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Analysis of Polymorphisms in Exons of the *LYZ* Gene and Effect on Growth Traits of Jinghai Yellow Chicken

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Abstract: Lysozyme plays important role in vertebrates and invertebrates for its unique protein structure and enzyme activity. In the present study, the potential physiological function of the chicken lysozyme gene (*LYZ*) was studied by SNP. 329 J^+ line Jinghai Yellow chicken were involved in this experiment. To determine gene mutations, DNA analysis of the *LYZ* gene, including Single Strand Confirmation Polymorphism (SSCP) and direct DNA sequencing, was performed. Chicken Body Weight (BW) were analyzed by GLM of SAS software. DNA sequencing demonstrated a G111A transition of exon 1, a T1426C transition and a C1492T transition of exon 2, all of them were silent mutations that caused no alteration in amino acid sequence. Statistical analysis indicated that, AA and CC genotype related with high BW, TT and CN genotype contributed to low BW. Therefore, results suggested that there was a possibility of the *LYZ* gene SNPs acting as a genetic marker for growth traits of Jinghai Yellow chicken.

Key words: Chicken, genotype, growth traits, *LYZ* gene, polymorphism, SSCP

INTRODUCTION

Lysozymes are defined as 1,4-β-N- acetylmuramidases cleaving the glycosidic bond between the C-1 of N-acetylmuramic acid (Mur N Ac) and the C-4 of N-acetylglucosamine (Glc N Ac) in the bacterial peptidoglycan (Lee and Brey, 1995). They are widespread enzymes in vertebrates, invertebrates as well as bacteria, phages and plants, plays an essential role in lives for its unique protein structure and enzyme activity (Schindler *et al*., 1977). Chicken egg white lysozyme is an important bio-enzyme used in foodstuff and fermentation industry. The chicken *LYZ* gene was purified by molecular cloning from two gene libraries, composed four exons (Baldacci *et al*., 1979), then the study of gene function far behind protein function. On *LYZ* gene, studies are progressing in gene expression (Akinalp *et al*., 2007) and preventive medicine (Sun *et al*., 2006), while mutation in the chicken *LYZ* gene has not been reported. Variant forms of human lysozyme are known to lead to hereditary non-neuropathic renal amyloidosis and so far, four different mutations of the *LYZ* gene have been reported, Ile56Thr, Asp67His (Pepys *et al*., 1993), Trp64Arg (Valleix *et al*., 2002) and Phe57Ile (Masahide *et al*., 2003). Despite extensive studies on the structure (Hideki, 2001), catalytic mechanism (Tsai, 1997), relationship between structure and activity (Tsuchiya *et al*., 2007), phylogeny (Li *et al*., 2005), immunology (Hultmark, 1996) and genetics (Phi-Van *et al*., 1990; Rogerio *et al*., 2003), the functions of *LYZ* gene in higher vertebrates are still unknown. The possibility that this enzyme may have important physiological functions had been suggested (Jolles and Jolles, 1984). To study gene function, candidate gene method is more frequently used and the candidate gene locus can be a direct target to study the linkage between polymorphism and traits (Short *et al*., 1997; Nagaraja *et al*., 2000).

In the present study, the potential physiological function of the chicken *LYZ* gene was studied by SNP. The association of SNP with chicken growth traits was evaluated.

MATERIALS AND METHODS

The sixth generation of J^+ line Jinghai Yellow chicken from Jinghai Group Corporation Yellow Chicken Breeding Center (Haimen, China) were used in the current study. Jinghai Yellow chicken is a new breed developed from local yellow chicken, which charactered by small body type. The J^+ line was established by crossing broiler sires for slow growth, with Chinese local yellow chicken. All birds were raised in floor pens and had free access to feed and water. Commercial corn-soybean-based diets that met all NRC requirements (National Research Council, 1994) were provided in the study. The BW of 329 (179 female, 150 male) chicken was measured in grams at hatch, 4, 8 and 12 week (wk), kilograms at 16 wk of age.

The DNA was extracted from blood cells. Chicken blood was homogenized in a lysis buffer [2M urea, 100 mM Tris-HCl (pH 8.0), 1%SDS, 100 mM EDTA], then treated with proteinase K, RNase A and TE [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH8.0)] at 55°C for 12 h, followed by phenol/chloroform extraction and ethyl alcohol precipitation. The genomic DNA in the pellet was dissolved in TE and used as a template for PCR.

