Original Article

Association of LOC101800257 gene with eggshell color in Leizhou black duck

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Abstract

LOC101800257 is a newly screened gene associated with eggshell color. The aim of this study was to explore association of LOC101800257 with eggshell color in Leizhou black duck. The real-time quantitative Polymerase Chain Reaction (RT-qPCR) was performed for expression pattern of LOC101800257 in the liver, oviduct and uterus of Leizhou black duck. The highest expression level of LOC101800257 was found in the liver and nearly no expression in other tissues. There were significant differences (P<0.05) in the expression between blue shell ducks (BSD) and white shell ducks (WSD) in the liver. There was fluctuation in the general expression of LOC101800257 from 27 to 59 weeks in the liver, with a significant turning point reported at 43 weeks. In addition, Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) was used to detect the single nucleotide polymorphisms (SNP) of LOC101800257. Two SNPs were identified in LOC101800257 (c.1406A>G and c.1642+16A>G) and both SNPs showed extensive genetic polymorphisms and were in Hardy-Weinberg non-equilibrium, and the GGGG combined genotypes were significantly correlated with the eggshell color (P<0.05). The above results suggested LOC101800257 may be involved in regulation of eggshell color through its function in the liver, and the GGGG combined genotype can serve as a novel genetic maker for eggshell color.

Keywords: Eggshell color, expression pattern, Leizhou black duck, LOC101800257, SNP

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Introduction

The Leizhou black duck is a famous local duck distributed in the Leizhou Peninsula of Guangdong Province of China with a high production rate, a large egg weight and a high rate of blue eggshell (Xu *et al*., 2016). The average thickness of egg weight, eggshell, egg yolk weight, protein height and Haugh unit of blue shell egg are higher than the parameters of white shell eggs in the Leizhou black duck (Huang, 2016). The Leizhou black duck does not only show excellent traits in blue shell laying but also blue shell eggs are more nutritious. A study has observed that customers prefer blue shell eggs (Dalirsefat, 2015). It has been reported that the color of the eggshell is related to eggshell quality (Gervais *et al*., 2016; Sirri, 2018). Aygun (2014) found that eggshell color was associated with eggshell strength and thickness. A correlation has been shown between eggshell color and the damage rate of eggs (Radwan *et al*., 2015). The eggshell color as an indicator to evaluate egg and eggshell quality should be considered important (Kim *et al*., 2014; Liang *et al*., 2017).

The LOC101800257 is one of the solute carriers (SLC) organic anion transporter polypeptide (OATP) family members and its encoded protein is OATP-1C1. It is a novel solute transporter polypeptide gene of duck based on the research of the chicken blue shell candidate gene SLCO1B3 (Hagenbuch *et al*., 2004). According to sequence alignment analysis, the LOC101800257 perform 70% homology with the SLCO1B3 and SLCO1C1 genes, indicating that they may have similar functions (Law *et al*., 2004). Moreover, LOC101792412, LOC101798111 and LOC101800520 genes were not only related to the color formation of duck egg shell, but also participated in the regulation of the calcium ion concentration in the calcium channel and affected the strength of egg shell through the transcriptome analysis of the glandular tissues of BSD and WSD (Liang, 2017). Wang *et al.* (2013) found that the insertion of EAV-HP in the promoter region of SLCO1B3 gene on chromosome 1 of hens made the SLCO1B3 gene specifically expressed in the egg glands of hens, indicating the specific formation mechanism of blue eggshells. Zhao *et al.* (2017) detected blue-shell in Dongxiang and White Leghorn chickens potential selected regions (PRS) by Eigen GWAS and efficient mixed-model association expedited methods, Gene Ontology and Quantitative Trait Locus analysis, revealed that there were a large number of SLCO1B3 genes in the PRS of Dongxiang chickens. They also found six related amino acids, lipid metabolism and signal transduction pathways. A study by Xu *et al.* (2018) analyzed the mRNA and miRNA expression profiles of ducks and found that there were 124 differential expressed genes between BSD and WSD, which were involved in ATP-binding cassette and the solute carrier. Currently, the research on eggshell color mainly focuses on SLCO1B3, SLCO2B1 and SLCO1C1, while the LOC101800257 as a candidate gene for duck eggshell color has less been documented. Therefore, this study used the Leizhou black ducks as research materials to explore the relationship between LOC101800257 and eggshell color, aiming to provide theoretical basis for the researches of poultry eggshell color.

