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Polymorphisms and Their Haplotype Combinations in the Lysozyme Gene Associated with the Production Traits of a Chinese Native Chicken Breed

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ABSTRACT

Animal lysozymes, which have been studied in many of invertebrate and vertebrate species, have been characterized and demonstrated to be immune-associated molecules, digestive enzymes and multifunctional molecules. The purpose of this study was to detect the connection between lysozyme-gene polymorphism and the production traits of a Chinese native chicken breed (Langshan chicken). Four single nucleotide mutation sites were identified: G345A, C1726T, G1836A, A1838G. By the linkage disequilibrium analysis, six haplotypes and 15haplotype combinations were depicted in the studied population. The statistical analysis demonstrated that the SNPs and the haplotype combinations are related to body weight at sixteen weeks of age in Langshan chickens ($p<0.05$), and those with combined haplotype Hap3-Hap6 (GA-TT-GG-AA) presented higher body weight. Our study demonstrated that the SNPs and their haplotype combinations in the lysozyme gene were associated with the chicken production traits, and that SNPs can be used as a molecular marker for chicken marker-assisted selection.

INTRODUCTION

Lysozyme is a ubiquitous enzyme defined as muramidase that catalyzes the hydrolysis of 1,4-betalinkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in bacterial peptidoglycan, a major component of the bacterial cell wall (Phillips, 1966). According to their type-specific amino acid sequence and their species of origin, lysozymes have been categorized into three main types in the animal kingdom: the c-type (chicken or conventional type), the g-type (goose-type), and the i-type (invertebrate type) lysozyme (Callewaert & Michiels, 2010; Nilsen & Myrnes, 2001). These animal lysozymes have been studied in many species of invertebrates and vertebrates and have been characterized and demonstrated to be immune-associated molecules (Roxström-Lindquist *et al.*, 2004), digestive enzymes (Grunclovà *et al.*, 2003), and multifunctional molecules (Ursic Bedoya *et al.*, 2005). C-type lysozymes are the main lysozymes produced by most vertebrates, including mammals. the mechanism of enzyme action has been studied most thoroughly in the c-type lysozyme from hen egg white, which has served as a model for studies on enzyme structure and function (Sassi *et al.*, 2011; Sugimoto *et al.*, 2011; Swaminathan *et al.*, 2001).

The chicken lysozyme gene, the first cloned and characterized eukaryotic lysozyme gene (Baldacci *et al.*, 1979; Jung *et al.*, 1980; Lindenmaier *et al.*, 1979), is one of the most thoroughly studied model genes for investigations of the molecular mechanisms involved in cell- and stage-specific transcriptional regulation (Peters *et al.*, 1989). The gene is expressed in the tubular gland cells of oviduct of laying chickens and it is under the control of steroid hormones (Bonifer *et al.*, 1990; Schiitz *et al.*, 1978). It is also constitutively expressed in mature macrophages (Peters *et*

al., 1989). This gene, mapped on chicken's chromosome 1, spans 3688bp and its sequence organization with four exons and three introns is homologous to the human lysozyme gene and the human α-lactalbumin gene (https://www.ncbi.nlm.nih.gov).

The objective of the research was to identify single nucleotide polymorphisms (SNPs) of the lysozyme gene in a Chinese well-known native chicken breed (Langshan chicken) and to implement the SNP haplotypes construction and relationship analysis to investigate the effects of the lysozyme gene on chicken production traits.

MATERIAL AND METHODS

Ethics statement

The Institutional Animal Care and Use Committee (IACUC) of the School of Life Science of Jiangsu Normal University approved the animal study proposal, with the permit number: SYXK (Su) IACUC 2011-0039. All chicken experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China.

Samples and data collection

In this study, 300healthy hens of a Chinese native chicken breed (Langshan chicken) reared in Rudong chicken farm, Jiangsu province, were randomly selected. These hens were reared in cages and were fed with commercial corn-soybean diets. Body weight at 16weeks of age (16-week BW) and the total number of eggs produced in 300 days(300-day EN) were recorded by the farm workers.

