# Polymorphism Analysis and Expression Profile of the Estrogen Receptor 2 Gene in Leizhou Black Duck

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**Research**

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

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## Abstract

**Background:** 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. **Method:** 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 (4) each of laying and non-laying Leizhou black ducks were selected to collect different tissues to analyze the ESR2 gene expression.

**Results:** 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 where 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.

**Conclusion:** This study provides molecular marker for selecting Leizhou black ducks for egg production and provides theoretical knowledge for the study of the related biological functions of the *ESR2* gene at the cellular level.

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

## Background

Estrogens belong to the gonadal steroid hormone family synthesized from cholesterol mainly in the ovaries, granulosa cells, and corpora lutea. They are also produced in other non-gonadal organs and tissues including the heart, liver, skin, brain, adipose tissue, and adrenal glands (1–4). 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 (1,3,5). Estrogens' biological and physiological functions are executed by binding to the cognate receptors known as estrogen receptors (ERs). The two main receptors found in poultry are estrogen receptor 1 (*ESR1/*ERα/*ER1*) and estrogen receptor 2 (*ESR2/ERβ*/*ER2*) which are found in the nuclear receptor superfamily (6–8). The ERs act as transcription factors to initiate gene transcription through estrogen response elements (EREs) in the target tissues and also interact with other transcription factors (9).

The female reproductive development and performance which includes 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 the production and release of eggs and serves as an endocrine gland to produce and discharge hormones. It regulates the production of proteins and steroid hormones for follicle development, ovulation, estrous cycle maintenance, secondary sex characteristics, and uterus preparation for implantation (10–14). 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 (11,15–20). The functional unit of the ovary is the follicles made up of germ cells (oocytes) and somatic cells (granulosa cells and theca cells) (12,21). 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 (13,22,23).

Using traditional breeding and selection methods, the reproductive performance of egg-laying ducks has progressively been enhanced, but additional improvement for maximum performance is very slow (11). The detection of single nucleotide polymorphisms (SNPs) has helped with the identification of 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 (24,25,34,35,26–33)

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 (36). As a high-quality local duck population, genetic diversity is an excellent genetic material to improve meat and egg performance and environmental adaptability. So far, there have been many reports on the research of Leizhou black duck (37–44), 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 (44). 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 HPG axis, oviduct, and non-reproductive 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).

A total of 100 female Leizhou black ducks from the same batch of the F4 generation were obtained from Hengcheng Breeding Professional Cooperative in Potou District, Zhanjiang city. All the ducks lived under the same housing, management, and feeding conditions as described in our previous work (41). The selected laying Leizhou black ducks were housed individually in pens and egg-laying traits which 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 weeks (E43W), number of eggs laid from the beginning to the end of 43 weeks were measured to use for marker-trait association analysis.

Blood samples were taken from the wings of 100 ducks into a syringe containing 2% EDTA used as an anticoagulant and stored at -80ºC for further experiment. Genomic DNA was isolated from the whole blood of each duck 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 and the UV spectrophotometer was used to detect the concentrations and the OD values of the DNA samples. The concentrations of the samples were about 600~800 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 weeks old and non-laying Leizhou black ducks at 16 weeks 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 non-reproductive 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, USA) at 260:280nmratio. 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, USA) and synthesized by Sangon Biotechnology (Shanghai, China). The detailed information of all primers used in this study is provided in table 1.

**Table 1. *ESR2* gene primer sequence**

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

**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, four (4) primers P1-P4 were selected for SNPs screening of 100 Leizhou black ducks (Table 1). The PCR amplification was performed in a 20$\mu$L total reaction volume containing 10 $\mu$L 2× Easy Taq SuperMix (TransGen Biotech, Beijing, China), 8 $\mu$L of ddH2O, 0.5 $\mu$L of each pair of primers and 1$\mu$L 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, China). 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., USA).

**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 (USA) fluorescence quantitative PCR. Three replicates for *ESR2* and β-actin were performed in every tissue. PCR reaction system: 10μL ChamQTM SYBR qPCR Master Mix, 0.5μL PCR Forward Primer (0.5μM), 0.5μL PCR Reverse Primer (0.5μM), 0.5μL cDNA, 8.5 μL ddH2O, a total volume of 20μL amplification reaction. Reaction procedure to amplify the template was 95°C, 30 s; 40 cycles (95°C, 10s; 56°C, 30s; lighting; 72°C, 25s);

95°C, 15s; 60°C, 1 min; 95°C, 15s. The relative expression levels of the genes test were calculated using the 2–ΔΔCt method (45).