Materials and Methods

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

Experimental animals: 120 female ducks (BSD 60, WSD 60) of the same heath, size, weight, nutrition level and feeding condition were provided by the Hengcheng Breeding Cooperative (Guangdong, China). All ducks were transferred to individual cage in a semiconfined house.

RNA isolation and RT-qPCR: Five each of BSD and WSD tissues including the uterus, oviduct and liver, were sampled at 27, 35, 43, 51 and 59 weeks. Total RNA from the above tissues was isolated by TRIZON Reagent kit (TAKARA, Dalian) to explore the expression profile of Leizhou black duck LOC101800257. The RNA quality was evaluated by 1.2% agarose electrophoresis and then was reverse transcribed by Prime Script TMRT reagent Kit with cDNA Eraser (TAKARA, Dalian) to synthetize cDNA. The cDNA was used as template to amplify the coding region of the LOC101800257. The RT-qPCR was performed according to YBR FSAT qPCR Kit (TAKARA, Dalian). The reaction procedure of RT-qPCR was 94°C, 35s; followed by 40 cycle (94°C, 20s; 57°C, 35s; lighting; 72°C, 25s)；95°C, 15s; 60°C, 1 min; 95°C, 15s. Primers for LOC101800257 and β-actin gene for RT-qPCR were designed by Primer Premier 5.0 (Table 1), according to the mRNA sequence (GenBank accession XM_005023034.1) of the Anas platyrhynchos LOC101800257 and β-actin gene (GenBank accession EF667345.1) published in GenBank.

DNA isolation and SNPs screening: The DNA from 120 female ducks at 27 weeks was extracted from 1 ml blood and was sampled to filter the SNPs of the Leizhou black duck LOC101800257. The 10 primers (Table 1) were designed by Primer Premier 5.0 to identify the SNPs of LOC101800257, according to complete sequence of LOC101800257 (GenBank accession NW_004677350). Randomly the DNA samples from 30 BSD and 30 WSD were selected for building mixed pools. The PCR amplification was conducted by the mixed pools as a template to amplify the exon and intron of LOC101800257. The reaction system of PCR contained 2 μl cDNA, 1 μl forward primer, 1 μl reverse primer, 25 μl Premix Taq (TAKARA, Dalian) and 21 μl double distilled water. PCR productions were detected by 1% agarose electrophoresis and then were carried out to purify and bi-directional sequence. The results of sequence were analyzed by Chromas and Blast software to screen the SNPs.

Table 1 The primer sequences for this study

Gene	Primer name	Primer sequence (5′ to 3′)	Fragments size (bp)	Annealing Temp (℃)	Application
LOC101800257	R1	F:CCGCTGGGTGGGTGAATGGT R:GTATGTGTCTTCCTGATGGCT	175	60	RT-qPCR
β-actin	R2	F: CGCAAATGCTTCTAAACC R:AGACTGCTGCTGATACCTT	167	60	Internal control
	P1	F: GAGCCAAACCTTTCCAGTG R:GTAATGCCAAATCTTGAATGAG	372	55	
	P2	F: CCAGTTTTCCTGTCCCTA R: TTTGTTGTCCAGAAGTTGAT	620	54	
	P3	F: TTTGTCACTATTCCCCATTCA R: CTTTCACTTTACCTCTGCTTCA	450	55	
	P4	F: GAAGCATCACTATCACTCCAC R: AAAACACTTGCCTTTCATCAT	257	60	
	P5	F: ACAACCCAAGGATTAGGC R: AGGCTGACACTCTGATGAC	500	54	Screening SNP
	P6	F: GGGCTTGTGAGATGGAT R: AAGGTCTATGTCTTCTATGCTG	457	54	
	P7	F: AGGTCCCACAAAATCCGTT R: GGAAAAGTTCAATCATCAGC	251	57	
	P8	F: GAGGGTATCCCCGCTTGA R: TGACAGACCGAGGAGTTTC	350	55	
	P9	F: CTTACTTGCTTGCGTTCCTTT R:AGATTTACTACCAGTTTGCCTTTC	298	57	
	P10	F: AAAAGTCTCCCTTAGCATTCTCG R: AAAAGTCTCCCTTAGCATTCTCG	265	59	
	M1	F: ACAAGCCAGGGACATCAGC R:TGGTTCTCACAGCCAACAAAA	255	60	Genotyping
	M2	F: CAAATGTGCCACCAATGT R: CTATGTCTGCTATGCTGTTCA	267	55	

