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Full Length Research Paper

Polymorphisms in 5'-upstream region of the myostatin gene in four chicken breeds and its relationship with growth traits in the Bian chicken

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Myostatin is a member of the transforming growth factor-β family with a key role in inhibition of muscle growth by negative regulation of both myoblast proliferation and differentiation. Single nucleotide polymorphisms (SNPs) of the 5'-upstream region of the myostatin gene were detected by single-stranded conformation polymorphism (SSCP) and DNA sequencing in the Bian, Jinghai, Youxi and Arbor Acre chickens. Four novel mutations (G673A, G985C, G1085A, and A1278T) were detected. Only one locus (A1278T) displayed polymorphism in the Bian chicken, and thus, three genotypes (QQ, QR, and RR) were formed. Association analysis showed that the Bian chicken of the RR and QR genotypes had significantly higher body weight than those of the QQ genotype (P < 0.05 or P < 0.01) from 14 to 18 weeks of age. The novel mutation (A1278T) could be a useful molecular marker for planning improvements in the growth traits of the Bian chicken.

Key words: Bian chicken, myostatin gene, polymorphism, growth traits.

INTRODUCTION

Myostatin is a member of the transforming growth factor-β family with a key role in inhibition of muscle growth by negative regulation of both myoblast proliferation and differentiation. Hence, myostatin acts to limit skeletal muscle mass by regulating both the number and growth of muscle fibers. The myostatin gene consists of three exons and two introns in all species studied (Bellinge et al., 2005), and it has a single long open reading frame that produces a 376 amino-acid-long protein. Similar to other TGF-β factors, the myostatin amino acid sequence includes a signal sequence for secretion, a proteolytic processing site and a carboxy-terminal region containing nine cysteines residues (McPherron and Lee, 1997). The effects of the myostatin gene were first described in mice. Later, an extreme form of muscularity (double muscling) seen in the Belgian Blue and Piedmontese cattle breeds was shown to result from mutations in the coding region of the myostatin gene (Kambadur et al., 1997; Wiener et al., 2009). In chickens, Gu et al. (2003) found out that the myostatin gene does not only regulate the skeletal muscle development, but also participate in the fat metabolism and disposition. Ye et al. (2007) evaluated the effects of several polymorphisms of the myostatin gene in three elite commercial broiler chicken lines on performance and mortality traits and suggested that the myostatin gene had pleiotropic effects on broiler performance**.**

Four chicken breeds (Bian, Jinghai, Youxi, and Arbor Acre chickens) were used in the current study. The Bian chicken is an eminent Chinese native breed raised for dual purposes (high-weight eggs and high-quality meat), which is characterized by its adaptability to poor quality feeds and cold. The Jinghai chicken is a national cultivated meat breed (minitype) and the Youxi chicken is a local breed raised for dual purposes. The Arbor Acre

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Table 1. The detailed information of the primers.

Primer	Primers sequence (5′-3′)	Position in AF346599	Annealing temperature (°C)	Fragment length (bp)
P1	F:GTTAGACAACCAAGCCACAG R: CCAGGGCACAGGTATTGAC	351 - 526	56	176
P2	F: GCCCTGGAAAGGCAATACA R: TGGGACCATGATGTCAGTTTAT	520 - 736	56	217
P3	F: GACATCATGGTCCCAACTTC R: TCTGTAGGCTCTGTATTATCCC	722 - 1008	56	287
P4	F: TATAAGCCATGACCTGTAAT R: CTTGTAAAAGACATAAACCA	899 - 1139	56	241
P5	F: AGATTTACTGACAGAAGGGATT R: ATAAGTGCTAACTATTGGACC	1094 - 1359	56	266

chicken, a commercial broiler selected for meat production, is well known for rapid growth. The objectives of this study were to identify the polymorphisms of the 5'-upstream region of the myostatin gene in the Bian, Jinghai, Youxi and Arbor Acre chickens using the method of polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and gene sequencing, and to evaluate the associations of the polymorphisms of the myostatin gene with growth traits in the Bian chicken.