Whole blood samples were collected from the wing vein in vacuum blood collection tube (anticoagulant EDTA) and submitted to the laboratory in an icebox. Genomic DNA was extracted according to method of Müllenbach *et al.* (1989). These DNA samples were then dissolved in sterile distilled water and stored at -18°C until analyses.

Mutation detection

Based on the chicken lysozyme gene sequence (GenBank accession number FJ542564), four pairs of primers to amplify three exons, partial intron regions, partial 5' untranslated regions (UTR), and partial 3' UTR (Table 1) were designed using the Primer Premier 6.0 software. Each amplification reaction was carried out in a 15-μL volume: 0.6μL genomic DNA (50 ng/μL), 0.6μL of each primer (10 pmol/μL), 7.5 μL 2× Reaction Mixture, and 0.12μL Taq DNA polymerase (2.5U/μL) (Tiangen, China), 5.58 μL nuclease-free water. PCR reactions were carried out using PTC-200PCR Thermal Cyclers (MJ Research, Inc., USA). Thermo-cycling comprised an initial step of 5 min at 95 ºC and 33 cycles of 50 s at 94 ºC for, 50 s at annealing temperature (Table 1), 50 s at 72 ºC, and a final extension at 72 ºC for 10 min.

Mutations were scanned using the single-strand conformation polymorphism (SSCP) method. Firstly, 5 μL denaturation reagent composed of xylene-cyanole (0.025%), bromophenol blue (0.025%), formamide (95%), and EDTA (25 mM) was mixed with 5 μL of the PCR products. Secondly, the mixture was heated at 98 ºC using PTC-200 PCR Thermal Cyclers (MJ Research, Inc.) for 10 min and immediately chilled on ice. Lastly, denatured DNA was loaded on 10% PAGE (polyacrylamide gel, 80×73×0.75 mm) in 1×tris-borate EDTA(TBE) buffer at constant voltage (200V) for 2.5 h, and then gels were stained with 0.1% silver nitrate. After the polymorphism was detected, the PCR fragments of different SSCP patterns were purified and sequenced in a DNA sequencer (ABI PRISM 377, Applied Biosystems, ThermoFisher Scientific, USA).

Data analyses

Genotypic frequencies, allelic frequencies and Hardy-Weinberg equilibriums (HWE) were directly

Table 1 - Genetic variants identified in the lysozyme gene of Langshan chicken population

Primer sets	Primer sequences (from 5' to 3')	Annealing temperature (°C)	Product length (bp)	Region of amplified fragment	SNPs[a]	Mutation location	Mutaition type
P1	F: TAAAAGAAGAGGCAGGTG R: TGTCTACATTCCAACATCA	58	301	Partial 5'-UTR, exon 1 and partial intron 1	g.345G>A	Exon 1	Silent
P2	F: TAAAATAATAATCTTTGAGG R: TTGTTCTGCTTTGTTCTA	55	317	Partial intron 1, exon 2 and partial intron 2	g.1726C>T g.1836G>A g.1838A>G	Exon 2 Intron2 Intron2	Silent Non-coding Non-coding
P3	F: GCCAACACCAACACGCACTG R: GAACCCCCCGCCACCTAC	68,5	161	Partial intron 2, exon 3 and partial intron 3		No SNPs	
P4	F: AGATCGTCAGCGATGGAAACG R: CGCGGCAGCTCCTCACAG	65	194	Partial intron 3, exon 3 and partial 3'-UTR		No SNPs	

[a]SNPs: single nucleotide polymorphisms

computed. Population diversity parameters, including gene heterozygosity (He), effective allele numbers (Ne), and polymorphism information content (PIC) were calculated. He and Ne were computed using POPGENE software (Version 3.2), while PIC was computed according to Botstein *et al.*, (1980).

