Polymorphism analysis and expression profile of the estrogen receptor 2 gene in Leizhou black duck

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ABSTRACT Our previous study on the ovarian transcriptomic analysis in Leizhou black duck revealed that the *ESR2* gene was involved in hormone regulation in reproduction and the estrogen signaling pathway related to reproductive performance was enriched. This suggested that *ESR2* may have a functional role in the reproductive performance of the Leizhou black duck. Thus, this study aimed at evaluating the polymorphism of the *ESR2* gene and its association with egg-laying traits and the distribution pattern of *ESR2* mRNA in laying and non-laying Leizhou black ducks. In this study, genomic DNA was extracted from blood samples of 101 Leizhou black ducks to identify single nucleotide polymorphisms (**SNPs**) of the *ESR2* gene to elucidate molecular markers highly associated with egg-laying traits. Four each of laying and non-laying Leizhou black ducks were selected to collect different tissues to analyze the ESR2 gene expression. A total of 23 SNPs were identified and association analysis of the single SNP sites showed that SNPs g.56805646 T>C and exon 3-20G>A were significantly ($P < 0.05$) associated with egg weight. Ducks with CT and AG genotypes had significantly higher ($P < 0.05$) egg weights than their respective other genotypes. Haplotype association analysis of g.56805646 T>C and exon 3-20G>A showed that the haplotypes were significantly associated with egg weight. Higher egg weight was seen in individuals with H3H4 haplotypes. In the hypothalamus-pituitary-gonadal (**HPG**) axis, the results of qRT/PCR showed that *ESR2* mRNA was significantly ($P < 0.05$) expressed in the ovaries of both duck groups than in the hypothalamus and pituitary. In the oviduct, *ESR2* was significantly ($P < 0.05$) higher in the infundibulum and magnum of laying and non-laying ducks respectively. This study provides a molecular marker for selecting Leizhou black ducks for egg production. In addition, it offers theoretical knowledge for studying the related biological functions of the *ESR2* gene at the cellular level.

Key words: *ESR2*, single nucleotide polymorphism, egg-laying traits, Leizhou black duck

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INTRODUCTION

Estrogens belong to the gonadal steroid hormone family synthesized from cholesterol mainly in the ovaries, granulosa cells, and corpora lutea. However, they are also produced in other nongonadal organs and tissues, including the heart, liver, skin, brain, adipose tissue, and adrenal glands (Knapczyk-Stwora et al., 2008; Cui et al., 2013; Nelson and Habibi, 2013; Fuentes and Silveyra, 2019). In the reproductive system, estrogens regulate oogenesis, ovulation, estrous behavior, uterine propagation, vitellogenesis, endometrial gland secretions, gonadotropin secretions, male and female sex organ development, and secondary sex characteristics (Nelson and Habibi, 2013; Hamilton et al., 2014; Fuentes and Silveyra, 2019). Estrogens' biological and physiological functions are executed by binding to the cognate receptors known as estrogen receptors (**ERs**). The two primary receptors in poultry are estrogen receptor 1 (*ESR1*/ERα/*ER1*) and estrogen receptor 2 (*ESR2*/*ER*β/*ER2*), which belong to the nuclear receptor superfamily (Murphy et al., 1998; Okat, 2018; Chen et al., 2019). The ERs act as transcription factors to initiate gene transcription through estrogen response elements (**EREs**) in the target tissues and interact with other transcription factors (Hall and McDonnel, 1999).

The female reproductive development and performance including ovary, oviduct, ovarian follicle development, egg production performance, and egg quality traits are of much concern to poultry breeders. The ovary is the female reproductive organ responsible for producing and releasing eggs and serves as an endocrine gland to produce and discharge hormones. In addition,

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it regulates the production of proteins and steroid hormones for follicle development, ovulation, estrous cycle maintenance, secondary sex characteristics, and uterus preparation for implantation (Luderer, 2014; Pan et al., 2014; Shimizu, 2016; Zhu et al., 2017; Asiamah Amponsah et al., 2019). Due to its inevitable functions and importance in poultry, several studies have focused on the ovary to identify and scrutinize main and differentially expressed genes (**DEGs**) that regulate its development and functions, including egg production and quality traits (Luan et al., 2014; Y. Wu et al., 2014; Ding et al., 2015; Zhu et al., 2017; C. Cui et al., 2019; Y. ming Cui et al., 2019; Mishra et al., 2020). The functional unit of the ovary is the follicles made up of germ cells (oocytes) and somatic cells (granulosa cells and theca cells) (Charlier et al., 2012; Luderer, 2014). The growth of the follicles is regulated by the hypothalamic (**GnRH**) and pituitary (follicle-stimulating hormone, **FSH** and luteinizing hormone, **LH**) hormones which promote the production of estradiol (main estrogen) by the granulosa cells to enhance the follicle development (Szenci et al., 2006; Zoheir and Ahmed, 2012; Shimizu, 2016).

Traditional breeding and selection methods to maximize the reproductive performance of egg-laying chickens are very slow (Zhu et al., 2017). The detection of single nucleotide polymorphisms (**SNPs**) has helped identify novel genetic markers to more precisely select animals for enhanced egg-production performance. The identification of SNPs in candidate genes and the correlation with egg-laying traits in chickens, geese, and ducks is an important technique used to genetically improve animal selection and production (Kang et al., 2012; Li et al., 2013; Wu et al., 2014, 2018; Kulibaba, 2015; Alsiddig et al., 2017; Mohamed et al., 2017; Niu et al., 2017; Xu et al., 2017; Ye et al., 2017; Feng et al., 2018; Bai et al., 2019)

Leizhou black duck is a duck breed widely distributed in the Leizhou Peninsula in China which has characteristics such as strong adaptability, strong disease resistance, long egg peak duration, early egg age, rich trace elements in eggs, and coarse feeding tolerance (Huang et al., 2014). Genetic diversity is an ideal genetic material for a high-quality local duck population to improve meat and egg performance and environmental adaptability. So far, there have been many reports on the research of Leizhou black duck (Meng et al., 2013, 2014a,b; Tang et al., 2013; Asiamah et al., 2020; Lu et al., 2020; Zou et al., 2019, 2020). However, no study has focused on the polymorphism of *ESR2* and its association with egg-laying traits and the expression profile of ESR2 in various tissues in Leizhou black ducks.

Recently, our study on the ovarian transcriptomic analysis in Leizhou black duck revealed that the *ESR2* gene was involved in hormone regulation in reproduction, and the estrogen signaling pathway related to reproductive performance was enriched (Zou et al., 2020). This suggested that *ESR2* may have a functional role in the reproductive performance of the Leizhou black duck. Thus, this study aimed at evaluating the polymorphism of the *ESR2* gene and its association with egg-laying traits, the distribution pattern of *ESR2* mRNA in the hypothalamic-pituitary-gonadal (**HPG**) axis, oviduct, and nonreproductive organs to identify genetic markers for duck selection to enhance egg production and to ascertain the expression profile of *ESR2* in various tissues of Leizhou black duck.

MATERIALS AND METHODS

Animals, Data Collection, and DNA Preparations

All the animals were maintained and studied following the National Institute of Health (**NIH**) guidelines for care and use of laboratory animals, and all protocols were approved in advance by the Animal Care and Ethics Committee of Guangdong Ocean University of China (No. NXY20160172).

