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Molecular cloning of the perilipin gene and its association with carcass and fat traits in Chinese ducks

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ABSTRACT. The perilipin (*PLIN*) gene is a candidate gene of carcass and fat traits in ducks. In order to study the molecular character of the *PLIN* gene and its function in different breeds of Chinese ducks, samples were obtained from the Chinese Academy of Agricultural Sciences Research Center for Birds, including 95 Peking ducks of the Z2 series, 91 Peking ducks of the Z4 series, 82 hybrid systems (Z2 x Z4), and 93 Cherry Valley ducks. We used RT-PCR and 3'-RACE to clone the duck *PLIN* gene, detect SNPs and analyze their associations with carcass and fat traits. A 2212-bp sequence was cloned with the complete coding region and a 3'-untranslated region. We found a nucleotide mutation (C→T) in exon 2 of the *PLIN* gene. There were no significant correlations between the 3 genotypes (CC, CT, TT) in breast muscle weight (BMW), leg muscle weight (LMW), subcutaneous fat weight (SFW), and intramuscular fat (IMF) in the Cherry Valley duck. The CC and CT genotypes had significant differences in carcass weight (CW),

carcass net weight (CNW), and percentage of abdominal fat weight (AFW); there were significant differences in AFW and percentage of SFW. In Z4, there were no significant correlations between the 3 genotypes (TT, CC, and CT) in CW, BMW, LMW, SFW, AFW, the percentage of SFW and AFW, and IMF. CNW was significantly different between TT, CC, and CT genotypes. In Z2 x Z4, there were no significant correlations between the 3 genotypes in CW, BMW, LMW, SFW, AFW, the percentage of SFW and AFW, and IMF, while the CC and CT genotypes had significant differences in CNW. In Z2, there were no significant differences between the 3 genotypes in all traits. We deduced that the *PLIN* gene is a potential major gene. It is linked to a major gene affecting meat quality traits. This SNP has potential as a molecular marker for marker-assisted selection.

Key words: Duck; *PLIN* gene; Clone; PCR-RFLP; SNP

INTRODUCTION

Nowadays, obesity has become a major disease impacting human health (Mokdad et al., 2003). In recent years, for undue pursuit of rapid growth, the problems of the duck fat, especially the excessive subcutaneous fat deposition to some extent, restrained the duck industry. The aim in meat duck production is to breed low-fat meat ducks in the future. Therefore, it is very necessary to study the related gene mechanism regulating fat metabolism in meat ducks.

Perilipin (PLIN) is a phosphoprotein that coats lipid droplets in adipocytes and can be hyperphosphorylated by protein kinase A. It was first found in epididymal fat cells in mice by Greenberg et al. (1991). PLIN is the most abundant phosphoprotein that coats lipid droplets in adipocytes (Xu et al., 2006) and has a dual regulatory role in triglyceride metabolism in adipose tissue (Lu et al., 2001; Souza et al., 2002; Londos et al., 2005; Granneman and Moore, 2008).

Numerous studies have shown that PLIN plays an important role in regulating lipid metabolism in humans, mice, cows, and chickens (Marcinkiewicz et al., 2006; Brasaemle, 2007; Miyoshi et al., 2007; Souza et al., 2007; Zhao et al., 2009; Fan et al., 2010), which are important references in the study of duck fat metabolism. Currently, researchers have cloned the *PLIN* gene of humans, mice, cattle, and chickens, but there are no reports about this gene in duck. cDNA fragments of the *PLIN* gene were cloned and sequenced to investigate the physiological role of the gene in fat deposition, lipid metabolism, and the meat quality of duck. At the same time, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing have been used to detect the SNP location of the *PLIN* gene in ducks; correlation analysis between the SNP loci and the carcass and fat traits in different duck species was used to provide a new means of theoretical methods and techniques to improve duck carcass quality and growth performance.