SNP, single nucleotide polymorphism; F, forward primer; R, reverse primer; RT-qPCR, real-time quantitative Polymerase Chain Reaction;

PCR-SSCP for genotyping: According to screening SNPs loci, the primers (Table 1) about 150~300bp DNA fragments were designed by Primer Premier 5.0. PCR amplification was carried out to amplify 150~300bp DNA fragments. PCR productions were detected by 1% agarose electrophoresis and then were genotyped by 10% polyacrylamide gel electrophoresis. The bands from genotyping were recycled by one lyse plasmid kit (Megen, Guangzhou) and transformed with T-vector to clone the DNA fragments. Finally, recombinant plasmids were isolated by one lyse plasmid kit (Megen, Guangzhou). The cloning fragments were sequenced and analyzed in Blast.

Data analysis: This study obtained data statistics of RT-qPCR whose method was referenced to 2-△△CT. The general linear model was used for analyzing the correlation SSCP genotype of LOC101800257 with the eggshell color of Leizhou black duck. The linear model is Yij = μ+G i +e ij (Yij, observation of different breeding traits; μ, average value of all population; G i, the effect of each genotype; e ij, random error).

Results

Expression pattern of LOC101800257 in BSD and WSD: The tissue expression level at 43 weeks is shown (Figure 1) due to peak laying days. The results show that LOC101800257 was expressed in most tissues, the highest expression was in the liver, the second expression was in the uterus and the lowest expression in the oviduct. Comparing the LOC101800257 expression levels of the same tissues between BSD and WSD, the BSD had higher expressions than WSD. There was a significant difference between BSD and WSD of the expression in the liver (P<0.05) while no significant difference was found in the uterus and oviduct (P>0.05). In addition, there was an extremely significant difference between the expression in uterus and oviduct and expression in the liver between BSD and WSD (P<0.01). The results show that LOC101800257 was not involved in major controls in the uterus and oviduct. Therefore, this study explored the temporal expression pattern of LOC101800257 in the liver.

This study examined the expression level of BSD and WSD LOC101800257 in the liver at 27, 35, 43, 51 and 59 weeks of experiment which displayed that there was fluctuation in the general expression level (Figure 2). In the BSD group, the highest expression of LOC101800257 was at 27 weeks (0.385), dropping to the lowest at 43 weeks (0.022), and then rising to 59 weeks (0.251). There was an extremely significant difference between 27 weeks and 43 weeks (P<0.01). In WSD group, the expression of LOC101800257 was 0.085 at 27 weeks, declining to the lowest at 43 weeks (0.006) and increasing to the highest at 59 weeks (0.103). There was a significant difference between 43 weeks and 59 weeks (P<0.05). The temporal expression trends of LOC101800257 in BSD and WSD declined from 27 weeks to 43 weeks and then increased from 43 weeks to 59 weeks, with 43 weeks as the lowest point. The LOC101800257 expression of BSD was higher than WSD at 27, 35, 43, 51 and 59 weeks of experiment, which was found by comparing BSD with WSD at

same weeks of experiment. There was a significant difference in the relative expression between the 2 groups at 27 weeks and 43 weeks (P<0.05), but no significant difference at 35, 51 and 59 weeks (P>0.05).

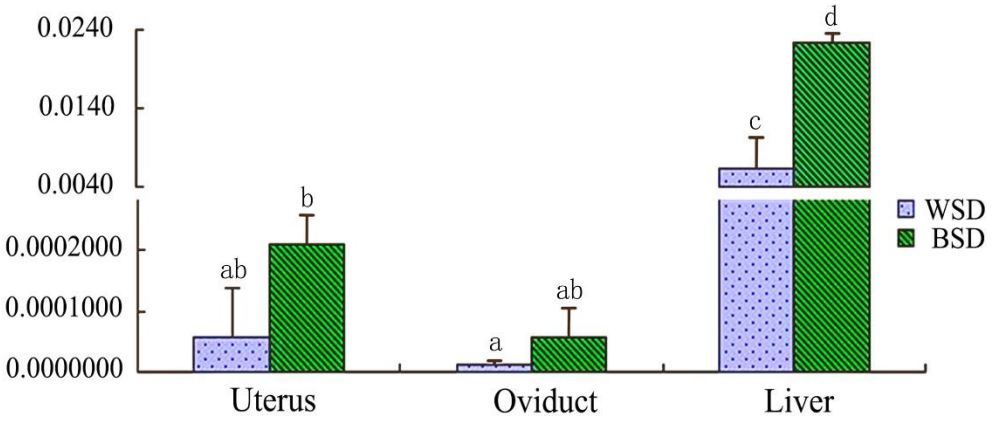

Figure 1 The different tissues expression of the LOC101800257 at 43 weeks of the Leizhou black duck.
abcd, Values bearing different superscripts significantly differ from each other; BSD, blue shell ducks; WSD, white shell ducks.