One hundred and one (101) female Leizhou black ducks from the same batch of the F4 generation were obtained from Hengcheng Breeding Professional Cooperative in Potou District, Zhanjiang city. As described in our previous work; all the ducks lived under the same housing, management, and feeding conditions (Asiamah et al., 2020). The selected laying Leizhou black ducks were housed individually in pens and egg-laying traits were measured for marker-trait association analysis. The egg-laying traits included; age at first egg (**AFE**), egg production rate of 50% ducks; bodyweight at first egg (**BWFE**), the weight of ducks at first egg; first egg weight (**FEW**), the weight of the first eggs laid, and egg number at 43 wk (E43W), number of eggs laid from the beginning to the end of 43 wk.

Blood samples were taken from the wings of 101 ducks into a syringe containing 2% EDTA used as an anticoagulant and stored at −80°C for further experiment. Genomic DNA was isolated from each duck's whole blood using Tiangen's blood DNA extraction kit (Beijing Tiangen) following the manufacturer's instructions. The quality of the extracted blood DNA of Leizhou black ducks was detected by 1.5% agarose gel electrophoresis. The UV spectrophotometer was used to detect the concentrations and the OD values of the DNA samples. The concentrations of the samples were about 600 to 800 ng/μL, and the OD value 260/280 was about 1.8. Then, the DNA samples were stored at −20°C for further use.

RNA Extraction and cDNA Synthesis

Four each of adult females laying ducks at 43 wk old and non-laying Leizhou black ducks at 16 wk old were selected and euthanized. A total of 14 tissues were quickly collected into tubes containing liquid nitrogen and stored in a refrigerator at −80°C for later use. The tissues were grouped as reproductive tissues (hypothalamus, pituitary, and ovary), reproductive tract or

oviduct tissues (infundibulum, magnum, isthmus, and uterus), and nonreproductive tissues (heart, liver, spleen, lung, kidney, breast muscle, and leg muscle).

Total RNA was extracted from each tissue using Magzol reagent (Beijing, Quanshijin), following the manufacturer's protocol. The quality and concentrations of the RNA were detected respectively by 1% agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) at 260:280 nm ratio. Then, reverse transcription was performed to synthesize cDNA using PrimeScript RT Reagent kit with gDNA Eraser (Beijing, Quanjin) according to the manufacturer's protocol.

Primer Design

Primers P1−P4 were designed for SNP screening, P5 and P6 were used for quantitative real-time PCR (**RT-qPCR**) analysis of ESR2 gene and duck β-actin gene (internal control) respectively.

All primers were designed using Primer Premier 6.0 (Palo Alto, CA) and synthesized by Sangon Biotechnology (Shanghai, China). The detailed information of all primers used in this study is provided in Table 1.

SNP Selection of Leizhou Black Duck ESR2 Gene

DNA samples from 30 Leizhou black ducks were chosen randomly to construct a DNA pool by mixing the same amount of DNA from each duck in a centrifuge tube. After PCR reaction and sequencing, 4 primers P1−P4 were selected for SNPs screening of 101 Leizhou black ducks (Table 1). The PCR amplification was performed in a 20 μL total reaction volume containing 10 μL 2 × Easy Taq SuperMix (TransGen Biotech, Beijing, China), 8 μL of ddH2O, 0.5 μL of each pair of primers and 1 μL DNA sample. The reaction conditions were denaturation at 95°C for 5 min, 35 PCR cycles (consisting of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s), and a final extension at 72°C for 5 min. The PCR products were detected by electrophoresis through a 1.5% agarose gel and confirming the length, the amplified PCR products were sequenced by a commercial service (Sangon Biotechnology). Finally, through the sequencing peak map returned by the company, each sample was screened for single-base mutations in the ESR2 gene using the Seqman sub-software in DNAstar ver. 7.1.0 software (DNAStar, Inc. Madison, WI).

Haplotype Analysis of SNPs

SNPs with a significant association with egg-laying traits were selected for haplotype analysis using Haploview 4.2 to obtain haplotypes for association analysis. One haplotype was randomly selected to represent haplotype combinations that had the same genotypes. Haplotype combinations that were not seen in ≥3 individuals were removed because they could not be used for the analysis.

Expression Profile of the Leizhou Black Duck ESR2 Gene

According to the ChamQTM SYBR qPCR Master Mix 7750 (Trans, Guangzhou) fluorescence quantification kit, the fluorescence quantification of each sample tissue was performed on the Applied Biosystems StepOnePlus (Foster City, CA) fluorescence quantitative PCR. Three replicates for *ESR2* and β-actin were performed in every tissue. PCR reaction system: 10 μL ChamQTM SYBR qPCR Master Mix, 0.5 μL PCR Forward Primer (0.5 μM), 0.5 μL PCR Reverse Primer (0.5μM), 0.5μL cDNA, 8.5 μL ddH2O, a total volume of 20 μL amplification reaction. Reaction procedure to amplify the template was 95°C, 30 s; 40 cycles (95°C, 10 s; 56°C, 30 s; lighting; 72°C, 25 s); 95°C, 15s; 60°C, 1 min; 95°C, 15 s. The relative expression levels of the genes test were calculated using the $2-\Delta\Delta$Ct method (Schmittgen and Livak, 2008).

Statistical, Genotyping, and Association Analyses

Statistical analyses of ESR2 mRNA expression data (fold changes) in various tissues were analyzed by one-way ANOVA and *t* test using SPSS 13.0 software. The data are presented as the mean ± the standard error of the mean (**SEM**) of each set of three independent

Table 1. *ESR2* gene primer sequence.

Gene	Primer name	Sequence (5'−3')	Annealing temperature (°c)	Product size (bp)	Application
ESR2	P1	**F:** TGTCATTGTACGGCTTATGTTCAC **R:** TTCCAGTCATTGCGAGTGTTTC	60	1149	SNP screening
	P2	**F:** GCATTTCCATTGTTAGGGTGA **R:** AAGCCTTAGGAGCAGGATGA	57	910	
	P3	**F:** GCCAGTATTGGAAACTGATGC **R:** AACCTTGCTCTAATTGCCTTGT	57.7	905	
	P4	**F:** CAATGTCCCATAGCAAGGAGT **R:** GATGCGTAATCACGAACCAG	56.5	1232	
	P5	**F:** CAGTGCTACCTGTGACCAGA **R**: TGCAGCCTTCACATGACCAG	60.0	168	RT/qPCR
B-actin	P6	**F**: CGCAAATGCTTCTAAACC **R**: AGACTGCTGCTGATACCTT	52.0	167	

experiments. A P value of ≤0.05 was considered statistically significant.

All SNP loci were found through the individual sequencing results; genotypes and alleles were recorded and calculated at each SNP site. Each polymorphism was evaluated for Hardy-Weinberg equilibrium using a Pearson's goodness-of-fit chi-square test (degree of freedom = 1). Gene homozygosity (***Ho***), heterozygosity (***He***), the effective number of alleles (***Ne***), and the polymorphism information content (***PIC***) were statistically analyzed using the POPGENE v. 1.32 software (Yeh and Boyle, 1997). In addition, haplotype analysis was performed for SNPs with a significant association with egg-laying traits using Haploview 4.2 software (BROAD, Cambridge, UK) (Barrett et al., 2005). Finally, association analyses of polymorphisms were performed with the measured egg-laying traits using SPSS 13.0 software.