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

Through the individual sequencing results, all SNP loci were found, genotypes and alleles recorded and calculated at each SNP site with each polymorphism 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 (46). Haplotype analysis was performed for SNPs of each primer using Haploview 4.2 software (BROAD, Cambridge, UK) (47). 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 a total of 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 is higher than that of allele T making allele G the dominant gene of the population. The genotype frequency of TT, TG, and GG 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 frequency 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).

Table 2 here

**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. 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).

**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 |

**NB: Different lowercase indicates significant difference (P < 0.05)**

**Haplotype analysis of single-SNPs of *ESR2* gene of Leizhou black duck**

Haploview 4.2 software was used for haplotype analysis for the SNPs that had an association with egg-laying traits and 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 four (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).

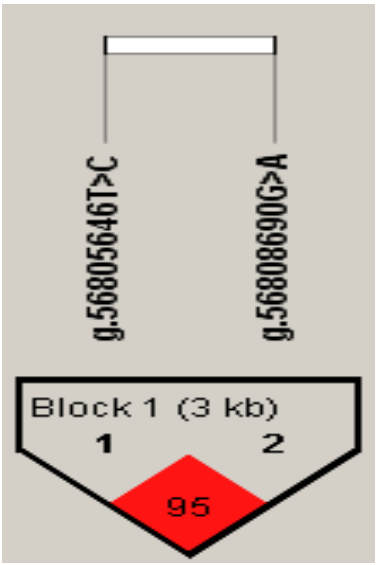


**Figure 1. T**he haplotype between g. 56805646 T>C and g. 56808690 G>A (Exon 3- 20 G>A). The linkage disequilibrium coefficient between mutations (D' and r2), the numbers are the $r^2$ value (%)

**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 |

## 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) five (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 other haplotype individuals 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, and H1H1, with H3H4 ducks have the lowest WFE. The highest ($P > 0.05$) NE300D were laid by H1H3 individuals whereas H2H3 individuals had the lowest NE300D (Table 5).

**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 |

**NB: Different lowercase indicates significant difference (P < 0.05)**

**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, fourteen (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* significantly (P < 0.05) was 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 (Figure 2A and B). 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).

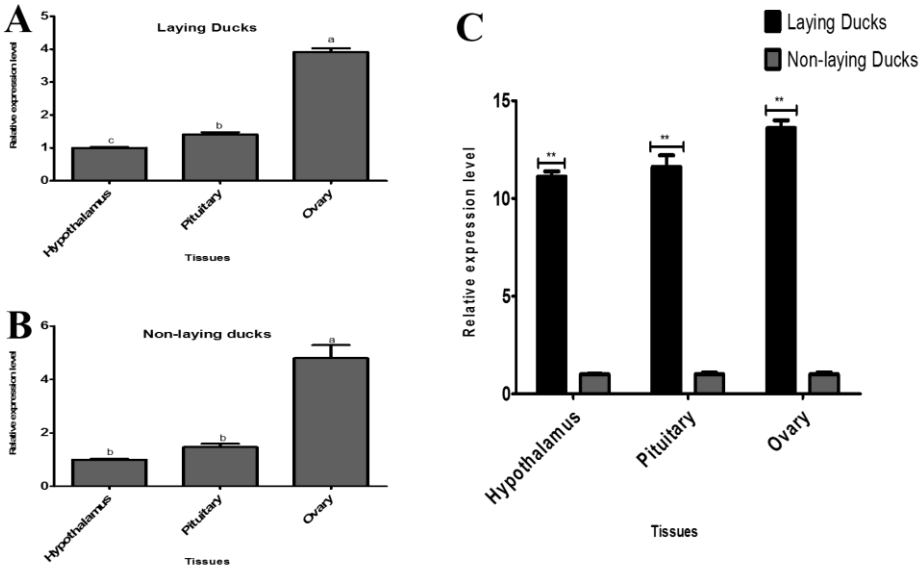

**Figure 2. Expression pattern of *ESR2* in the HPG of laying and non-laying Leizhou black ducks.**

**NB: A- expression pattern in laying ducks; B- expression pattern in non-laying ducks; C- comparative expression pattern of *ESR2* in HPG axis of laying and non-laying Leizhou black ducks. Different lower cases show a significant difference (P < 0.05; 0.01). ** show an extremely significant difference (P < 0.01)**

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 significant ($P < 0.01$) expression of the *ESR2* gene in the infundibulum, magnum, and uterus of laying ducks than that of non-laying ducks. Also, *ESR2* was highly ($P < 0.05$) expressed in the isthmus of laying ducks than that of non-laying ducks (Figure 3C).