MATERIAL AND METHODS

Animal source and preparation of DNA samples

Tissue samples used to clone the *PLIN* gene were obtained from adipose tissue of

8-week-old Peking ducks. Dissected tissues were placed in liquid nitrogen and stored at -80°C. Total RNA was isolated (Trizol reagent), and its concentration and purity were determined using a UV/Vis spectrophotometer. The RNA was diluted to 1 μg/μL for a stock solution, and the first-strand cDNA was synthesized (RevertAid^{TM} First-Strand cDNA Synthesis kit, Fermentas Co.).

Samples for trait association analysis were obtained from the Chinese Academy of Agricultural Sciences Research Center for Birds, where a total of 361 ducks were used. A random sampling method was adapted to select 95 Peking ducks of the Z2 series, 91 Peking ducks of the Z4 series, 82 hybrid systems (Z2 x Z4), and 93 Cherry Valley ducks. They were fed for 6 weeks under the same conditions, after which 5 mL blood was taken from the wing vein (ACD anticoagulant) and stored at -80°C. Carcass weight (CW), breast muscle weight (BMW), leg muscle weight (LMW), eviscerated weight (carcass net weight, CNW), subcutaneous fat weight (SFW), and abdominal fat weight (AFW) were determined after slaughter, simultaneously; SFW percentage, AFW percentage, and intramuscular fat (IMF) content were also calculated.

All experimental protocols and animal care were according to authorization granted by the Chinese Ministry of Agriculture. Genomic DNA was isolated from 2% heparin-treated blood samples and stored at -80°C.

Primer design

Primer Premier 5.0 was used to design the primers to amplify the coding area of the *PLIN* gene in duck according to the mRNA sequence of the chicken *PLIN* gene (GenBank accession No. NM_001127439): F-5'-ACGGCGAAGAAGAATCAG-3', R-5'-CCGTAGAGG TTGGCGTAG-3'.

The primers used to clone the 3'-UTR of the *PLIN* gene was designed according to the cloned sequence by Primer Premier 5.0:

GSP1: 5'-ACAACCTCTACCGCAGCCCGGCCTT-3', GSP2: 5'-ATGTACAGCCGG ACCTACTACGCCAAC-3'.

The primers used to amplify exon 2 of the *PLIN* gene were also designed by Primer Premier 5.0 according to the cloned sequence and the genomic sequence of the chicken *PLIN* gene (173 bp in length): F-5'-ATCTCGACCTGCGAAAGCC-3', R-5'-TTGGAGGACTCACACTG CGG-3'.

Amplification of the coding region of the *PLIN* gene

cDNA was amplified by PCR in a 15-μL reaction volume containing 1.5 μL 10X PCR buffer, 1 μL 25 mM $MgCl_2$, 1.5 μL 2.5 mM dNTPs, 0.2 μL 5 U/μL Taq DNA polymerase, 1 μL cDNA, 0.5 μL of each primer (10 pM), and 5.8 μL ddH_2O. PCR amplifications were performed under the following conditions: initial denaturation at 95°C for 4 min, followed by 38 cycles of 30 s at 94°C for denaturing, 30 s at 55°C for annealing and 30 s at 72°C for extension, and finally 72°C for 10 min. The PCR product was analyzed on a 1.5% agarose gel.

3'-RACE of the *PLIN* gene

According to 3'-RACE, we used the Peking duck fat tissue total RNA as template and adapter primer as primer for reverse transcription of the first chain of cDNA, the GSP1 and

UPM as primers, and annealing at 67°C for the first-round PCR amplification.

The product of the first-round PCR amplification was diluted 100 times; 1 µL was taken as model, and GSP and NUP were used as primers, with annealing at 67°C, for the second-round PCR amplification. The reaction volume and program were the same as with the amplification of the coding region, but the annealing temperature was different. The PCR product was analyzed on a 1.5% agarose gel after the reaction.