Two different programs were used in sequence to construct haplotypes from SNP genotypes of the chicken population. HAPLOVIEW software (Version 4.2) was used to reconstruct haplotypes (Barrett *et al.*, 2005), while PHASE computer program (Version 2.1) was used to obtain haplotypes and haplotype frequencies (Huang *et al.*, 2010; Stephens *et al.*, 2001).

The effects of the SNPs and haplotypes on the production traits were calculated using the general linear model (GLM) procedure of SPSS software (Version 17.0) (Yan *et al.*, 2013). The model was:

$$Trait_{ij} = \mu + Xi + Fj + \mathcal{E}_{ij}$$

where $Trait_{ij}$=16-week BW and 300-day EN; μ = mean of the trait in the population; X_i=fixed effect of genotype or combined haplotype; F_j= fixed effect of hatch and $\mathcal{E}_{ij}$=the random residual.

Least-squares means of each genotype and combined haplotype and the corresponding standard errors were calculated. Variance homogeneity of all the data was tested before multiple comparisons. Homoscedastic data were submitted to Duncan's multiple-range test. When data were not homoscedastic, Dunnett's T3 test was applied.

RESULTS AND DISCUSSION

Identification of genetic variants

We amplified four loci of the Langshan chicken lysozyme gene, including all four exons, 5'UTR-exon boundaries, exon-intron boundaries, and intron-3'UTR boundaries (Table 1). Polymorphisms were detected at the P1 and P2 loci of the lysozyme gene using the SSCP method. Three genotypes (named GG, GA, and AA) were clearly discerned at the P1 locus (Figure 1), while five patterns (designated AA, AB, BB, AC and CC) were observed at P2 locus (Figure 2). The polymorphic DNA amplification fragments were sequenced. Compared with the sequence previously reported in the GenBank (GenBank Accession number:FJ542564), four SNPs (SNP1 g.345G>A; SNP2 g.1726C>T; SNP3 g.1836G>A; SNP4 g.1838A>G) were identified in these chickens (Figure 3). The mutation location and type of the four SNPs are shown in Table 1. The SNP1 resulting in a synonymous mutation, GCG (28Ala)>GCA (28Ala), was located in exon 1. The SNP2 in exon 2 was also a synonymous mutation, TAC(71Tyr)>TAT(71Tyr). The other two SNPs (SNP3 and SNP4) in intron 2 were the non-coding mutations. Most of the previous studies focused on two aspects: 1) the association analyses between the lysozyme protein and the chicken growth traits, 2) the structure and regulation mechanism of the chicken's lysozyme gene. There are few studies on the effects of the lysozyme gene mutation on production traits of chickens. Hou *et al.* (2010) found three SNPs in the lysozyme gene of the Jinghai yellow chicken population (another Chinese indigenous chicken breed). Two of the three SNPs identified (g.345G>A in exon1, g.1726C>T in exon2) were identical to those found in the present study. However, the other mutation (g. 1660T>C in exon2) was not detected in the chicken breed studied here. These results show a difference in the structure of the lysozyme gene between the two Chinese native breeds.

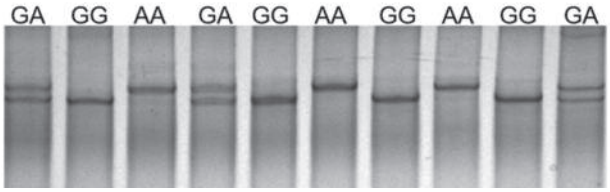

Figure 1 – PCR-SSCP patterns of P1 locus of the Langshan chicken lysozyme gene.

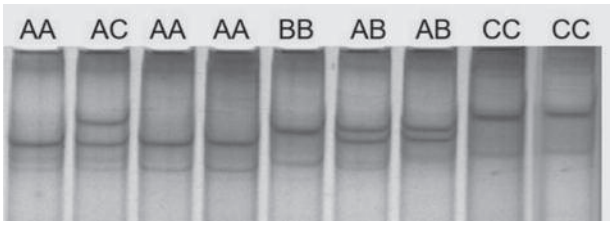

Figure 2 – PCR-SSCP patterns of P2 locus of the Langshan chicken lysozyme gene.