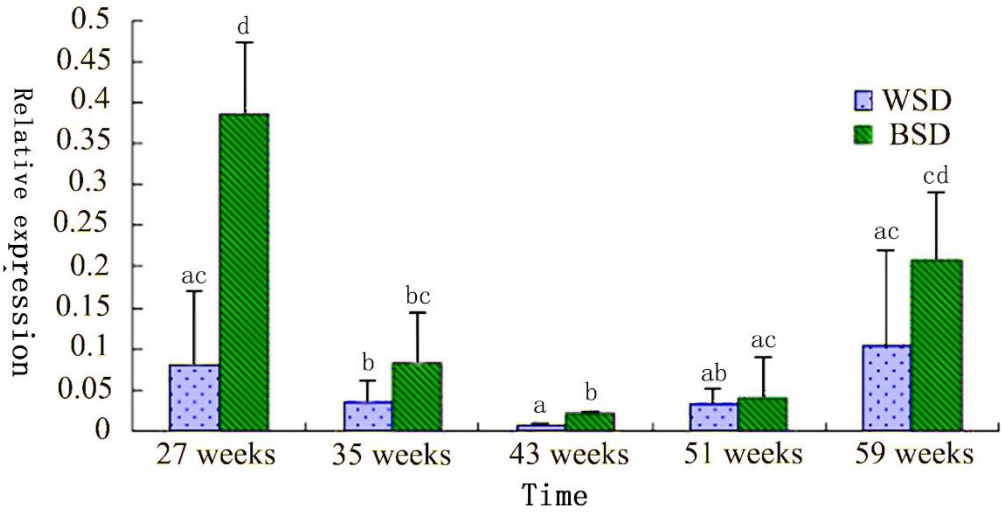

Figure 2 The temporal expression of the LOC101800257 in the liver of the Leizhou black duck.
abcd, Values bearing different superscripts significantly differ from each other; BSD, blue shell ducks; WSD, white shell ducks.

Polymorphisms of LOC101800257 gene: There were two mutation loci in 13815bp (exon 9) and 15325bp (intron 11) of LOC101800257 (Figure 3), which were named c.1406A>G （A→G）and c.1642+16A>G （A→G）respectively (Dunnen et al., 2001). Using 10% polyacrylamide gel electrophoresis and sequencing, three different band types (AA, GG and AG) were detected at c.1406A>G（A→G）and c.1642+16A> G (Figure 4, 5, 6). The c.1406A>G caused original encoding amino acid isoleucine (ILe) to proline (Val) in the mutation; and the mutated amino acid site was in the MFS region of the protein. The c.1642+16A>G located in intron and was a synonymous mutation.

Data statistic: The study obtained heterozygous AG as the dominant type in the c.1406A>G locus, and wild-type AA was dominant type in the c.1642+16 A>G. Both SNPs loci had moderate polymorphism (0.25<PIC<0.5) and were in the Hardy-Weinberg non-equilibrium state (P<0.01). There existed higher heterozygosity at both SNPs loci, indicating their genetic polymorphisms were abundant (Table 2).
There was no correlation between the six genotypes (c.1406A>G: AA, GG, AG; c.1642+16A>G: AA, GG, AG) of LOC101800257 and eggshell color traits (P>0.05) (Table 3). However, association analysis showed that there was a correlation between combinative genotype GGGG and eggshell color (P<0.05) but no correlation between other combinative genotypes (AAAA, AAAG, AAGG, AGAA, AGAG, AGGG, GGAA, GGAG) and eggshell color traits (P>0.05) (Table 3).