Sequencing of the PCR products

PCR products were purified using an agarose gel DNA extraction kit and cloned into pMD18-T vector. The positive clones of this gene were sequenced. The sequencing results were analyzed by online sequence analysis tools.

Amplification of the *PLIN* exon 2

The *PLIN* gene exon 2 was amplified by PCR in a 15-µL reaction volume containing 1.5 µL 10X PCR buffer, 1 µL 25 mM $MgCl_2$, 1.5 µL 2.5 mM dNTPs, 0.2 µL 5 U/µL Taq DNA polymerase, 0.5 µL cDNA, 0.5 µL of each primer (10 pM), and 6.3 µL ddH_2O. PCR amplifications were performed under the following conditions: initial denaturation at 95°C for 4 min, followed by 38 cycles of 30 s at 94°C for denaturing, 30 s at 57°C for annealing and 30 s at 72°C for extension, and finally 72°C for 10 min. The PCR product was analyzed on a 3% agarose gel.

PCR-RFLP

The total reaction volume was 10 µL containing 5 µL PCR products, 2 U *Bsp*1286I, 1.0 µL 10X PCR buffer and sterile water added to 10 µL. The reaction tubes were incubated at 30°C overnight, and the results were analyzed on a 3% agarose gel. A photograph was taken with the Gel Imaging System camera and recorded.

Statistical analysis

The individual genotype was determined according to the PCR-RFLP results, calculating the allele frequency, genotype frequency, homozygosity, heterozygosity, effective number of alleles, and polymorphism information content (PIC) of different breeds, analyzing their genetic diversity.

The association between genotypes and the traits was analyzed using the general linear model procedure according to the following model:

$$Y = \mu + G + S + G \times S + e$$

where Y is the phenotypic value of target trait; μ is the population mean; G is the genotype effect; S is the sex effect; $G \times S$ is the interaction of genotype and sex; and e is the random error. The SPSS16.0 software was used to analyze the data, the linear model was fitted with the least square method, and the significance test was done for the phenotype of different genotypes.

RESULTS

Cloning of the *PLIN* gene in Peking ducks

The amplification of *PLIN* code region was analyzed on a 1.5% agarose gel, and a single band appeared, which was the same as expected. It was 1562 bp in length after sequencing (Figure 1). The electrophoresis results of the *PLIN* gene 3'-RACE by RT-PCR are shown in Figure 2.

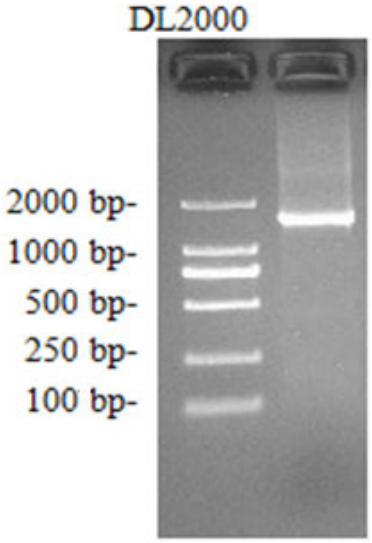

Figure 1. Electrophoresis of the *PLIN* gene by RT-PCR.

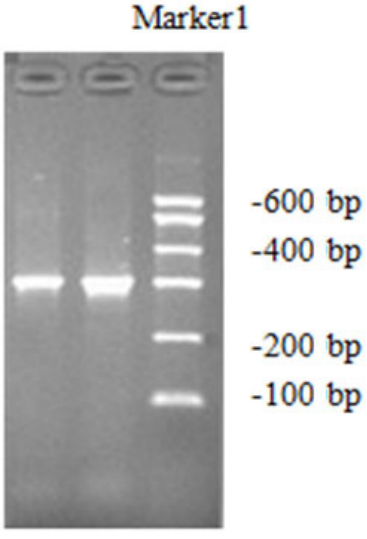

Figure 2. Electrophoresis of the *PLIN* gene 3'-RACE by RT-PCR.