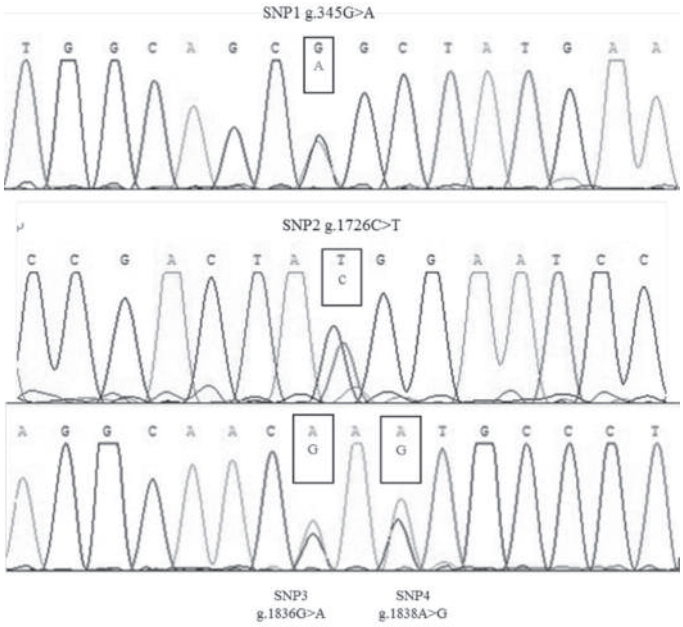

Figure 3 – Sequence results of the four SNPs within the lysozyme gene in Langshan chicken.

Diversity analysis

Genotype frequencies, allele frequencies, value of $\chi 2$ test, He, Ne, and PIC for the P1 and P2 loci were are listed in Table 2. At the P1 locus, allele G was the dominant allele, and the genotype GG was the dominant genotype. At the P2 locus, allele A was the dominant allele and the genotype AA was the dominant genotype. In order to estimate the quality or informativeness of a gene locus as a genetic marker, He and PIC are often used, and PIC has become the most widely used measure since its first application (Nagy *et al.*, 2012). At the two loci (P1 and P2), the Langshan chicken population belonged to intermediate genetic diversity (PIC classification: PIC< 0.25, low polymorphism; 0.25<PIC<0.5, intermediate polymorphism; and PIC>0.5, high polymorphism). In addition, the Chi-square test demonstrated that, at the two loci, this native chicken breed was not in Hardy–Weinberg equilibrium ($p<0.05$), which may be possibly explained by the fact that, as a native breed specially protected and developed by the local government during the last few years, the Langshan chicken population has been under high selection pressure. The sustained artificial selection by the government eventually resulted in an increase in the gene frequency of alleles favored by selection, such as the lysozyme gene (Yan *et al.*, 2015).

Haplotype analysis and extent of linkage disequilibrium

In Figure 4, extent of linkage disequilibrium (LD) between every two of the four SNPs was estimated, and indicated that the LD between pairs of loci varied from complete disequilibrium to almost no disequilibrium.