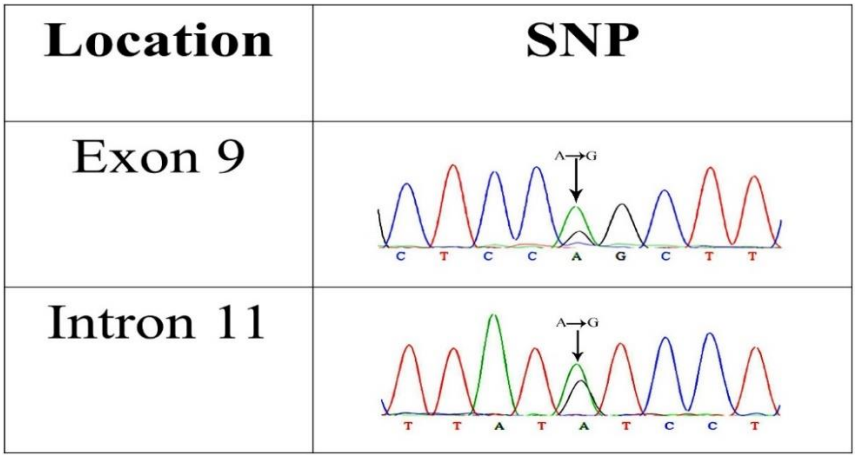

Figure 3 The SNPs in exon 9 and intron11 of LOC101800257 gene.
SNP, single nucleotide polymorphism.

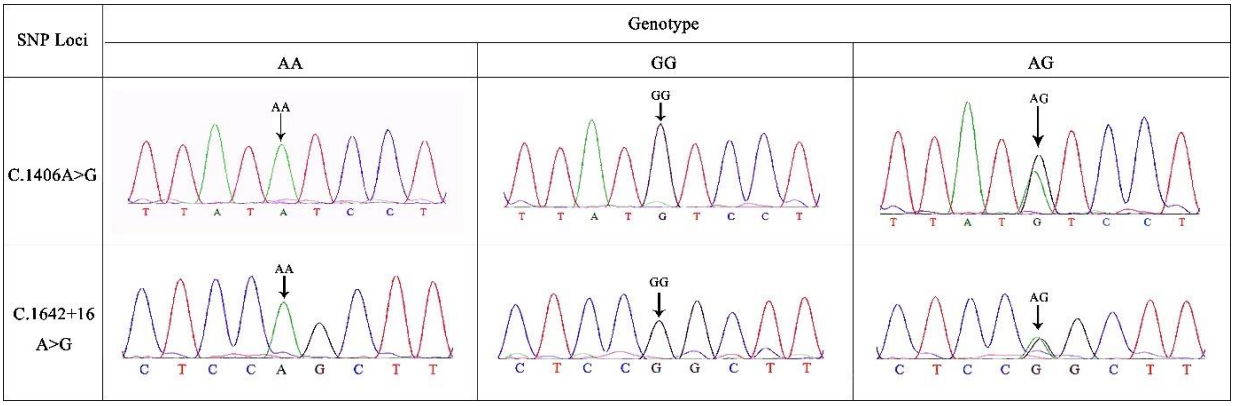

Figure 4 The sequence of different genotypes in c.1406A>G and c.1642+16A>G of LOC101800257 gene.
SNP, single nucleotide polymorphism.

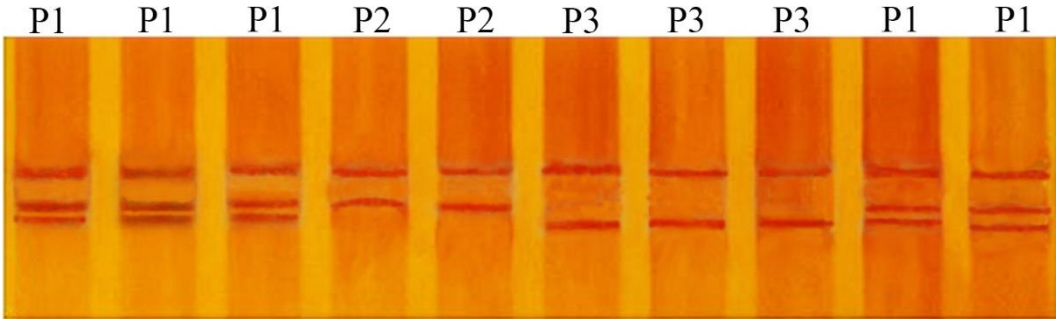

Figure 5 The PCR-SSCP pattern of c.1406A>G of LOC101800257 gene.
There were 3 different electrophoresis strips in c.1406A>G of LOC101800257 gene.
PCR-SSCP, Polymerase Chain Reaction-Single Strand Conformation Polymorphism; P1, genotype AG; P2, genotype GG; P3, genotype AA.