Comparison of the duck *PLIN* sequencing with genome and mRNA *PLIN* sequence in chicken, respectively, showed that it had a complete coding region and 3'-non-coding region of *PLIN* in ducks and that it was 2212 bp in length. Furthermore, we found that there were 1590 nucleotides in the opening reading frame, with an initiation codon of ATG and terminator of TGA, encoding 530 amino acids and including 8 exons and 7 introns in the *PLIN* gene. The molecular weight of the protein was 57.23 kDa. According to the comparison, the sequence similarity of the *PLIN* gene between ducks and chicken was 85%.

SNP analysis

Sequencing and PCR-RFLP

Using the primers of the *PLIN* gene exon 2, we obtained a fragment of 173 bp. A C/T mutation was found at 135 bp. Furthermore, there was a restriction enzyme cutting site of *Bsp*1286I at this location. With only a C at 135 bp, it would be cut into 2 fragments of 134 and 39 bp by *Bsp*1286I, recorded as the CC type, while with a T at 135 bp, it could not be cut and only one fragment of 173 bp was recorded as the TT type, and with both C and T present, 3 fragments of 173, 134, and 39 bp were recorded as the CT type. The digestion results of the PCR product of exon 2 of the *PLIN* gene with *Bsp*1286I are shown in Figure 3. The 39-bp fragment was too short to show. The sequencing results are shown in Figure 4.

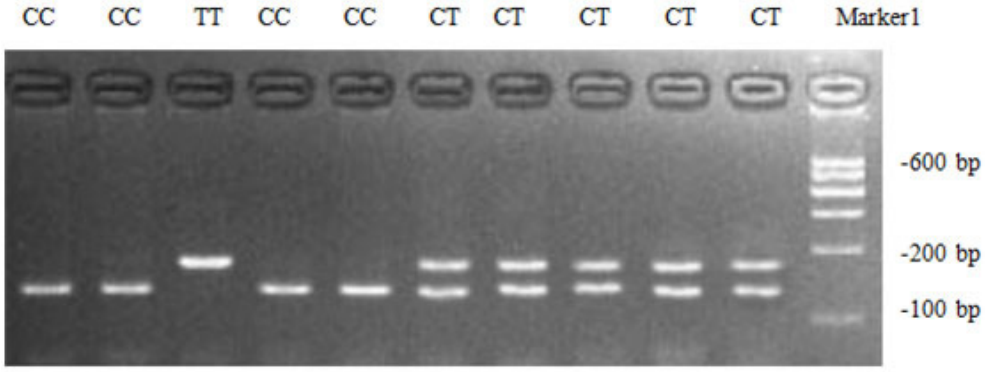

Figure 3. Digestion of PCR product of the *PLIN* gene exon 2 with *Bsp*1286I.

Genetic analysis of the polymorphic site of *PLIN*

The polymorphism of the *Bsp*1286I locus in the *PLIN* gene exon 2 was analyzed in 4 breeds of ducks. We calculated the genotype, allele frequency, heterozygosity, effective number of alleles, PIC, and the χ^2 test (Table 1) of 4 breeds after typing. The results showed that in the 4 duck populations, there were more heterozygous individuals than homozygous ones. The allele frequency results showed that C was the dominant allele and CT was the dominant genotype, while the PIC value showed that it was a moderate polymorphism. The χ^2 test showed that the mutation of this location in the 4 populations was not in Hardy-Weinberg equilibrium.