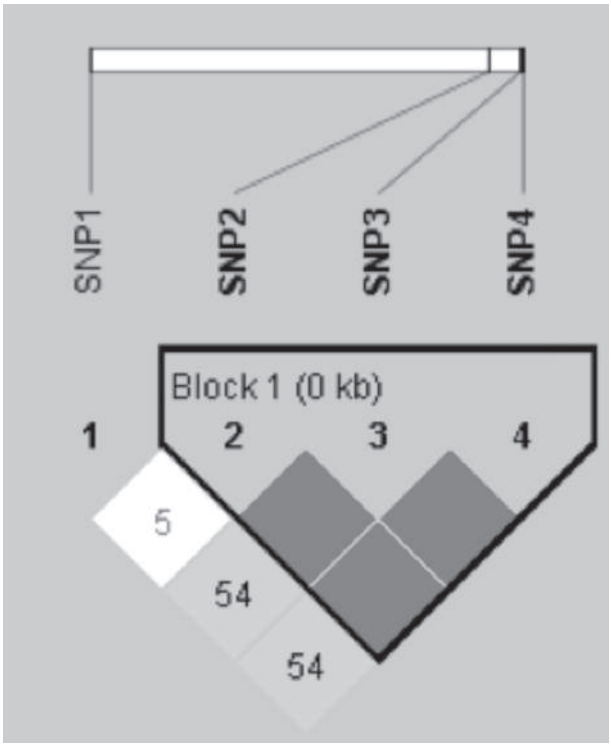

Figure 4 – LD across the SNPs visualized by the Haploview software. Each diamond contained the level of LD measured by "Standard (D'/ LOD)" between the SNPs specified. Redder tones correspond to increasing levels of D'. Values in the cells represented pairwise D' values (%), while empty cells indicated that pairwise D' equalled to one between the corresponding SNPs.

Color scheme was demonstrated according to Haploview standard (D'/LOD) scheme, and a haplotype block formed with the three SNPs (SNP2, SNP3 and SNP4) was identified in the study. The estimated values of linkage disequilibrium analysis among the four SNPs are shown in Table 3. The D' values varied from 0.050 to 1.000, while the r^2 values ranged from 0.002 to 1.000. The D' value between any two of the three SNPs (SNP2, SNP3 and SNP4) was 1.000. Only the r^2

Table 2 – Genotype frequencies, allele frequencies, value of $\chi 2$ test, He, Ne and PIC of the lyzosyme gene in Langshan chickens

Loci	Genotype frequencies(Number)					Allele frequencies			$\chi 2$ (HWE)[a]	He[b]	Ne[c]	PIC[d]
P1	GG		GA		AA	G		A	30.4056(p<0.05)	0,2133	1,4533	0,2630
	0.700(210)		0.213(64)		0.087(26)	0,807		0,193				
P2	AA	AB	BB	AC	CC	A	B	C	29.1865(p<0.05)	0,2700	1,7451	0,3860
	0.547(164)	0.220(66)	0.053(16)	0.150(45)	0.030(9)	0,732	0,163	0,105				

[a]$\chi 2$ (HWE): Hardy–Weinberg equilibrium $\chi 2$ value,H ardy–Weinberg disequilibrium ($p<0.05$); [b]He: gene heterozygosity
[c]Ne: effective allele numbers; [d]PIC: polymorphism information content

Table 3 – Linkage values estimated in the disequilibrium analysis among four SNPs identified in the lysozyme gene of the studied population

SNPs[a]	SNP 1/2	SNP 1/3	SNP 1/4	SNP 2/3	SNP 2/4	SNP 3/4
D'	0,050	0,546	0,546	1,000	1,000	1,000
LOD	0,200	10,670	10,670	5,180	5,180	73,990
r^2	0,002	0,146	0,146	0,023	0,023	1,000

[a]SNPs: estimated LD values (D', LOD score and r2) between polymorphism pairs. SNP 1: g.345G>A; SNP 2: g.1726C>T; SNP 3: g.1836G>A; and SNP 4: g.1838A>G

Table 4 – Haplotype (Hap), haplotype frequency and standard srror of four SNPs in the lysozyme gene within the studied population

Haplotype	SNPs[a]				Frequency	Standard Error
	SNP 1	SNP 2	SNP 3	SNP 4		
Hap 1	G	C	G	A	0,6461	0,0042
Hap 2	G	C	A	G	0,0379	0,0029
Hap 3	G	T	G	A	0,1227	0,0028
Hap 4	A	C	G	A	0,0872	0,0042
Hap 5	A	C	A	G	0,0655	0,0029
Hap 6	A	T	G	A	0,0406	0,0028