RESULTS

Polymorphisms of Leizhou Black Duck ESR2 Gene (Genotype Frequency, Allele Frequency, Ne, PIC, and Hardy Weinberg's Law)

After PCR amplification and sequencing, 23 SNP sites were finally identified of which 2 SNPs were found in the exon and 21 SNPs in the introns.

The genotype and allele frequencies, Ne, and PIC of the 23 SNP loci of *ESR2* gene were calculated, and Hardy-Weinberg equilibrium was evaluated using the chi-squared test (Table 2). For the locus g. 56800546T>G, the gene frequencies of alleles T and G were 40.1 and 59.9%, respectively. The gene frequency of allele G was higher than that of allele T, making allele G the dominant gene of the population. TT, TG, and GG genotype frequencies were 16.5, 47.2, and 36.3%, respectively. Considering Exon 2-160 C>T locus, the gene frequencies of alleles C and T are 58.3 and 41.7% making allele C higher and dominant over allele T in the population. The genotype frequencies of CC, CT, and TT were 32.3, 52.1, and 15.6%, respectively. Gene homozygosity was higher than the heterozygosity for all the 23 SNP loci, with the number of effective alleles ranging from 1.3 to 2. PIC analysis results indicated that all the SNPs displayed moderate polymorphism (0.30 < PIC < 0.40) except g.56808450 G>A (PIC < 0.25) which showed a low polymorphism. The mean PIC for all the SNPs was 0.36, which is a moderate polymorphism. The chi-square test results indicated that all 23 SNPs were in Hardy-Weinberg equilibrium (Table 2).

Association Analysis Between SNPs of ESR2 Gene and Egg-Laying Traits of Leizhou Black Duck

Association analysis between *ESR2* genotypes and egg-laying traits of Leizhou black duck was performed. The result showed that the SNP g. 56805646 T>C was significantly ($P < 0.05$) associated with egg weight. Furthermore, ducks with CT genotype had significantly ($P < 0.05$) higher egg weight than those with CC genotypes (Table 3). Also, SNP exon 3-20 G>A was associated with egg weight, where individuals with AG genotypes had significantly higher ($P < 0.05$) egg weight than AA genotype ducks (Table 3).

Haplotype Analysis of Single-SNPs of ESR2 Gene of Leizhou Black Duck

Haploview 4.2 software was used for haplotype analysis for the SNPs (g. 56805646 T>C and exon 3- 20 G>A) associated with egg-laying traits. The linkage disequilibrium analysis indicated a high linkage block between g. 56805646 T>C and exon 3- 20 G>A (g. 56808690 A>G) for *ESR2* gene (Figure 1) with 4 different kinds of related data hap 1, hap 2, hap 3, and hap 4 respectively for H1, H2, H3, and H4 and their frequencies. The combined genotype present at the highest frequency was H1 (TG; 0.511), with H2 (CA) being the next most frequent (0.445), followed by H3 (CG; 0.033) and H4 (TA; 0.011) (Table 4). Each of the 4 haplotypes was paired with itself and each other to form 10 combinations. Since H1H2 and H3H4 combinations had the same genotypes, H3H4 was randomly selected to represent both combinations. The data of egg-laying traits of individuals that had the combined haplotypes were used for the association analysis. Haplotype combinations that were not seen in ≥3 individuals were taken out because they could not be used for the analysis.

Association of g. 56805646 T>C and Exon 3-20G>A Haplotype Combinations With Egg-Laying Traits

In the linkage between g. 56805646 T>C and exon 3-20G>A (g. 56808690 G>A) 5 research significant combinations (combinations with the number of individuals greater than or equal to 3) were formed from consecutive SNPs to reveal their association with egg-laying traits. The results showed that the haplotypes were significantly associated with egg weight. Higher egg weight was seen in individuals with H3H4 haplotypes, followed by HIH3, H1H1, H2H3, with the lowest egg weight in H2H2 haplotype individuals. Individuals with haplotype H3H4 had significantly ($P < 0.05$) higher egg weight than H2H2 individuals (Table 5). There was no difference ($P > 0.05$) in the egg weight of H1H1, H1H3, H2H2, and H2H3 individuals. Individuals with H1H1 haplotypes had lower FEA than the others but the difference was not significant ($P > 0.05$). H1H3 individuals had the highest ($P > 0.05$) WFE compared to the other individuals followed by H2H2, H2H3, H1H1, with H3H4 ducks having the lowest WFE. The highest ($P > 0.05$) NE300D

Table 2. Genotype frequency, allele frequency, and Hardy Weinberg's law data of SNPs of ESR2 gene in Leizhou black duck.