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Table 1: Primers designed for the exons of the *LYZ* gene

Primer name	Primer sequence (5' ➔ 3')	Annealing temperature	Amplicon
L1	F: CTGGCAACATGAGGTCTTT	56°C	Exon 1
	R: CATTCCAACATCACGCAGA		(243 bp)
L4	F: CTTTGAGGGTTTTGTTTTC	54°C	Exon 2
	R: GCATTCGTTGCCTTGAGA		(232 bp)
L6	F: GACATAACAGCGAGCGTGAA	58°C	Exon3
	R: CGGCAGCCTCTGATACAC		(206 bp)
L10	F: CAGACATAACAGCGAGCGTGAA	62°C	Exon 4
	R: AACTGCCAAGCGGGTAGCG		(288 bp)

Primers for PCR were designed in exon regions to amplify the putative lysozyme coding sequence based on the nucleotide sequences in the GenBank databases of NC006088 (Table 1).

The PCR reaction volume was 20 μL, which consisted of 1 μL of the template, 2 μL of 10 x buffer [100 mM Tris-HCl (pH8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin], 2.2 μL of $MgCl_2$ (25 mM), 0.8 μL dNTPs, 1 μL of the forward primer (10 pmol), 1 μL of the reverse primer (10 pmol), 0.2 μL of Taq polymerase (5U/μL) (Sangon Biotechnology Co.) and 11.8 μL of Dnase free distilled water. The mixture was preheated for 6 min at 95°C and subjected to 30 cycles of 1 min at 94°C, 30s at the required temperature for each primer pair (Table 1), 30s at 72°C and a final 10 min incubation at 72°C. The PCR products were analyzed by electrophoresis on a 1.0% agarose gel and visualized by UV irradiation of the ethidium bromide-stained gel. SSCP analysis was performed using 3 μL of the PCR product. Samples were electrophoresed at a 10% polyacrylamide gel for 11 h (150V).

PCR products of polymorphisms observed by SSCP analysis were sent to Sangon Biotechnology Co. for sequencing. The DNA sequences of samples were compared with that in the GenBank databases of NC006088.

The data were analyzed by a randomized complete block design. The association between the genotype and BW was analyzed by GLM of SAS software (SAS Institute, 2001). The model was fitted with the genotype (G) and Sex (S) as fixed effects, Family (F) as random effects, G*S as interaction of G by S effect, as follow:

$$Y = \mu+G+S+F+G*S+e$$

Where Y is the dependent variable, μ the population mean and e the random error. Significant differences in the mean were compared by Least Significant Difference (LSD). All values were statistically significant at $p<0.05$ and were presented as least square means±standard error means.

RESULTS

SSCP analysis revealed three patterns of migrating bands in exon 1 (Fig. 1), named GG, GA, AA and that five patterns of migrating bands in exon 2, named CC, TT, CT, CN and TN (Fig. 2). Direct DNA sequencing of exon 1 and 2 showed three single base transition at the 111, 1426 and 1492 bp position of the LYZ gene, G111A, T1426C, C1492T.

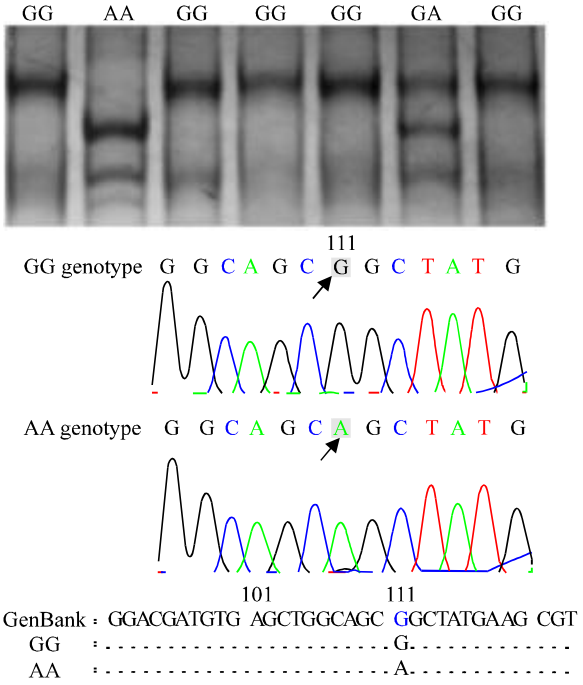

Fig. 1: Polyacrylamide gel electrophoresis picture of exon 1. There was one single nucleotide polymorphisms of G111A. Three genotypes (GG, GA and AA) were observed from the gel picture. According to sequencing, GG bands represented G111G, AA bands represented A111A, GA bands represented G111A