MATERIALS AND METHODS

Chicken populations

Blood samples of 249 fowl of the four breeds (Bian, Jinghai, Arbor Acre and Youxi chickens) were collected randomly by investigators from the Institute of Animal Husbandry and Veterinary of Shanxi Academy of Agricultural Sciences, Jiangsu Jinghai Poultry Industry Group Co., Ltd., and the National Gene Bank for local chickens in Poultry Institute, Chinese Academy of Agricultural Sciences. The body weight of each female Bian chicken (n = 137) was measured in grams at hatching, 6, 8, 10, 12, 14, 16, and 18 weeks. These birds were hatched on the same day, reared in the pens, and transferred to the laying pens at the age of 10 weeks. Birds had access to feed (commercial corn-soybean diets meeting the National Research Council's [NRC] requirements) and water *ad libitum*. Genomic DNA was extracted from the whole blood using the phenol-chloroform method.

PCR amplifications and genotyping

Five pairs of primers (Table 1) were designed to amplify the 5'-upstream region of the myostatin gene according to the chicken DNA sequence of the myostatin gene (GenBank Accession No. AF346599). PCR was carried out in 25 µl reactions consisting of 2 µl template DNA (50 ng/µl), 1 µl each primer (10 µmol/L), 2.5 µl 10 ×buffer, 1.5 µl Mg^{2+} (25 mmol/L), 0.2 µl Taq DNA polymerase (5 U/µl), 2 µl dNTPs (2 mmol/L), and 14.8 µl sterilized water. The amplification conditions were: denaturation at 94°C for 6 min; followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, as well as a final elongation at 72°C for 10 min. The PCR process was completed by DNA Engine® Peltier Thermal cycler.

For single-strand conformation polymorphism (SSCP) analysis, 2 µl of each amplification product was mixed with 7 µl denaturing buffer: 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanole FF, 10 mmol/L ethylenediaminetetraacetic (EDTA) (pH 8.0), and 10% glycerol. The samples were heat-denatured at 98°C for 10 min, and were chilled in ice for 5 min. Electrophoresis was performed at 150 V on 10% non-denaturing polyacrylamide gel (29:1) for 11 to 13 h at 16°C. The electrophoresis process was completed by DYCZ-24A type electrophoresis meter and the area of the gel is 170 × 170 mm. The gels were stained with 0.1% silver nitrate. Genotypes were recorded according to band patterns.

PCR products of each homozygote were purified with a DNA Fragment Quick Purification/Recover Kit. The purified PCR products were ligated to pGEM-T easy vector, and transformed into DH5-α *Escherichia coli*. Three positive recombinant colonies for each genotype were chosen. Sequencing was performed on an ABI-PRISM3730 sequencer to identify the mutation site.

Statistical analysis

Marker-trait association analyses were performed with the SPSS general linear model procedure. The genetic effects were analysed using the following model:

$$yij = \mu + Gi + e$$

where, yij are the growth traits; µ is the overall mean; Gi is the genetic effect of myostatin gene and e is the residual error.