	SNP	Genotype frequency	Gene frequency	Effective allele numbers	Homo zygosity	Hetero zygosity	PIC	HWE	
								X^2	P
1	g. 56800546G>T	TT(0.164835) TG(0.472527) GG(0.362637)	T(0.4011) G(0.5989)	1.9247	0.5196	0.4804	0.365027	0.044039	0.833781
2	g. 56800575C>T	CC(0.362637) CT(0.472527) TT(0.164835)	C(0.599) T(0.401)	1.9247	0.5196	0.4804	0.365027	0.044039	0.833781
3	g.56800841A>G	AA(0.351648) AG(0.483516) GG(0.175824)	A(0.5934) G(0.4066)	1.9326	0.5174	0.4826	0.366124	0.833781	0.97318
4	g. 56800870 C>T	CC(0.362637) CT(0.483516) TT(0.153846)	C(0.6044) T(0.3956)	1.9165	0.5218	0.4782	0.363863	0.97318	0.97318
5	g. 56800876G>A	AA(0.164835) AG(0.483516) GG(0.351648)	A(0.4066) G(0.5934)	1.9326	0.5174	0.4826	0.366124	0.00113	0.97318
6	g. 56800878 T>C	CC(0.164835) CT(0.483516) TT(0.351648)	C(0.4066) T(0.5934)	1.9326	0.5174	0.4826	0.366124	0.00113	0.97318
7	g. 56800880 C>T	CC(0.351648) CT(0.483516) TT(0.164835)	C(0.5934) T(0.4066)	1.9326	0.5174	0.4826	0.366124	0.00113	0.97318
8	g. 56801022 G>C	CC(0.164835) GC(0.461538) GG(0.373626)	C(0.3956) G(0.6044)	1.9165	0.5218	0.4782	0.363863	0.148486	0.699986
9	g. 56805646 T>C	CC(0.239583) CT(0.46875) TT(0.291667)	C(0.474) T(0.526)	1.9946	0.5014	0.4986	0.374321	0.407954	0.52301
10	g. 56805648 C>T	CC(0.34375) CT(0.5) TT(0.15625)	C(0.5938) T(0.4062)	1.9321	0.5176	0.4824	0.366056	0.093535	0.759731
11	g. 56805668 T>C	CC(0.145833) CT(0.520833) TT(0.333333)	C(0.4062) T(0.5938)	1.9321	0.5176	0.4824	0.366056	0.531627	0.465924
12	Exon 2- 160 C>T	CC(0.322917) CT(0.520833) TT(0.15625)	C(0.5833) T(0.4167)	1.9459	0.5139	0.4861	0.367959	0.420891	0.516493
13	g. 56805900 G>C	CC(0.15625) CG(0.5) GG(0.34375)	C(0.4062) G(0.5938)	1.9321	0.5176	0.4824	0.366056	0.093535	0.759731
14	g. 56806025 T>A	AA(0.15625) AT(0.510417) TT(0.333333)	A(0.4115) T(0.5885)	1.9392	0.5157	0.4843	0.367037	0.227338	0.633504
15	g. 56806052 T>C	CC(0.15625) CT(0.510417) TT(0.333333)	C(0.4115) T(0.5885)	1.9392	0.5157	0.4843	0.367037	0.227338	0.633504
16	g. 56806132 G>T	TT(0.15625) TG(0.5) GG(0.34375)	T(0.4062) G(0.5938)	1.9321	0.5176	0.4824	0.366056	0.093535	0.759731
17	g. 56806168 G>A	AA(0.15625) AG(0.510417) GG(0.333333)	A(0.4115) G(0.5885)	1.9392	0.5157	0.4843	0.367037	0.227338	0.633504
18	Exon 3- 20 G>A	AA(0.217822) AG(0.49505) GG(0.287129)	A(0.4653) G(0.5347)	1.9904	0.5024	0.4976	0.373793	0.010299	0.919167
19	g. 56808646 A>G	AA(0.287129) AG(0.485149) GG(0.227723)	A(0.5297) G(0.4703)	1.993	0.5018	0.4982	0.374116	0.09859	0.753528
20	g. 56808531A>G	AA(0.376238) AG(0.485149) GG(0.138614)	A(0.6188) G(0.3812)	1.8931	0.5282	0.4718	0.360488	0.055317	0.814057
21	g. 56808450G>A	AA(0.029703) AG(0.217822) GG(0.752475)	A(0.1386) G(0.8614)	1.3137	0.7612	0.2388	0.210272	0.889832	0.345523
22	g. 56810074 C>T	CC(0.27) CT(0.52) TT(0.21)	C(0.53) T(0.47)	1.9928	0.5018	0.4982	0.374098	0.150035	0.698502
23	g. 56810329 C>G	CC(0.26) CG(0.48) GG(0.26)	C(0.5) G(0.5)	2	0.5	0.5	0.375	0.202731	0.652525

Table 3. Association of two (2) SNPs in *ESR2* gene and egg-laying traits of Leizhou black duck.

SNP	Genotypes	Traits (Mean ± SD)			
		FEA	WFE	EW	NE300D
g. 56805646 T>C	CC	141.95 ± 20.00	1330.84 ± 152.30	45.4 ± 9.99[a]	121.86 ± 21.27
	CT	138.79 ± 22.32	1296.61 ± 132.93	50.10 ± 7.43[b]	123.33 ± 26.07
	TT	137.68 ± 23.39	1320.25 ± 102.22	48.7 ± 8.68[ab]	118.91 ± 21.99
Exon 3- 20 G>A	AA	139.47 ± 20.99	1338.99 ± 146.16	45.96 ± 9.97[a]	125.11 ± 24.27
	AG	138.79 ± 22.58	1299.40 ± 130.24	50.53 ± 8.08[b]	122.30 ± 25.18
	GG	135.22 ± 22.38	1323.39 ± 112.36	47.56 ± 7.36[ab]	122.48 ± 22.38

[ab]Different lowercase indicates significant difference ($P < 0.05$).Abbreviations: EW, egg weight; WFE, weight at first egg.

were laid by H1H3 individuals whereas H2H3 individuals had the lowest NE300D (Table 5).

Expression Profile of ESR2 Gene in Various Tissues of Laying and Non-laying Leizhou Black Ducks

To evaluate the expression pattern of *ESR2* in Leizhou black ducks, 14 different tissues were selected from the ducks and detected by RT-qPCR. The results showed that the ESR2 gene was expressed in all the studied tissues. In the reproductive tissues (hypothalamus, pituitary, and ovary) of both laying and non-laying ducks, the *ESR2* gene significantly ($P < 0.01$) expressed in the ovary compared to the other tissues (Figure 2). *ESR2* was significantly ($P < 0.01$) expressed in the pituitary than in the hypothalamus in laying ducks, but no difference ($P > 0.05$) was found in the non-laying ducks for the two tissues (Figures 2A and 2B). Comparatively, there was a significant ($P < 0.01$) expression of the *ESR2* gene in all three tissues of laying ducks than that of non-laying ducks (Figure 2C).

In the oviduct (infundibulum, magnum, isthmus, and uterus), the greatest expression level of *ESR2* was found in the infundibulum compared to other tissues, followed by the uterus and isthmus with the lowest expression level in the magnum in the laying ducks (Figure 3A). There was no significant ($P > 0.05$) difference in the expression of the *ESR2* gene in the infundibulum and uterus. *ESR2* was significantly ($P < 0.01$, $P < 0.05$) expressed in infundibulum than in magnum and isthmus (Figure 3A). *ESR2* was highly expressed ($P < 0.01$) in the uterus compared to the magnum. There was no significant ($P > 0.05$) difference in the expression of *ESR2* between the uterus and isthmus and between the isthmus and magnum (Figure 3A). In non-laying ducks, the highest expression level of *ESR2* was found in the magnum compared to other tissues followed by the infundibulum and uterus with the lowest expression level in the isthmus. *ESR2* significantly ($P < 0.01$) expressed in magnum compared to the three other tissues (Figure 3B). There was no significant ($P > 0.05$) difference in the expression of the *ESR2* gene in the infundibulum, isthmus, and uterus (Figure 3B). Comparatively, there was a significantly higher ($P < 0.01$, $P < 0.05$) expression of the *ESR2* gene in the oviduct of laying ducks than that of non-laying ducks (Figure 3C).

In non-reproductive tissues (heart, liver, spleen, lung, kidney, breast muscle, and leg muscle), the highest ($P < 0.01$) expression level of *ESR2* was found in the spleen compared to other tissues in both laying and non-laying ducks (Figures 4A and 4B). Evident *ESR2* mRNA expression was discovered in the heart, liver, lung, and kidney with lower expression levels in breast and leg muscles. *ESR2* was significantly expressed ($P < 0.01$) in the lung and kidney than in the heart, liver, breast, and leg muscles of laying ducks (Figure 4A). A similar pattern was recorded in non-laying ducks except for the liver which did not differ significantly ($P > 0.05$) from lung and kidney (Figure 4B). Whereas *ESR2* expression

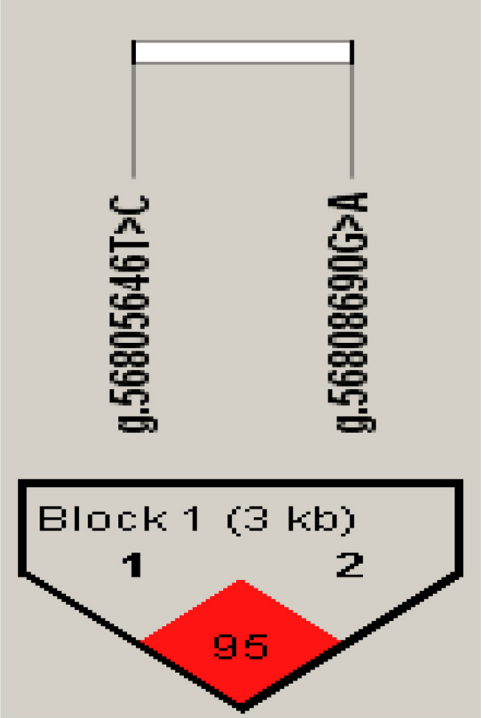

Figure 1. The haplotype between g. 56805646 T>C and g. 56808690 G>A (Exon 3- 20 G>A). The linkage disequilibrium coefficient between mutations (D' and r^2), the numbers are the r^2 value (%)

Table 4. Haplotype frequency g. 56805646 T>C and exon 3-20G>A of *ESR2* gene.