[a] SNPs: SNP 1: g.345G>A; SNP 2: g.1726C>T; SNP 3: g.1836G>A; and SNP 4: g.1838A>G

value between SNP3 and SNP4, however, was equal to 1.000. The other two r^2 values (between SNP2 and SNP3, between SNP2 and SNP4) were equal to 0.023. In this situation, r^2 and D' act differently, with D' still equal to 1, but r^2 can be much smaller. It indicates that mutations (SNP2, SNP3 and SNP4) occur in different lineages, but with no recombination between them (between SNP2 and SNP3, between SNP2 and SNP4) (Flint-Garcia *et al.*, 2003). The LOD score is a statistical test often used for linkage analysis in human, animal, and plant populations. Positive LOD scores indicate the presence of linkage. Furthermore, a LOD score greater than 3 is considered and evidence of linkage (Morton, 1955). All of the LOD scores here were positive, and, except for LOD between SNP1 and SNP 2, all of which were greater than 3. This demonstrated the linkage among these SNPs.

Using PHASE computer program, haplotypes were reconstructed in the studied population. Six different haplotypes were detected in the population (Table 4). The dominant haplotype was haplotype 1 (GCGA), with the highest frequency (0.6461). Haplotype 3 (GTGA), with a frequency of 0.1227, ranked second. The frequency of haplotype 2 (GCAG) was the lowest (0.0379).

Effects of individual SNPs and haplotypes on the production traits

The effects of the individual SNPs on the two production traits were analyzed (Table 5). At the P1 locus, the chickens with genotype GA had significantly higher16-week BW than those with GG and AA ($p<0.05$), while individuals with the genotype GG had significantly higher16-week BW than those with AA ($p<0.05$). It demonstrates that the allele G and heterozygote at this locus may have the positive effects on16-week BW in the Langshan chicken population. The results are different from the previous research (Hou *et al.*, 2010). It may be concluded that the breed factor affected the effects of lysozyme gene polymorphisms on production traits. At the P2 locus, the individuals with the genotypes AC (the SNPs combination is CC-GA-AG) and BB (the SNPs combination is TT-GG-AA) presented significantly higher 16-week BW than those with CC (the SNPs combination is CC-AA-GG). Neither of the two loci (P1 and P2) of the lysozyme gene were associated with the total numbers of eggs produced in 300 days in the Langshan chicken population ($p>0.05$). In this study, protein structure and sequence were not changed by the mutations detected, but these mutations did affect the chicken's production traits. There were many other similar studies were reported (Komar, 2007a,b). For example, a silent mutation of the MDR1 gene led to substrate specificity change (Kimchi-Sarfaty *et al.*, 2007), synonymous mutations of the BMP7 gene affected cattle's body weight (Huang *et al.*, 2013), a synonymous mutation of theIGF2 gene was found to be connected with body weight and egg production in Langshan chicken population (Yan *et al.*, 2015). Accordingly, it would be interesting to explore the molecular mechanism of the relationship of the synonymous mutations with the animals' productive performance.

Table 5 – Associations between the four SNPs and two production traits in the studied Langshan chicken population

Loci	Genotypes	Number	Traits (mean ± standard error)	
			16-week BW (kg)	300-day EN
P1 (SNP1)	GG	210	1.551±0.0103[b]	87.880±1.3970
	GA	64	1.611±0.0133[a]	93.080±2.2430
	AA	26	1.503±0.0286[c]	95.310±3.3460
	[1]p value		p=0.001	p=0.054
P2 (SNP 2/3/4)	AA	164	1.560±0.0116[ab]	88.240±1.5490
	AB	66	1.529±0.0174[ab]	90.390±2.4090
	BB	16	1.591±0.0398[a]	92.250±5.9910
	AC	45	1.605±0.0169[a]	92.130±2.7260
	CC	9	1.500±0.0408[b]	92.110±5.6230
	[1]p value		p=0.043	p=0.725