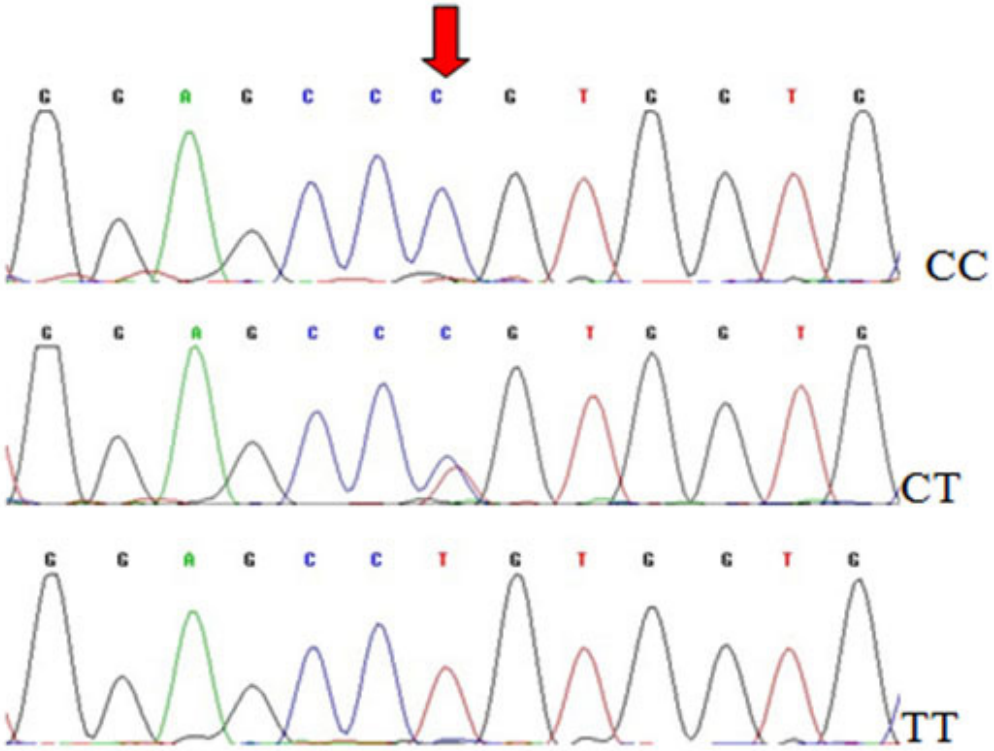

Figure 4. Sequence comparisons of CC, CT, and TT genotypes.

Table 1. Genetic diversity analysis of the *PLIN* gene.

Breed	Genotype	Genotype frequency	Allele frequency	H	N_E	PIC	χ^2
Z2	CC	0.432	0.716	0.407	1.686	0.324	21.034
	CT	0.568					
	TT	0	0.284				
Z4	CC	0.209	0.571	0.490	1.960	0.370	8.271
	CT	0.7259					
	TT	0.066	0.429				
Z2 x Z4	CC	0.354	0.652	0.454	1.830	0.351	31.970
	CT	0.598					
	TT	0.049	0.348				
Cherry Valley	CC	0.215	0.597	0.481	1.928	0.365	14.977
	CT	0.763					
	TT	0.022	0.403				

H = heterozygosity index; N_E = effective number of alleles; PIC = polymorphism information content.

Association analysis

The polymorphism results of the *PLIN* gene exon 2 and its relationship with carcass and adipose traits in the 4 duck types studied are shown in Table 2.

Table 2. Polymorphism of the *PLIN* gene exon 2 and its relationship with carcass and adipose traits in the 4 duck types studied.