Haplotype	g. 56805646 T>C	g. 56808690 A>G	Frequency
H1	T	G	0.511
H2	C	A	0.445
H3	C	G	0.033
H4	T	A	0.011

Table 5. Association of haplotype combinations (number of individuals ≥3) egg-laying traits.

Haplotypes	Traits (Mean ± SD)			
	FEA	WFE	EW	NE300D
H1H1	135.71 ± 23.30	1313.64 ± 102.31	47.55 ± 7.82[ab]	120.58 ± 22.02
H1H3	137.0 ± 16.97	1358.9 ± 236.88	48.35 ± 0.92[ab]	151.0 ± 5.66
H2H2	141.24 ± 21.09	1332.72 ± 153.54	45.02 ± 9.60[a]	122.59 ± 23.26
H2H3	145.0 ± 16.79	1322.85 ± 169.68	47.0 ± 13.0[ab]	118.75 ± 10.72
H3H4	138.78 ± 23.35	1292.33 ± 126.63	50.73 ± 7.57[b]	121.19 ± 26.8

[ab]Different lowercase indicates significant difference ($P < 0.05$).

was significantly ($P < 0.01$) higher in the liver than in the heart of laying ducks (Figure 4A), it did not differ in non-laying ducks (Figure 4B). A similar pattern was seen in breast and leg muscles in both duck groups (Figure 4A and 4B). Comparatively, there was significantly higher ($P < 0.01$) expression of the *ESR2* gene in all tissues of laying ducks compared to non-laying ducks (Figure 4C).

DISCUSSION

Genetic Polymorphism of ESR2 Gene

To elucidate the possible relationship between the *ESR2* gene and egg-laying traits, we designed 4 different primers and examined SNPs in coding and non-coding regions. Each of the four (primers 1,2,3, and 4) were found to have 8, 9, 4, and 2 SNP sites respectively, a total of 23 SNP sites. Out of the 23 SNP sites, only 2 of them, exon 2-160C>T (primer 5) and exon 3-20G>A (primer 6) were found in the coding region. SNPs mostly occur in the non-coding regions to affect gene splicing, non-coding RNAs, and transcription factor binding (Barreiro et al., 2008); thus, most of the SNPs found in this study were located in the non-coding region. Only 4% of the over 1.4 million SNPs are located in the coding regions with a few causing amino acid changes (Kassam et al., 2005). In this study, the 2 SNPs found in the coding regions caused no effect on the amino acid sequence.

Homozygosity in a population indicates that individuals possess 2 identical forms of a particular gene. In this study, the homozygosity of all the SNP sites identified was higher than the heterozygosity, which may be due

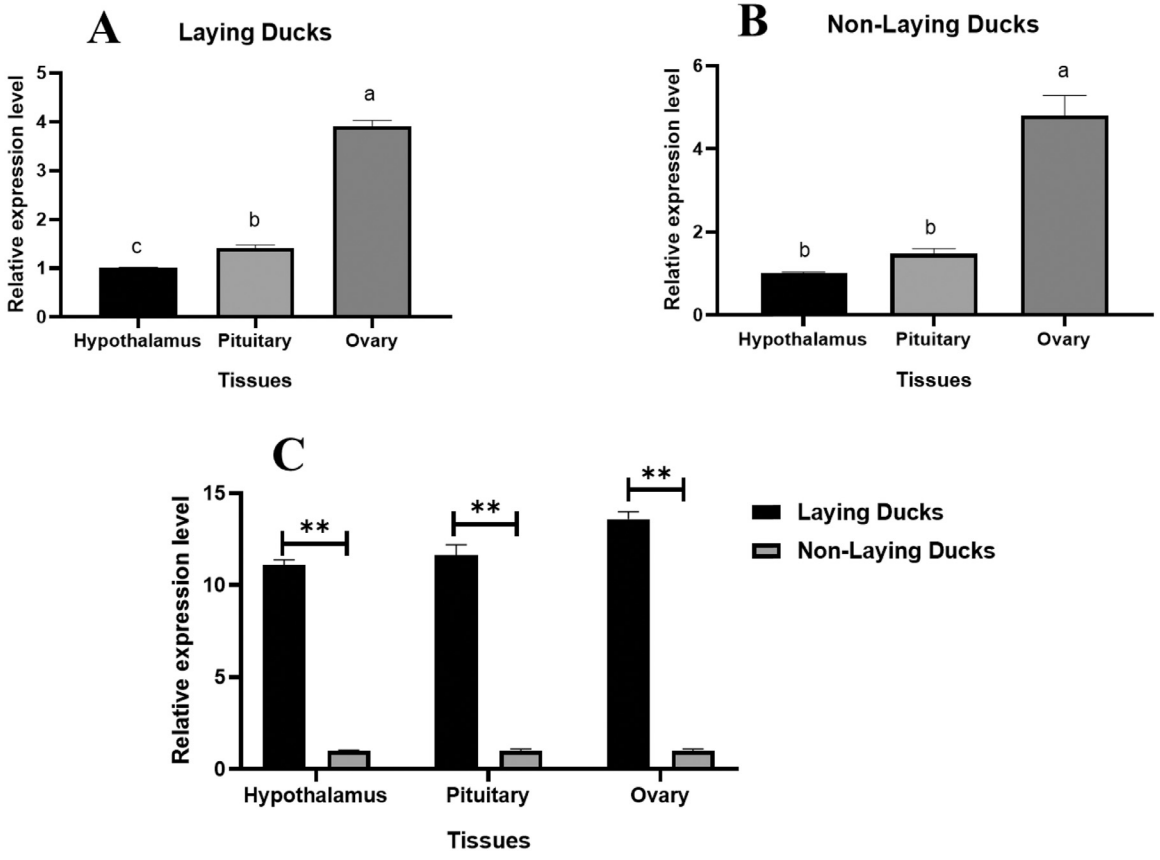

Figure 2. Expression pattern of *ESR2* in the HPG-axis of Leizhou black ducks. (A) Expression pattern of ESR2 in HPG-axis of laying ducks; (B) Expression pattern of ESR2 in HPG-axis of non-laying ducks; (C)comparative expression pattern of *ESR2* in HPG axis of laying and non-laying Leizhou black ducks. In both A and B, ESR2 was significantly expressed in the ovaries than the other tissues. In C, ESR2 was highly expressed in all tissues of laying ducks than non-laying ducks. NB: Different lower cases show a significant difference ($P < 0.01$). ** show an extremely significant difference ($P < 0.01$)