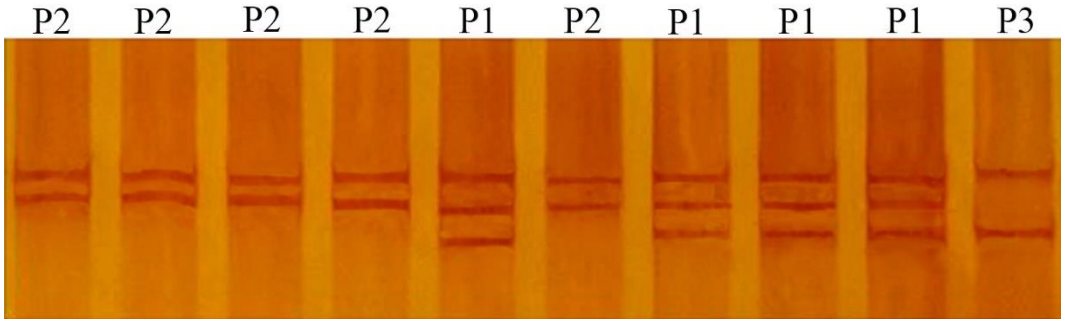

Figure 6 The PCR-SSCP pattern of c.1642+16A>G of LOC101800257 gene.
There were 3 different electrophoresis strips in c.1642+16A>G of LOC101800257 gene.
PCR-SSCP, Polymerase Chain Reaction-Single Strand Conformation Polymorphism; P1, genotype AG; P2, genotype GG; P3, genotype AA.

Figure 7 The multispecies alignment of LOC gene protein sequence.
Missense mutation of ILe435Val is indicate by red box. The wild type ILe was homologous to the white-tailed pheasant lepturus, and the mutant Val was homologous to phalacrocorax carbo.

Table 2 The allele frequency and genetic polymorphism of LOC101800257 SNP in Leizhou black ducks

SNP loci	Genotype and frequency			Allele frequency		χ^2	H_o	He	H_O	Ne	PIC
	Genotype	Individual number	Frequency	A	G						
c.1406 A>G	AA	40	0.333	0.554	0.446	1.353	0.506	0.494	0.442	1.977	0.372
	AG	53	0.442								
	GG	27	0.225								
c.1642+ 16 A>G	AA	57	0.475	0.683	0.317	0.166	0.567	0.433	0.417	1.763	0.339
	AG	50	0.417								
	GG	13	0.108								

SNP, single nucleotide polymorphism; χ^2, Chi-square test; H_o, genetic homozygosity; He, expected heterozygosity; H_O, observed heterozygosity; Ne, effective number of alleles; PIC, polymorphism information content.

Table 3 The correlation analysis on single SNP and blue eggshell trait

SNP loci	Genotype	Blue eggshell traits		*P*-Value and χ^2
		BSD number	WSD number	
c.1406A>G	AA	19	21	*P*=0.636 χ^2=0.905
	AG	29	24	
	GG	12	15	
c.1642+16 A>G	AA	25	32	*P*=0.169 χ^2=3.552
	AG	30	20	
	GG	5	8	
Combination genotypes	AAAA	15	18	*P*=0.728
	AAAG	1	3	-
	AAGG	3	0	-
	AGAA	8	10	*P*=0.815
	AGAG	19	13	*P*=0.377
	AGGG	2	1	-
	GGAA	2	4	*P*=0.678
	GGAG	10	4	*P*=0.180
	GGGG	0	7	*P*=0.016*

SNP, single nucleotide polymorphisms; BSD, blue shell ducks; WSD, white shell ducks; χ^2, Chi-square test. -, no statistical significance; *, differ significantly at $P \leq 0.05$ level.