For exon 1, the results indicated that the G111A SNP was significantly associated with BW at 12 and 16 wk of age ($p<0.05$). The BW of genotype AA was higher than that of GG and GA genotype at 4-16 wk of age (Table 2). For exon 2, statistical analysis revealed that the BW of genotype TT was significantly associated with low BW at 4 and 8 wk of age ($p<0.05$), genotype TN and CC contributed to high BW ($p<0.05$) (Table 3). Table 4 showed all genotype combainations constructed from the 3 SNPs. Nine genotype combainations were

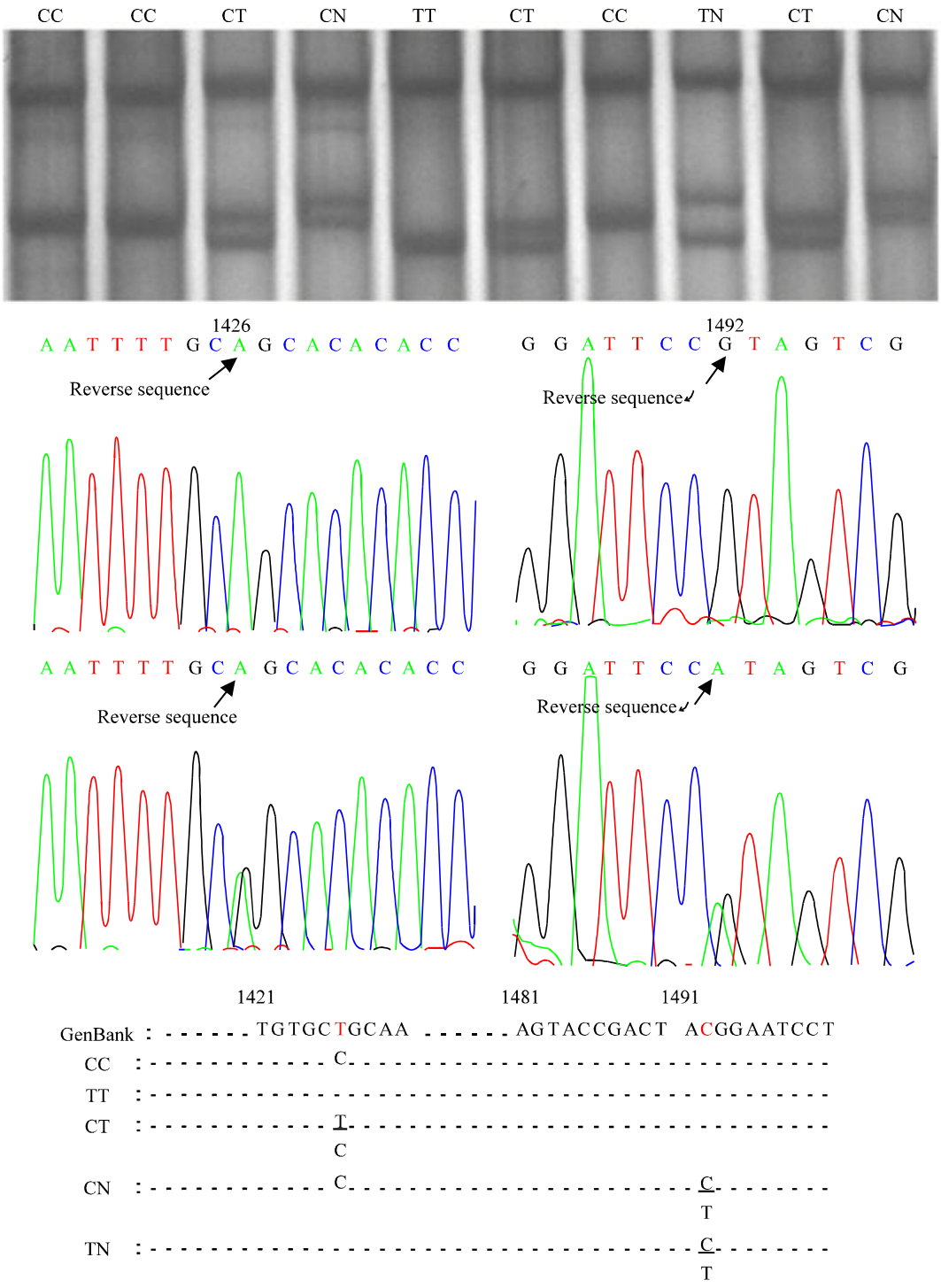

Fig. 2: Polyacrylamide gel electrophoresis picture of exon 2. There were two single nucleotide polymorphismes of T1426C and C1492T. Five genotypes (CC, TT, CT, CN and TN) were observed from the gel picture. According to sequencing, CC bands represented C1426C/C1492C, TT bands represented T1426T/C1492C, CT bands represented T1426C/C1492C, CN bands represented C1426C/C1492T and TN bands represented T1426T/C1492T. For the T1426C SNP, CC and CN represented C1426C genotype, TT and TN represented T1426T genotype and CT represented T1426C genotype. For the C1492T SNP, CC, TT and CT represented C1492C genotype, CN and TN represented C1492T genotype

Table 2: Least square means of BW for growth traits in Jinghai Yellow chicken with SNP genotypes in exon 1 of the *LYZ* gene

			BW				
Genotype	N	Frequency	Hatch/g	4 wk/g	8 wk/g	12 wk/g	16 wk/kg
GG	260	0.79	32.21±0.8	154.15±23.2	475.03±70.7	821.69±104.6^{b}	1.22±0.22^{b}
GA	49	0.15	31.94±0.6	158.12±15.3	478.53±43.1	851.29±79.8^{b}	1.21±0.17^{b}
AA	20	0.06	29.50±0.5	170.007±13.1	557.50±37.4	996.00±96.8^{a}	1.48±0.15^{a}

^{ab}Means within lines with different superscripts differ significantly (p<0.05)