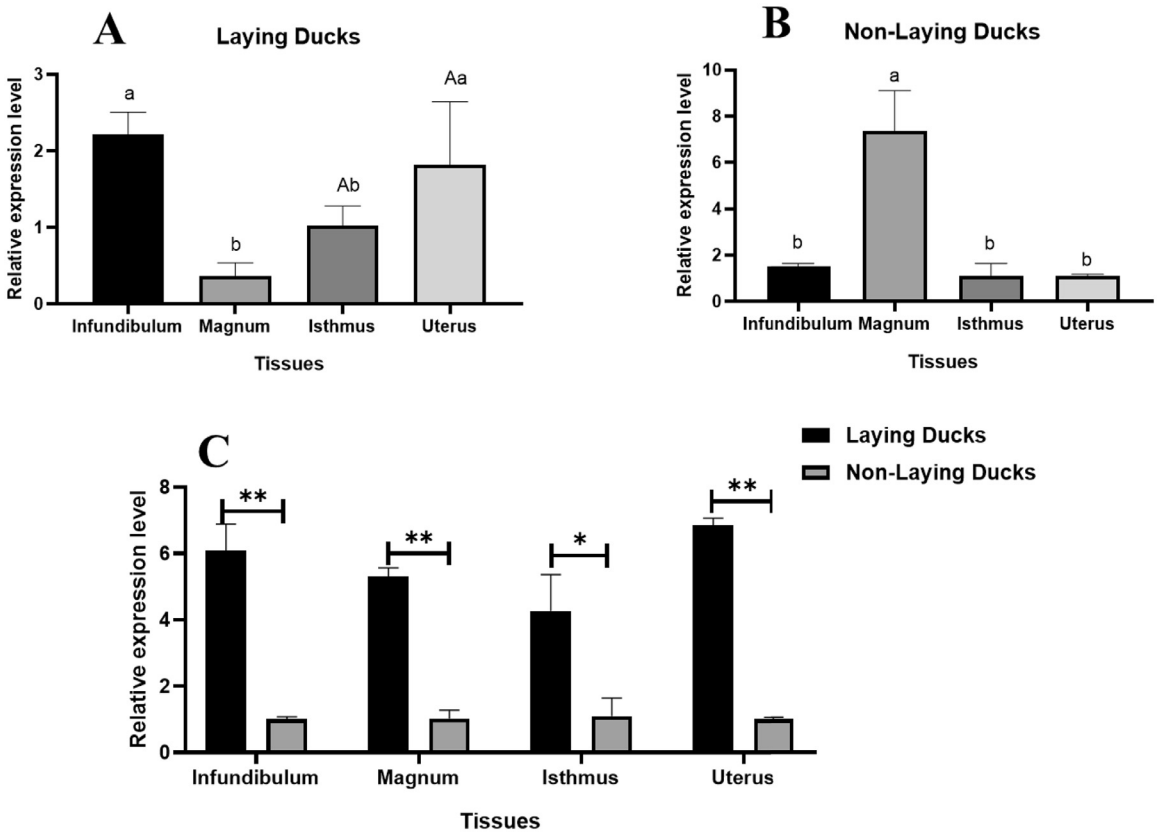

Figure 3. Expression pattern of *ESR2* in the oviduct of Leizhou black ducks. (A) Expression pattern of ESR2 in the oviduct of laying ducks; (B) expression pattern of ESR2 in the oviduct of non-laying ducks; (C) comparative expression pattern of *ESR2* in the oviduct of laying and non-laying Leizhou black ducks. The highest expression levels of ESR2 were found in the infundibulum and magnum of A and B, respectively while in C, the expression level were significantly higher in the oviduct of laying ducks than non-laying ducks. NB: Different lower and upper cases show a significant difference ($P < 0.05$; 0.01); * show a significant difference ($P < 0.05$), ** show an extremely significant difference ($P < 0.01$).

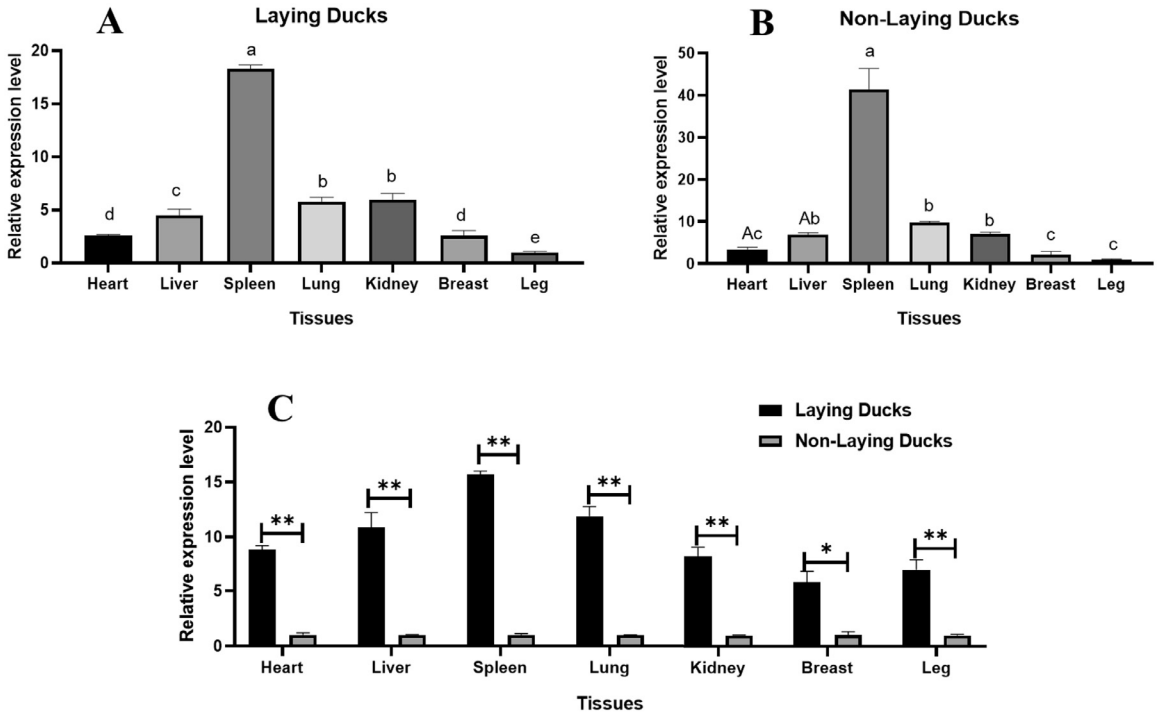

Figure 4. Expression pattern of *ESR2* in various tissues of Leizhou black ducks. (A) Expression pattern of ESR2 in various tissues of laying ducks; (B) expression pattern of ESR2 in various tissues of non-laying ducks; (C) comparative expression pattern of *ESR2* in various tissues of laying and non-laying Leizhou black ducks. The highest expression of ESR2 was seen in the spleen of both A and B and in C, ESR2 was highly expressed in laying ducks than non-laying ducks. NB: Different lower and upper cases show a significant difference ($P < 0.05$; 0.01). * show a significant difference ($P < 0.05$), ** show an extremely significant difference ($P < 0.01$)

