genes, with estimated densities of 365 and 203 bp per SNP, respectively. It was indicated that the higher variations of the *GHRL* and *GHSR* genes were found in chicken and duck compared with those of human and mouse. Moreover, as far as these two species were concerned, higher SNP densities were found in duck compared to chicken, as the nucleotide diversity of the *dGHRL* (3.10×10^{-3}) and *dGHSR* (3.56×10^{-3}) genes were much higher than those of *cGHRL* (1.83×10^{-3}) and *cGHSR* (1.56×10^{-3}). Our recent study on the chicken and duck *THRSP α* genes also showed higher variations in the duck genome (data not shown).

4.2. Effects of the GHRL and GHSR Genes on Fat Deposition. As indicated in this study, the *GHRL* and *GHSR* genes were associated with fatness traits in chicken and duck, which is consistent with some previous studies in humans. Many studies showed associations of the *GHRL* gene with adiposity [10–12, 38] and Type 2 diabetes [39] in humans. Additionally, some polymorphisms in the *GHSR* gene were found to be associated with obesity, bulimia nervosa, and pharmacological abnormalities in humans [40–43]. In chickens, the association of *cGHRL* polymorphisms with growth traits has been reported [22, 23]. Moreover, significant association of the *cGHSR* gene with several fatness traits was reported by a recent study [21]. In this study, the *GHRL* and *GHSR* genes were found to be associated with several fatness traits like AFW, CPCLM, and SFT in both chicken and duck.

Analysis of mRNA levels also showed the effects of the *GHRL* and *GHSR* genes on fat deposition in poultry. Even though the *GHRL* gene was predominantly expressed in the proventriculus, high mRNA levels of the *GHRL* and *GHSR* genes were detected in subcutaneous fat and abdominal fat in both chicken and duck (Figure 4). This suggests that both *GHRL* and *GHSR* genes may be involved in fat mobilization in poultry. Moreover, much higher *GHRL* mRNA levels were found in LY (2.07 ± 0.06) rather than HB (0.07 ± 0.05) chickens; higher levels were found in SW (0.78 ± 0.01) compared to those in PT (0.13 ± 0.01) ducks (Supplementary Table 1). SW ducks and LY chickens are two fast-growing commercial breeds with higher fat contents compared with those of the other two native breeds of PT ducks and HB chickens. The higher *GHRL* mRNA

in higher-fat breeds suggested that GHRL was probably advantageous for fat deposition in poultry.

5. Conclusion

From these investigations, the variations and expression patterns of the *cGHRL*, *cGHSR*, *dGHRL,* and *dGHSR* genes were characterized, and some SNPs of these genes were related to fatty traits.

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