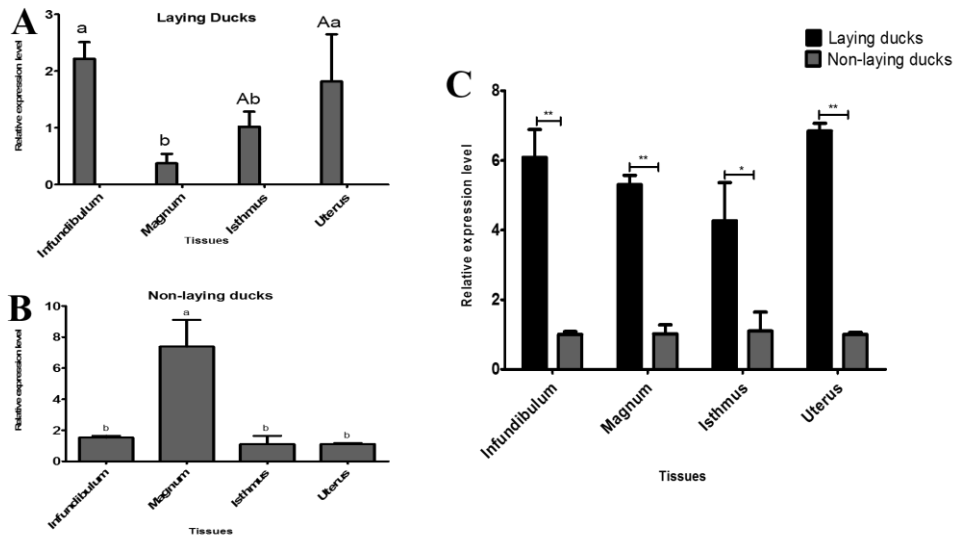


**Figure 3. Expression pattern of *ESR2* in the oviduct of laying and non- laying Leizhou black ducks.**

**NB: A- expression pattern in laying ducks; B- expression pattern in non-laying ducks; C- comparative expression pattern of *ESR2* in the oviduct of laying and non-laying Leizhou black ducks. Different lower and upper cases show a significant difference (P < 0.05); * show a significant difference (P < 0.05), ** show an extremely significant difference (P < 0.01).**

In non-reproductive tissues (heart, liver, spleen, lung, kidney, breast muscle, and leg muscle), the highest expression level of *ESR2* was found in the spleen compared to other tissues in both laying and non-laying ducks followed by the lung (Figure 4A and B). Obvious *ESR2* mRNA expression was discovered in the heart, liver, and kidney with lower expression levels in breast and leg muscles. In laying ducks, the *ESR2* gene was significantly ($P < 0.01$) expressed in the spleen compared to the other tissues except for the lung (Figure 4A). *ESR2* was significantly expressed ($P < 0.05$) in the lung and heart compared to the breast and leg muscles. There was no significant ($P > 0.05$) difference in the expression of *ESR2* in the liver, kidney, breast, and leg

muscles (Figure 4A). In non-laying ducks, there was a significant difference ($P < 0.01$) in the expression level in the spleen compared to the other tissues except for the lung. Also, the *ESR2* gene was significantly ($P < 0.05$) higher in the lung and heart than breast and leg muscles (Figure 4B). There was no difference ($P > 0.05$) in the expression level in liver, kidney, breast, and leg muscles. Comparatively, there was significant ($P < 0.01$) expression of the *ESR2* gene in all tissues of laying ducks compared to non-laying ducks (Figure 4C).