to genetic drift that causes loss in genetic diversity due to loss of alleles caused by inbreeding (Oldenbroek and Liesbeth, 2014). Earlier studies have shown that PIC and Ne are important genetic parameters that indicate the level of intrapopulation genetic variation (Abdalhag et al., 2015; Niu et al., 2017). The results of Ne and PIC in this study showed that 22 of 23 SNPs displayed moderate polymorphism with the mean PIC value of 0.36. A study in chickens showed that the *ESR2* gene SNP exhibited a low PIC value of 0.226, lower than that in this study (Niu et al., 2017). Though the allele homozygosity of 22 SNPs was higher than the heterozygosity, it was less than 0.55, signifying that the dominant allele has been moderately subjected to selection. However, allele homozygosity of one SNP (g. 568088450G>A) was higher than 0.7 which indicates that the allele has been subjected to high selection, which was similar to a study on *ESR2* in chicken with high homozygosity of 0.74 (Niu et al., 2017). All the SNPs were found to be in Hardy-Weinberg equilibrium.

Association Analysis Between ESR2 Gene Polymorphism and Egg-Laying Traits

AFE is an essential trait that indicates sexual maturity and egg-laying performance even though it has a negative correlation with the number of eggs laid (Savegnago et al., 2011; Niknafs et al., 2012; Shann-Ren et al., 2018; Tongsiri et al., 2019). In this study, the average AFE of Leizhou black ducks was 20 wk which indicates the sexual maturity of the entire population; thus, EW, WFE, and NE300D were qualified in this study. However, AFE is controlled by polygenes with low to moderate heritability ranging from 0.13 to 0.20, making the traditional breeding method ineffective (Hu et al., 2004; Goraga et al., 2012; Lin et al., 2016). Given this, SNP as a molecular marker is a powerful tool to improve egg production traits.

As reported earlier, estrogens are primarily found in the ovary and regulate several functions of the reproductive system such as ovulation, oogenesis, vitellogenesis, estrous behavior among others (Gustafsson, 2003; Heldring et al., 2007; Nelson and Habibi, 2013; Hamilton et al., 2014) indicating that estrogen participates in egg-laying performance by binding to its receptors. Therefore, *ESR2* may be a possible marker for selecting ducks for egg-laying performance. Several candidate genes such as GH, PRL, OIH, MTNR, FSHR, IGF, and DRD2 have as well been studied to have an association with egg-laying traits in ducks (X. Wu et al., 2014; Xu et al., 2017; Ye et al., 2017; Feng et al., 2018; Wu et al., 2018; Bai et al., 2019) but none is known about polymorphism of *ESR2* and association with egg-laying traits in ducks.

Similar to this study, a previous study in Chinese Dagu chickens showed that the SNP G1755A of the *ESR2* gene was significantly associated with EW at 30 wk. Furthermore, eggs produced by chickens with AG genotype had a higher weight than those with GG genotypes (Niu et al., 2017). This finding indicates that SNPs g. 56805646 T>C and exon 3-20 G>A of the *ESR2* gene may affect egg weight and can be used as novel molecular markers to increase egg weight in Leizhou black ducks.

Haplotype analysis for the 2 single-SNPs that had a significant association with egg weight showed that the region was in linkage disequilibrium. The haplotypes H1 (TG) and H2 (CA) reached 51 and 44%, respectively indicating that the haplotypes may be important for the Leizhou black ducks egg weight trait. Like the current study, an earlier study reported the highest frequency of 56% H1 combined genotype of *ESR1* and *ESR2* (Niu et al., 2017).

Association analysis of the haplotype showed that the haplotype-SNP of *ESR2* was significantly associated with EW. Individuals with haplotype H3H4 had the highest EW compared to the other haplotypes. This haplotype association analysis was consistent with the significant effect detected by the single-SNP association analysis, which was similarly reported in chickens (Niu et al., 2017).

These results demonstrate a strong association between the *ESR2* gene and egg-laying traits and can be used as a marker for selecting Leizhou black ducks for egg production.

ESR2 Distribution Pattern in the HPG Axis of Laying and Non-laying Leizhou Black Ducks

The HPG axis regulates follicle development and ovulation which influence egg-laying performance. GnRH is released from the hypothalamus into the pituitary to excite the production and discharge of gonadotropins, FSH and LH. The gonadotropins then stimulate the growth of follicles and estrogen production by the granulosa cells in the ovary (Szenci et al., 2006; Zoheir and Ahmed, 2012; Shimizu, 2016).

Given this, we focused on the reproduction-related organs; the hypothalamus, pituitary, and ovary to examine the expression pattern of *ESR2* in these organs. The results disclosed that *ESR2* was expressed in all the organs mentioned above. *ESR2* was significantly expressed in the ovary, followed by the pituitary with the lowest in the hypothalamus in both duck groups. Similarly, studies have revealed that *ESR2* was highly expressed in the ovary than in the pituitary and brain of Fathead Minnow fish, goldfish, yellow perch fish, hagfish, and teleost fish (Socorro et al., 2000; Choi and Habibi, 2003; Filby and Tyler, 2005; Lynn et al., 2008; Nishimiya et al., 2017). After feeding Zhedong White Geese with phytoestrogen daidzein to examine its effect on mRNA levels in the HPG axis, *ESR2* was significantly found in the ovary where estrogen is mainly localized (Zhao et al., 2013). Again, when laying geese were fed with dietary energy concentration, estrogen mRNA levels were higher in the ovaries of animals fed with a sufficient energy diet than those fed with deficient energy diets (Liu et al., 2019).

In this study, *ESR2* in the hypothalamus, pituitary, and ovary of laying ducks was significantly higher than in non-laying ducks. This may be because an increase of estrogen levels in the ovary at the end of the follicular phase in laying Leizhou black duck may exert a positive feedback effect on the hypothalamus to trigger a preovulatory GnRH surge which in turn excites secretion of gonadotropins in the pituitary for preovulatory development, maturation and oviposition of follicles in the ovary (Christian and Moenter, 2010; Y. ming Cui et al., 2019; Zhu et al., 2019). After treating ewes with estradiol, there was a significant increase concentration of GnRH receptor mRNA in the hypothalamus to influence pituitary gonadotropins (Turzillo et al., 1998). The expression level of *ESR2* in the ovaries of laying Leizhou black duck in this study was similar to that discovered in the ovaries of Jingjiang and Shaoxing ducks at 500 days old (Y. Wu et al., 2014). The study showed a significantly higher expression of *ESR2* in duck ovaries in all 3 laying stages (age at first egg, 180 d, and 500 d). The level of *ESR2* mRNA increased progressively from age at first egg through to 500 d (Y. Wu et al., 2014). In Zi geese, the expression profile of *ESR2* in the ovaries was unraveled on d 1 and 1, 2, 3, 4, 5, and 8 mo. It was disclosed that *ESR2* was comparatively higher at 1 to 5 and 8 mo than that of d 1 with the greatest expression level at eight months (Kang et al., 2011). This was similar to what was discovered in Leizhou black ducks where *ESR2* expression in the ovaries was higher in laying ducks than non-laying ducks. The highest expression at a later age indicates that *ESR2* plays a vital role in ovarian function, maintenance, and reproduction (Knapczyk-Stwora et al., 2008). *ESR2* levels in laying ducks indicate that *ESR2* may play essential roles in the ovary during follicle development and egg-laying in Leizhou black ducks (Kang et al., 2011). In prepubertal ducks (Anas platyrhynchos), the expression of *ESR2* in the ovary at developmental stages (1-day-old, 30-day-old, 60-day-old, and 90-day-old) was elucidated. It was revealed that *ESR2* mRNA increased gradually from D1 to D60 and declined on D90, suggesting that *ESR2* may mediate the physiological role of estrogen in the ovary and regulate prepubertal follicular development in ducks (Ni et al., 2007). This signifies that *ESR2* is predominantly expressed in the ovaries, primarily localized in the granulosa cells of the follicles essential for follicle development and ovulation (Drummond et al., 2002; Jefferson et al., 2002; Kazeto et al., 2011). The findings in this study demonstrate that the *ESR2* gene may be a predominant and important gene found in the ovaries of Leizhou black duck for egg production.