Breed	Genotype	Trait								
		CW (g)	BMW (g)	LMW (g)	CNW (g)	SFW (g)	AFW (g)	SFW (%)	AFW (%)	IMF (%)
Z4	CC (19)	2753.7 ± 57.9	241.7 ± 9.5	136.7 ± 4.4	1243.6 ± 24.4^{a}	694.5 ± 25.6	58.1 ± 2.7	55.9 ± 2.0	4.7 ± 0.2	7.2 ± 0.4
	CT (66)	2717.9 ± 31.1	235.3 ± 5.1	133.7 ± 2.4	1241.5 ± 13.1^{a}	669.7 ± 13.7	54.6 ± 1.4	54.1 ± 1.1	4.4 ± 0.1	6.5 ± 0.2
	TT (6)	2737.7 ± 103	251.5 ± 16.8	134.8 ± 7.8	1352.3 ± 43.4^{b}	675.4 ± 45.5	62.2 ± 4.8	49.9 ± 3.5	4.6 ± 0.4	7.0 ± 0.7
Z2 x Z4	CC (29)	2389.4 ± 43.3	173.4 ± 5.9	124.1 ± 3.1	1083.2 ± 17.4^{a}	598.1 ± 15.6	48.5 ± 1.9	55.4 ± 1.4	4.5 ± 0.2	5.3 ± 0.4
	CT (49)	2454.7 ± 33.3	180.7 ± 4.6	122.0 ± 2.4	1127.2 ± 13.4^{b}	620.8 ± 12.0	51.6 ± 1.5	55.2 ± 1.0	4.6 ± 0.1	5.6 ± 0.3
	TT (4)	2419.5 ± 117	188.7 ± 16.1	117.1 ± 8.5	1118.8 ± 47.0	615.4 ± 42.1	53.5 ± 5.3	55.3 ± 3.7	4.8 ± 0.5	4.3 ± 1.0
Cherry Valley	CC (20)	2332.2 ± 118^{A}	267.4 ± 10.7	137.9 ± 3.5	1216.7 ± 140.9^{A}	450.0 ± 14.2	31.6 ± 2.0^{a}	38.5 ± 1.3^{a}	2.7 ± 0.2^{A}	4.1 ± 0.2
	CT (71)	1705.2 ± 63^{B}	283.6 ± 5.7	142.1 ± 1.9	2110.1 ± 74.8^{B}	449.1 ± 7.6	26.6 ± 1.1^{b}	35.4 ± 0.7^{b}	2.1 ± 0.1^{B}	4.2 ± 0.1
	TT (2)	1878.9 ± 373	255.7 ± 33.9	143.1 ± 11.2	1864.3 ± 445.6	431.0 ± 45.1	23.1 ± 6.4	33.8 ± 4.2	1.8 ± 0.6	3.8 ± 0.6
Z2	CC (41)	2334.3 ± 26.6	165.4 ± 4.1	148.7 ± 17.7	1085.4 ± 11.1	570.7 ± 12.3	45.8 ± 1.9	52.8 ± 1.2	4.2 ± 0.2	4.9 ± 0.2
	CT (54)	2345.0 ± 23.2	173.8 ± 3.6	123.3 ± 15.4	1079.0 ± 9.7	577.8 ± 10.8	47.2 ± 1.6	53.6 ± 1.0	4.4 ± 0.2	4.6 ± 0.2

Data followed by different superscript letters within the same row differ significantly (capital letters <0.01, small letters <0.05). CW = carcass weight; BMW = breast muscle weight; LMW = leg muscle weight; CNW = carcass net weight; SFW = subcutaneous fat weight; AFW = abdomen fat weight; IMF = intramuscular fat weight.

The association between different genotypes and carcass was analyzed in Peking duck (Z2, Z4, Z2 x Z4) and Cherry Valley duck. In Cherry Valley duck, there were no significant correlations between the 3 genotypes and BMW, LMW, SFW, and IMF ($P > 0.05$). CW, CNW, and the percentage of AFW were very significant between CC and CT ($P < 0.01$). AFW and the percentage of SWF were significant between CC and CT ($P < 0.05$). In the Z4 population, there were no significant correlations between the 3 genotypes and CW, BMW, LMW, SFW, AFW, the percentage of SFW and AFW, and IMF ($P > 0.05$). CNW was significant between TT, CC, and CT ($P < 0.05$). In the Z2 x Z4 population, there were no significant correlations between the 3 genotypes and CW, BMW, LMW, SFW, AFW, the percentage of SFW and AFW, and IMF ($P > 0.05$). CNW was significant between CC and CT ($P < 0.05$). In the Z2 population, there were no significant correlations between the 3 genotypes and any of the traits studied ($P > 0.05$).