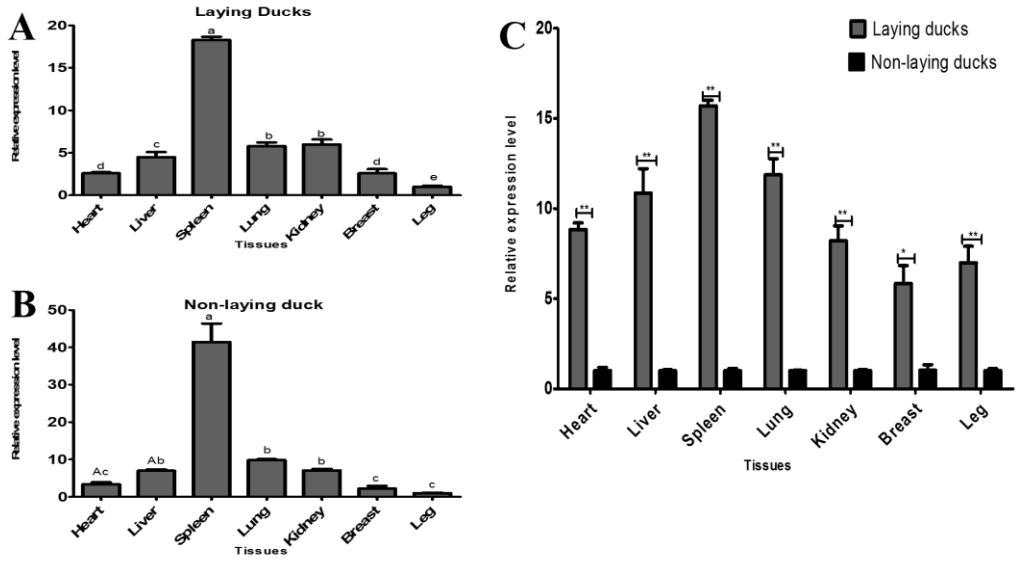


**Figure 4. Expression pattern of *ESR2* in various tissues of laying and non-laying Leizhou black ducks.**

**NB: A- expression pattern in laying ducks; B- expression pattern in non-laying ducks; C- comparative expression pattern of *ESR2* in various tissues of laying and non-laying Leizhou black ducks. Different lower and upper cases show a significant difference ($P < 0.05$). * show a significant difference ($P < 0.05$), ** show an extremely significant difference ($P < 0.01$)**

## Discussion

### Genetic polymorphism of *ESR2* gene

To elucidate the possible relationship between the *ESR2* gene with egg-laying traits, we designed four (4) different primers and examined SNPs in both coding and non-coding regions. Each of the four (primers 1,2,3, and 4) were found to have eight (8), nine (9), four (4), and two (2) SNP sites respectively, a total of 23 SNP sites. Out of the 23 SNP sites, only two (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 (48), 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 change in the amino acid (49). In this study, the two SNPs found in the coding regions caused no effect on the amino acid sequence.

In this study, for SNP at the locus g. 56800546G>T, the allele frequency of allele G was higher than that of allele T and the genotype frequency of GG was higher than that of TT. For locus g. 56805646 T>C, the allele frequency of T was higher than that of allele C and the genotype frequency of TT was higher than that of CC. For locus exon 3-20 G>A, the allele frequency of G was greater than that of allele A and the genotype frequency of GG was greater than that of AA. Also for locus g. 56810074 C>T of primer 7, allele C had higher allele frequency than allele T and CC genotype frequency was higher than TT.

Homozygosity in a population indicates the size of allele frequencies. In this study, the homozygosity of all the SNP sites identified was higher than the heterozygosity which may be due to genetic drift that causes loss in genetic diversity due to loss of alleles caused by inbreeding (50).

Earlier studies have shown that PIC and Ne are important genetic parameters that show the level of intra-population genetic variation (33,51). The results of Ne and PIC in this study showed that 22 out 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 which was lower than that in this study (33). Even 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 which reported high homozygosity of 0.74 (33). All the SNPs were found to be in Hardy-Weinberg equilibrium.

**Association analysis between *ESR2* gene polymorphism and egg-laying traits**

Age at first egg (AFE) is an important trait that indicates sexual maturity and egg-laying performance even though it has a negative correlation with the number of eggs laid (52–55). In this study, the average AFE of Leizhou black ducks was 20 weeks 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 (56–58). 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 (3,5,59,60) 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 (24,28–32) but none is known about polymorphism of *ESR2* and association with egg-laying traits in ducks.