ESR2 Distribution Pattern in the Oviduct of Laying and Non-laying Leizhou Black Ducks

The oviduct is a complex and dynamic organ that provides a convenient biological environment for fertilizing ovulated oocyte and egg formation. Therefore, it is of much concern to egg producers as an interruption in its activities and pathological changes directly affect egg quality and eventually decrease the economic value of the eggs (Chousalkar and Roberts, 2008). The oviduct is divided into 5 parts: infundibulum, magnum, isthmus, uterus, and vagina, and each has distinctive roles in egg formation and production. Several hormones, proteins, and genes have been identified in the oviduct to regulate the processes and functions of the oviduct in egg formation and production (Brionne et al., 2014; Hrabia et al., 2014; Atikuzzaman et al., 2015; Du et al., 2015; Zhao et al., 2016; Rath et al., 2017; Socha et al., 2017).

Herein, we studied the expression pattern of the *ESR2* gene in four parts of the oviduct excluding vagina in both laying and non-laying ducks. In laying ducks, *ESR2* was highly expressed in the infundibulum followed by the uterus, isthmus, with the least expression in the magnum. The highest expression in the infundibulum may be due to the proximity of the infundibulum to the ovary containing follicles where *ESR2* is primarily localized. A study in mice revealed detectable levels of *ESR2* in the oviduct (Couse et al., 1997) which is consistent with the current studies where *ESR2* was expressed in the parts of the oviduct.

In non-laying ducks, *ESR2* was expressed in all the parts of the oviduct studied with the highest expression in the magnum followed by infundibulum, isthmus, and uterus. Estrogen is essential in the development of young and immature laying chicks. A study revealed that estrogen injection into sexually immature chicks stimulated massive growth in the oviduct (Munro and Kosin, 1943; Seo et al., 2009) and caused an eightfold increase in the wet gain of the magnum in the first three days of treatment which increased to 40 g in laying hens from 1.58 g in young chicks (Palmiter and Wrenn, 1971). In Zebra finch chick, oral administration of estrogen significantly increased the weight of the oviduct compared to the control, and oviduct was differentiated such that they had tubular glands and pseudostratified, ciliated epithelium (Millam et al., 2002). These findings demonstrate that estrogens are involved in the proliferation and differentiation of the oviduct. Estrogens execute their functions by binding to their receptors (Nelson and Habibi, 2013; Chen et al., 2019); thus, the presence of *ESR2* in non-laying ducks shows that *ESR2* regulates proliferation and differentiation of the oviduct.

Comparatively, *ESR2* was highly expressed in all the parts of the oviduct of laying ducks than non-laying ducks. Estrogen induces the expression of ovalbumin, ovostatin, and pleiotrophin responsible for oviduct development and egg formation (Lim et al., 2011), thus the higher levels of *ESR2* in laying ducks than non-laying ducks. In chicken, diethylstilbestrol (**DES**), an analog of estrogen regulated and increased the expression of Alpha 2 macroglobulin (**A2M**) and Serine protease inhibitor B12 (**SERPINB12**) genes in the oviduct of DES-treated chicks. It was observed that these genes were highly expressed in the infundibulum, magnum, isthmus, and uterus of DES-treated chicks compared to control (Lim et al., 2011; Jo et al., 2014).

ESR2 Distribution Pattern in Nonreproductive Organ Systems of Laying and Non-laying Leizhou Black Ducks

Even though estrogen binding to its receptors plays pivotal roles in functions of the reproductive system (Heldring et al., 2007; Carré et al., 2011), we sought to investigate the expression profile of *ESR2* mRNA in 7 different tissues and compare the expression of the gene in tissues of laying and non-laying Leizhou black ducks.

In this study, the tissue distribution of *ESR2* mRNA expression was similar in both duck groups. The expression of the *ESR2* gene was highest in the spleen, followed by kidney, lung, liver, heart, breast, with the least expression in the leg in both duck groups. A previous study that unraveled the genome-wide transcription in rats after oral administration of lavender oil revealed that *ESR2* gene was surprisingly one of the key genes found in the spleen of the rats (Kubo et al., 2015). This finding indicates that *ESR2* plays a function in the spleen of Leizhou black ducks. The different expression patterns of *ESR2* in different tissues have been shown in other studies in fish (Socorro et al., 2000; Choi and Habibi, 2003; Filby and Tyler, 2005; Nishimiya et al., 2017), rats (Kuiper et al., 1997), mice (Couse et al., 1997), and yellow perch (Lynn et al., 2008).

Similar to our findings, a study in teleost fish showed that *ESR2* was higher in the kidney than in the liver, heart, and muscles (Socorro et al., 2000). Contrary to our study, *ESR2* was higher in muscles than in liver and heart in hagfish (Nishimiya et al., 2017). In female goldfish, *ESR2* expression in the liver and heart was not significantly different (Choi and Habibi, 2003) which contrasts with what was recorded in female yellow perch (Lynn et al., 2008) and this study. Contrary to this study, *ESR2* was highly expressed in the liver than in the spleen and kidney in female yellow perch (Lynn et al., 2008).

Comparatively, expression of the *ESR2* gene was significantly higher in all 7 tissues of laying ducks than non-laying ducks. This may be because laying hens are in active egg production, regulated by the ovary where estrogen is primarily located (Carré et al., 2011). Thus *ESR2* mRNA may be linked to function in other tissues as more estrogens are produced during reproduction.

These results provide theoretical knowledge for the in-depth study of the related biological functions of the *ESR2* gene and its application at the cellular level. Also, this study demonstrates a strong association between the *ESR2* gene and egg-laying traits. Therefore, it can be used as a novel molecular marker for selecting Leizhou black ducks for egg production.

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DISCLOSURES

No conflict of interest exists in the submission of this manuscript, and all authors approve it for publication. Furthermore, all authors declare that the work described is original research that has not been published previously, and is not under consideration for publication elsewhere, in whole or in part.

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