Table 3: Least square means of BW for growth traits in Jinghai Yellow chicken with SNP genotypes in exon 2 of the *LYZ* gene

			BW				
Genotype	N	Frequency	Hatch/g	4 wk/g	8 wk/g	12 wk/g	16 wk/kg
CC	188	0.57	32.20±0.9	156.35±19.5^{a}	488.29±65.6^{ab}	841.80±103.7	1.24±0.18
TT	11	0.33	32.40±0.6	105.00±21.4^{b}	325.50±42.3^{c}	707.40±82.5	1.09±0.08
CT	97	0.30	32.12±0.5	150.31±22.9^{a}	461.87±54.2^{abc}	809.47±80.4	1.17±0.13
CN	22	0.07	32.13±0.5	131.67±16.3^{ab}	408.17±25.9^{bc}	761.00±97.8	1.08±0.07
TN	11	0.03	30.67±0.2	175.00±11.3^{a}	555.50±54.1^{a}	809.33±70.4	1.18±0.07

$^{a-c}$Means within lines with different superscripts differ significantly (p<0.05)

Table 4: Least square means of BW for growth traits in Jinghai Yellow chicken with SNP genotype combinations of the *LYZ* gene

			BW				
Genotype combination	N	Frequency (%)	Hatch/g	4 wk/g	8 wk/g	12 wk/g	16 wk/kg
GG/CC	135	41.03	32.27±0.8	155.97±11.5^{ab}	486.55±38.4^{a}	834.59±85.5^{ab}	1.22±0.12^{ab}
GG/CT	89	27.05	32.17±0.5	149.04±14.7^{ab}	459.84±45.6^{ab}	812.66±71.9^{b}	1.18±0.08^{b}
GA/CC	33	10.03	32.07±0.5	157.36±18.5^{ab}	490.62±63.3^{a}	876.29±108.8^{ab}	1.25±0.06^{ab}
GG/CN	14	4.26	32.14±0.7	150.71±14.4^{ab}	413.00±24.3^{ab}	704.71±96.6^{b}	1.09±0.08^{bc}
GG/TN	11	3.34	30.67±0.5	175.00±17.3^{a}	555.50±58.1^{a}	809.33±54.2^{b}	1.18±0.05^{b}
GG/TT	11	3.34	32.40±0.4	154.00±23.4^{ab}	325.50±42.3^{b}	707.40±92.7^{b}	1.09±0.03^{bc}
AA/CC	20	6.08	29.50±0.9	170.00±14.1^{a}	557.50±38.9^{a}	998.00±106.8^{a}	1.47±0.09^{a}
GA/CN	8	2.43	32.23±0.7	125.16±10.4^{b}	384.54±68.7^{b}	735.91±79.4^{b}	0.99±0.05^{c}
GA/CT	8	2.43	31.61±0.4	180.79±11.3^{a}	551.19±54.5^{a}	734.56±86.8^{b}	1.05±0.07^{bc}