Values with different superscript letters (a, b, ab, c) within the same column differ significantly at ($p<0.05$) .[1] The F-test for genotype effect. BW: body weight, EN: egg number

Table 6 – Associations between combined haplotypes (Hap) of four SNPs and two production traits of Langshan chickens

ID	Combination of haplotypes	Number of combination	Traits (mean ± standard error)	
			16-week BW (kg)	300-day EN
1	Hap3-Hap6	6	1.680±0.0536[a]	89.830±9.7820
2	Hap1-Hap5	24	1.630±0.0168[ab]	91.080±3.5030
3	Hap1-Hap2	12	1.606±0.0318[ab]	92.000±6.5750
4	Hap1-Hap4	18	1.599±0.0252[ab]	95.560±4.2370
5	Hap6-Hap6	2	1.595±0.1550[ab]	110.500±7.5000
6	Hap1-Hap6	12	1.581±0.0339[ab]	98.420±4.6050
7	Hap1-Hap1	140	1.559±0.0129[ab]	87.200±1.6860
8	Hap2-Hap5	4	1.540±0.0679[ab]	82.750±10.4430
9	Hap4-Hap5	8	1.536±0.0624[ab]	97.750±5.3910
10	Hap3-Hap3	8	1.523±0.0536[ab]	89.500±9.3240
11	Hap1-Hap3	48	1.521±0.0214[ab]	88.310±2.9220
12	Hap4-Hap6	6	1.488±0.0507[ab]	91.000±7.8660
13	Hap5-Hap5	3	1.470±0.0902[b]	103.670±4.6310
14	Hap2-Hap2	2	1.465±0.0450[b]	93.500±6.5000
15	Hap4-Hap4	7	1.464±0.0485[b]	88.290±7.5200
	[1]p value		p=0.043	p=0.573

Values with different superscript letters (a, b, ab) within the same column differ significantly at ($p<0.05$). [1]F-test for combined haplotype effect. BW: body weight, EN: egg number

There were six unique haplotypes for the lysozyme gene (Table 4), and 15 haplotype combinations were identified in the studied population (Table 6). In addition, the relationships between these 15 haplotype combinations with the Langshan chicken's production traits are demonstrated in Table 6. No associations were found between haplotype combination and 300-day EN ($p>0.05$), while the effects of these haplotype combinations on 16-week BW were found to be significant ($p<0.05$). The chickens with combined haplotype Hap3-Hap6 (GA-TT-GG-AA) presented the highest body weight. This result is consistent with previous findings on the effect of individual SNPs on chicken's body weight, and the SNP1-GA and SNP2,3,4-BB genotypes (TT-GG-AA) (chickens with AC genotype were the heaviest, but the difference between AC and BB chickens was not significant) were associated with higher body weight (Table 5). The interactions between the individual SNPs may influence the effect of haplotypes, and the inheritance of combined haplotype was more efficient than that of individual SNP (Fallin *et al.*, 2001). Therefore, the combined haplotype Hap3-Hap6 (GA-TT-GG-AA) may be used as a molecular marker in the future for the selection for higher body weight in Langshan chicken. In addition, neither the individual SNPs nor the haplotype combinations in the lysozyme gene were found to be connected with 300-day EN. However, because other egg parameters, such as egg weight and age at first egg, were not included in the research, further studies are needed before it is confirmed that the lysozyme gene has no effects on the chicken's egg production.

CONCLUSIONS

In summary, four SNPs, six haplotypes and 15 combined haplotypes of the lysozyme gene of a Chinese native chicken breed (Langshan chicken) were reported for the first time, and their associations with the chicken's productive parameters were analyzed. The results revealed that the combined haplotype Hap3-Hap6 (GA-TT-GG-AA) may be used as a molecular marker in the future for the selection of higher body weight in Langshan chickens. However, before confirming the lysozyme gene as a selection marker for all chicken populations, further studies on the molecular mechanisms and on the bioactivity of this gene in other chicken breeds are required.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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