In this study, two (2) SNPs g. 56805646 T>C and exon 3-20 G>A of *ESR2* were significantly associated with EW in Leizhou black ducks. Ducks with CT genotype (56805646 T>C) had the highest egg weight than ducks with CC and TT genotypes. Also, ducks with AG genotype (exon 3-20 G>A) produced eggs with the highest weight than those with AA and GG genotypes.

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 weeks. Eggs produced by chickens with AG genotype had a higher weight than those produced by chickens with GG genotypes (33). 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 two single-SNPs that had a significant association with egg weight showed that the region was in linkage disequilibrium. The frequencies of 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. Similar to the current studies, an earlier study reported the highest frequency of 56% H1 combined genotype of *ESR1* and *ESR2* (33).

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 (33).

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 hypothalamic-pituitary-gonadal (HPG) axis of laying and non-laying Leizhou black ducks

The HPG axis regulates follicle development, 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 the production of estrogen by the granulosa cells in the ovary (13,22,23).

Given this, we focused on the reproduction-related organs which are 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 above-mentioned organs. In both duck groups, *ESR2* was significantly expressed in the ovary followed by the pituitary with the lowest in the hypothalamus. Similarly, a study 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 (61–65). 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 (66). 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 (67).

In this study, *ESR2* in the hypothalamus, pituitary, and ovary of laying ducks were significantly higher than that 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 (15,68,69). After treating ewes with estradiol, there was a significant increase concentration of GnRH receptor mRNA in the hypothalamus to influence pituitary gonadotropins (70). 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 (20). The study showed a significantly higher expression of *ESR2* in duck ovaries in all three laying stages (age at first egg, 180 days, and 500 days). That is, the level of *ESR2* mRNA increased progressively from age at first egg through to 500 days (20). In Zi geese, the expression profile of *ESR2* in the ovaries was unraveled on days 1 and 1, 2, 3, 4, 5, and 8 months. It was disclosed that *ESR2* were comparatively higher at 1 to 5 and 8 months than that of day 1 with the greatest expression level at 8 months (71) and 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 (4). *ESR2* levels were higher in laying ducks indicate that *ESR2* may play essential roles in the ovary during follicle development and egg-laying in Leizhou black ducks (71). 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 decline on D90 suggesting that *ESR2* may mediate the physiological role of estrogen in the ovary and regulate prepubertal follicular development in ducks (72). 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 (73–75). 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 the fertilization of ovulated oocyte and egg formation. 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 (76). The oviduct is divided into five (5) parts which are 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 (77–83).

Herein, we studied the expression pattern of the *ESR2* gene in four parts of the oviduct which are infundibulum, magnum, isthmus, and uterus 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 (84) 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 (85,86) 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 (87). In Zebra finch chick, oral administration of estrogen greatly increased the weight of the oviduct compared to the control and oviduct was differentiated such that they had tubular glands and pseudostratified, ciliated epithelium (88). These findings demonstrate that estrogens are involved in the proliferation and differentiation of the oviduct. Estrogens execute their functions by binding to their receptors (3,6), 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 (89), thus the higher levels of *ESR2* in laying ducks than non-laying ducks. In chicken, diethylstilbestrol (DES), an analog of estrogen-regulated ovostatin gene to increase its expression in the oviduct of DES-treated chicks. It was observed that ovostatin was highly expressed in the infundibulum, magnum, and isthmus (89).

### *ESR2* distribution pattern in non-reproductive 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 (60,90), we sought to investigate the expression profile of *ESR2* mRNA in seven (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,

456 breast with the least expression in the leg in both duck groups. Similar to our study, a previous
457 study identified the *ESR2* gene in rats as the ninth ranking molecule and in network 1 as a central
458 molecule that mediates transcriptional activation (91). This finding indicates that *ESR2* plays a
459 function in the spleen of Leizhou black ducks. The different expression patterns of *ESR2* in
460 different tissues have been shown in other studies in fish (61,62,64,65), rats (92), mice (84), and
461 yellow perch (63).

462 Similar to our findings, a study in teleost fish showed that *ESR2* was higher in the kidney than
463 in the liver, heart, and muscles (65). Contrary to our study, *ESR2* was higher in muscles compared
464 to that of the liver and heart in hagfish (64). In female goldfish, *ESR2* expression in the liver and
465 heart was not significantly different (62) which is in contrast with what was recorded in another
466 study in female yellow perch (63) and this study. Contrary to this study, *ESR2* was highly
467 expressed in the liver than in the spleen, kidney, muscle, and heart in female yellow perch (63).