$^{a-c}$Means within lines with different superscripts differ significantly (p<0.05)

identified, there was significant association of genotype combination with BW. Genotype combainations, AA/CC, GA/CC and GG/CC, were significantly associated with high BW at 4-16 wk of age (p<0.05), while GG/CN, GA/CN and GG/TT contributed to low BW at the same ages (p<0.05). Genotype combination GG/TN contributed to high BW at 4 and 8 wk of age (p<0.05).

DISCUSSION

Growth is a comprehensive reflection of development of various parts of a chicken body and its final expression is the result of interaction among genetic, nutritional and environmental factors (Scanes *et al*., 1984). Uncovering the molecular mechanisms of growth will contribute to more efficient selection for growth in broiler chickens (Deeb and Lamont, 2002). The present study elucidated the direct or indirect effect of the *LYZ* gene on chicken growth.

Chicken *LYZ* gene mutation has not been reported, however, in the present study , 3 polymorphisms, G111A within exon 1, T1426C and C1492T within exon 2, were found by direct sequencing. From statistical of genotypes, the frequency of AA, GA, CN and TN were quite low, while GG and CC shared more than half. The genotype distribution was extremely unbalance. This indicated that the chicken had been selected out off equilibrium in exon1 and 2 of the *LYZ* gene. From sequencing, T1492T mutation was not found. These may due to breed or line selection for commercial profit. Mutations found in this study were silent mutations that caused no alteration in amino acid sequence, for their locations were in the third seat of codon.

Chicken egg white lysozyme is an well studied bio-enzyme. Despite of extensive structural, physico-chemical, crystallographic, immunological and evolutionary studies devoted to lysozymes, their biological role is still not exactly known (Jolles and Jolles, 1984). Significant associations of the SNPs and growth were present by one factor analysis in this study. Genotype AA, TN and CC contributed to high BW, genotype CN and TT contributed to low BW. Association of genotype combination with BW analysis proved the former discoveries, BW of AA/CC genotype combination was the highest and GA/CN was the lowest. The current study was the first to report such a relationship between the *LYZ* gene and growth in Jinghai Yellow chicken. Research on feedstuff which added lysozyme revealed that BW of broiler fed with high lysozyme feed higher (Zhang *et al*., 2006; Lu *et al*., 2007). This may be explained by its function in immune system.

Lysozyme is an antimicrobial innate immune molecule (Shimada *et al*., 2008), chicken with high expression of lysozyme may be healthier and grow faster. Study of gene polymorphism is one of the primary methods to

determine whether specific genes are related to economic traits in farm animals. Haplotype of 2 polymorphisms of the chicken apolipoprotein B gene affected body growth and fatness traits (Zhang *et al*., 2006). Polymorphisms in the promoter region of chicken prolactin associated with egg production (Cui *et al*., 2006). The results from the current study indicated that the *LYZ* gene polymorphisms was associated with BW. The *LYZ* gene is, therefore, a potential marker for use in marker-assisted selection programs. Select AA/CC individuals may lead to fast growing breed, while select GA/CN chicken may develop low growing beed.

Conclusion: In the present study , 3 SNPs were found in exons of the *LYZ* gene by sequencing and significant effect of genotype on growth traits is demonstrated. Results of the statistical analysis indicated that the polymorphisms may have direct impact on the function of the chicken *LYZ* gene and thus on the growth traits, or were linked with potential major loci or genes affecting the body growth. To make further progress, there is a need to evaluate polymorphism and growth traits in other different breeds of chicken.

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