DISCUSSION

Cloning of the *PLIN* gene

In this study, the cDNA partial sequence of the *PLIN* gene in Peking duck was cloned. A sequence of 2212 bp was cloned with complete coding region and 3'-untranslated region (GenBank accession No. FJ862531). The similarity of the duck *PLIN* gene sequence with that of human, mouse, and chicken was 62, 25, and 87%, respectively, which shows the substantial variation of this gene in different species. Furthermore, sequence analysis results showed that there were several small hydrophobic areas in the amino acid sequence. Transmembrane structure prediction showed that there was no transmembrane structure. The small hydrophobic regions based on the analysis were evidently not significant.

Genetic analysis of the *PLIN* gene exon 2

In this study, DNA sequencing and RFLP were used to analyze the genetic diversity of the *PLIN* gene exon 2 in Peking ducks (Z2, Z4, Z2 x Z4) and Cherry Valley ducks. The results show that there was one SNP location in the 4 breeds; in addition, it was a synonymous mutation that did not change the amino acid sequence. The mutation of this location in 4 populations was not in Hardy-Weinberg equilibrium. The reasons for this may be the small population size, the sample size, the data organization, and the breed. The samples need to be increased in future research.

PIC and heterozygosity are measures of the genetic variation within populations, where the size of their value reflects the homogeneity of individuals within the group, i.e., the greater the genetic variation, the higher the value. As seen in Table 1, in exon 2 of the *PLIN* gene, the heterozygosity index, effective number of alleles and PIC were the highest in the Peking duck Z4 series, while the lowest were in the Z2 series. It means that there was large genetic variation between the Z4 and Z2 series. Vaiman et al. (1994) pointed out that $PIC > 0.5$ shows highly polymorphic loci, $0.5 > PIC > 0.25$ shows the moderately polymorphic loci, $0.25 > PIC$ shows the low polymorphic loci. From Table 1, it can be seen that the PIC values of the 4 groups all showed moderately polymorphic loci. It was shown that the site may be used as a potential molecular marker. However, further research is needed to determine if the locus can

be a suitable molecular marker to be used for marker-assisted selection.

Polymorphism of the *PLIN* gene and its relationship with carcass and adipose traits

Research on the polymorphism of the *PLIN* gene has focused on humans and the gene positioned near the predisposing genes of fat, diabetes, and hyperlipidemia, showing that it has a regulatory role in lipid metabolism diseases in humans (Nishiu et al., 1998; Atshaves et al., 2001; Grönke et al., 2003; Mottagui-Tabar et al., 2003; Qi et al., 2004).

There are dozens of SNP loci of the *PLIN* gene in humans, and most of them have a relationship with fat and some lipid parameters (Mottagui-Tabar et al., 2003). In addition, research on the polymorphism of the *PLIN* gene in cattle and chickens has been reported (Vaiman et al., 1994; Nishiu et al., 1998; Atshaves et al., 2001; Tansey et al., 2003; Grönke et al., 2003; Mottagui-Tabar et al., 2003; Qi et al., 2004; Zhao, 2008; Fan et al., 2010).

However, the study of the polymorphism of the *PLIN* gene in ducks has not been reported. In this study, we detected the SNPs of the *PLIN* gene in ducks, and found a polymorphic locus that has a significant effect on carcass traits in 3 duck groups, excepting the Peking duck Z2 series. Additionally, this locus has a significant effect on the adipose traits in Cherry Valley ducks; the above research lays the foundation for further research into ducks.

In this study, we found only 2 genotypes, CC and CT, in the Peking duck Z2 series; this might have been a result of the small sample size. Meanwhile, this locus had no significant effect on the carcass and adipose traits; it might have been related to the small population size.

Based on this research, we speculate that the *PLIN* gene may be a major gene that affects the duck carcass and adipose traits or that is linked with a QTL controlling these traits.

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