468 Comparatively, expression of the *ESR2* gene was significantly higher in all seven (7) tissues of
469 laying ducks than non-laying ducks. This may be because laying hens are in active egg production
470 which is regulated by the ovary where estrogen is primarily located (90), thus *ESR2* mRNA may
471 have a link to function in other tissues as more estrogens are produced during reproduction.

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

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## Abbreviations

ESR2: Estrogen receptor 2; SNPs: single nucleotide polymorphisms; HPG-axis: hypothalamus, pituitary and gonadal axis; AFE: age at first egg; BWFE: bodyweight at first egg; FEW: first egg weight; E43W: egg number at 43 weeks.

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## Authors' contribution

**CAA:** Conceptualization, design, experimentation, data curation and analysis, writing-original draft; writing-review & editing. **YL:** Experimentation, methodology, data curation and analysis, software. **RY:** Experimentation and data curation. **YP**: Experimentation and data curation**. LL:** Experimentation, methodology, data curation and analysis, software. **YS:** Conceptualization, funding acquisition, methodology, project administration; supervision; writing-review & editing. **ZZ:** Funding acquisition, project administration, supervision; Writing-review & editing. All authors read and approved the final manuscript.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Declarations

### Ethics approval and consent to participate

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).

### Consent for publication

Not applicable.

### Competing Interest

The authors declare that they have no competing interests.

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772 **Table 2. Genotype frequency, allele frequency, and Hardy Weinberg's law data of SNPs of**

773 **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) | C(0.5934) T(0.4066) | 1.9326 | 0.5174 | 0.4826 | 0.366124 | 0.00113 | 0.97318 |

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|---|---|---|---|---|---|---|---|---|---|
| | | TT(0.164835) | | | | | | | |
| 8 | g. 56801022 G>C | CC(0.164835)<br>GC(0.461538)<br>GG(0.373626) | C(0.3956)<br>G(0.6044) | 1.9165 | 0.5218 | 0.4782 | 0.363863 | 0.148486 | 0.699986 |
| 9 | g. 56805646 T>C | CC(0.239583)<br>CT(0.46875)<br>TT(0.291667) | C(0.474)<br>T(0.526) | 1.9946 | 0.5014 | 0.4986 | 0.374321 | 0.407954 | 0.52301 |
| 10 | g. 56805648 C>T | CC(0.34375)<br>CT(0.5)<br>TT(0.15625) | C(0.5938)<br>T(0.4062) | 1.9321 | 0.5176 | 0.4824 | 0.366056 | 0.093535 | 0.759731 |
| 11 | g. 56805668 T>C | CC(0.145833)<br>CT(0.520833)<br>TT(0.333333) | C(0.4062)<br>T(0.5938) | 1.9321 | 0.5176 | 0.4824 | 0.366056 | 0.531627 | 0.465924 |
| 12 | Exon 2- 160 C>T | CC(0.322917)<br>CT(0.520833)<br>TT(0.15625) | C(0.5833)<br>T(0.4167) | 1.9459 | 0.5139 | 0.4861 | 0.367959 | 0.420891 | 0.516493 |
| 13 | g. 56805900 G>C | CC(0.15625)<br>CG(0.5)<br>GG(0.34375) | C(0.4062)<br>G(0.5938) | 1.9321 | 0.5176 | 0.4824 | 0.366056 | 0.093535 | 0.759731 |
| 14 | g. 56806025 T>A | AA(0.15625)<br>AT(0.510417)<br>TT(0.333333) | A(0.4115)<br>T(0.5885) | 1.9392 | 0.5157 | 0.4843 | 0.367037 | 0.227338 | 0.633504 |
| 15 | g. 56806052 T>C | CC(0.15625)<br>CT(0.510417)<br>TT(0.333333) | C(0.4115)<br>T(0.5885) | 1.9392 | 0.5157 | 0.4843 | 0.367037 | 0.227338 | 0.633504 |
| 16 | g. 56806132 G>T | TT(0.15625)<br>TG(0.5) | T(0.4062)<br>G(0.5938) | 1.9321 | 0.5176 | 0.4824 | 0.366056 | 0.093535 | 0.759731 |

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|---|---|---|---|---|---|---|---|---|---|
| | | GG(0.34375) | | | | | | | |
| 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 |

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