Discussion

Temporal and tissues expression pattern: The main components of eggshell pigments, including protoporphyrin and biliverdin, are intermediates in the synthesis or metabolism of heme in the body (Wang *et al*., 2013). It has been reported that OATP1A2, OATP1B1 and OATP1B3 are involved in mediating the transport of biliverin on the basolateral membrane of the liver (Hagenbuch *et al*., 2010). The formation of blue eggshell regulated by polygene was closely interrelated to the transport of biliverdin (Guang *et al*., 2017). Therefore, the liver is a necessary organ for the formation of blue eggshells. The results from tissues expression showed that LOC101800257 was mainly expressed in the liver, and there was significant difference between BSD and WSD (P<0.05). Surprisingly, SLCO1C1, SLCO2B1 and SLCO1B3 of the same family as LOC101800257 can regulate the transport of biliverdin in different ways to control the formation of blue eggshells (Wang *et al*., 2013; Wang *et al*., 2017; Yu *et al*., 2016). Therefore, LOC101800257 may be involved in the regulation of eggshell color through its function in the liver.

However, temporal expression results showed that the expression trend of LOC101800257 was high-low-high like a cyclic form from 27 weeks to 59 weeks. The high expression level of LOC101800257 was at 27 weeks and 59 weeks and the low expression was at the peak period of laying (35, 43, 51 weeks). The results of the temporal expressions of LOC101800257 were inversely correlated with the biliverdin transport. Numerous reports have shown that increase in blue shell eggs is dependent on the increased amount of biliverdin transport (Liu *et al*., 2009; Badás *et al*., 2017).

In other words, the more blue shell eggs, the more the transport of biliverdin, and the higher the expression of LOC101800257. In actual production, the color of the blue shell egg may become lighter with the production of the blue shell egg increasing (Wang *et al*., 2013; David *et al*., 2013; Zheng *et al*., 2014). Moreover, the function of LOC101800257 may have different strengths at different times. Thus, the expression level of LOC101800257 may be normal, and its specific regulatory mechanism still needs further research. Nevertheless, it is undeniable that LOC101800257 was involved in the regulation of eggshell color, according to the differential expression between WSD and BSD. Liu *et al.* (2009) proposed that the duck eggshell color was observed due to the transport of biliverdin from the liver to the eggshell gland through the bile duct to the uterus. Therefore, the bile duct as an important channel for transporting biliverdin and other ion in the liver may be an important research object in the future.

Polymorphism and association analysis of eggshell color: LOC101800257 is a member of the SLC family and may be related to the transport of biliverdin. A study has reported that the expression level of SLC family members is regulated by SNP sites and it plays an important regulatory role in eggshell color formation (Shen *et al*., 2017). Eggshell color is a micro-effect multi-gene determined quality trait, and this trait is regulated by multiple sites (Liu *et al*., 2017; Tuiskulahaavisto *et al*., 2018). Wang *et al*., found mutation site g.67419892-67419904del13 of SLCO1C1 caused variation of Ala528Glu, and the mutation site significantly correlated with the transport of biliverdin of chickens (Wang *et al*., 2017). EAV-HP inserted into startup area of SLCO1B3 caused biliverdin transport abnormalities in chickens to form blue shell eggs (Wang *et al*., 2013). The results of this study showed GGGG combined genotype significantly correlates with the eggshell color ($P<0.05$), while other genotypes were not related to the eggshell color ($P>0.05$). The WSD (11.7%) was much higher than BSD (0%) in the GGGG genotype frequency ($P<0.05$). The c.1406A>G caused original encoding amino acid isoleucine (ILe) to proline (Val) in the mutation; and the mutated amino acid site is in the MFS region of the protein. A study of melamine mutation has observed that proline affects the affinity of the GABA receptor complex (Tian *et al*., 2013). Whether the mutation site correlates with the biliverdin transport function remains to be further studied. The c.1642+16A>G is located in intron but there are inducible enhancer actions or expression regulatory elements of mRNA alternative splicing on introns affecting gene expression indirectly (Nott *et al*., 2003). Several researches have indicated that, single based mutations in introns affect the expression of functional genes (Reen *et al*., 2017). Moreover, comparing different species of amino acid sequence of LOC genes (solute carrier organic anion transporter family member 1C1-like protein), found that the amino acid sequence of the duck was highly homologous to other birds, but there were many mutations in the amino acid at the 435th position (Figure 7). The above-suggested SNPs identified in this study may have an effect on the mutation of LOC101800257 and may have certain significance for the evolution of the Leizhou black duck.

In conclusion, LOC101800257 may be involved in the regulation of eggshell color through its function in the liver, but the function strength may vary at different periods. Moreover, the GGGG combined genotype can serve as a novel genetic marker for eggshell color, which may have certain significance in the evolution of the Leizhou black duck.

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***Conflict of interest*:** The authors declare that they have no competing interests.

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