RESULTS

SSCP analysis

Analysis of the PCR products of primers P2, P4 and P5

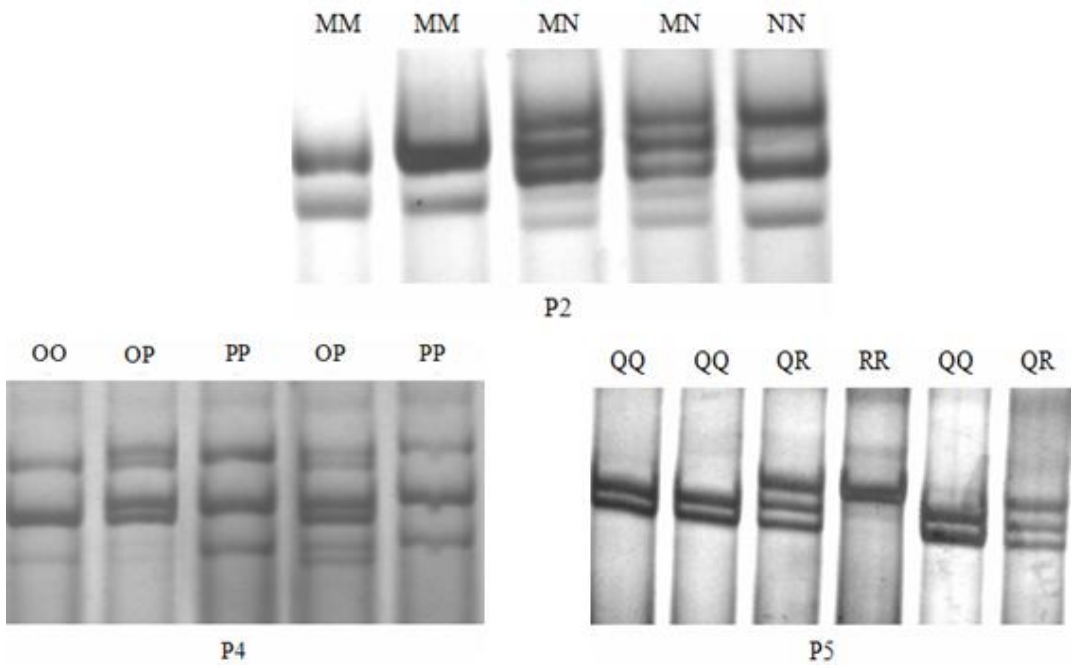

Figure 1. SSCP analysis of PCR amplification in different chicken breeds.

displayed polymorphisms (Figure 1). For primer P2, two chicken breeds (Jinghai and Youxi chickens) displayed three genotypes (MM, MN, and NN). For primer P4, two chicken breeds (Jinghai and Youxi chickens) displayed three genotypes (OO, OP, and PP), while the Bian and Arbor Acre chickens displayed monomorphism for both primers P2 and P4. For primer P5, four chicken breeds all displayed three genotypes (QQ, QR and RR).

Sequence analysis

The PCR products of the MM, NN, OO, PP, QQ and RR genotypes were cloned and sequenced (Figure 2). Sequencing revealed one nucleotide mutation (G673A) in the 5'-upstream region of the myostatin gene between MM and NN genotypes. Sequencing revealed two nucleotide mutations (G985C and G1085A) in the 5'-upstream region of the myostatin gene between OO and PP genotypes. Sequencing revealed one nucleotide mutations (A1278T) in the 5'-upstream region of the myostatin gene between QQ and RR genotypes.

Genotype and allele frequencies

Genotype and allele frequencies of the myostatin gene are presented in Table 2. For primer P2, allele M was fixed in the Bian and Arbor Acre chickens. Allele M was predominant in the Jinghai and Youxi chickens with frequencies of 0.950 and 0.783, respectively. For primer P4, allele O was fixed in the Bian and Arbor Acre chickens. Allele O was predominant in the Jinghai and Youxi chickens with frequencies of 0.960 and 0.817, respectively. For primer P5, allele Q was predominant in the four chicken breeds with frequencies of 0.912, 0.710, 0.967 and 0.797, respectively.

Marker-trait association analysis

The results of the general linear model (GLM) analysis of the association between the myostatin gene polymorphisms and growth traits for the female Bian chickens are presented in Table 3. The body weight of the Bian chickens of the QQ genotypes were the lowest from hatching to 18 weeks. Bian chickens of the QR genotype had higher body weight than those of the QQ genotype ($P < 0.05$ or $P < 0.01$) from 8 to 12 weeks of age. Chickens of the RR and QR genotypes had higher body weight than those of QQ genotype from 14 to 18 weeks of age. The differences for growth traits between the QR and RR genotypes were not significant ($P > 0.05$) from hatch to 18 weeks.

DISCUSSION

Polymorphisms of the myostatin gene

Although, the myostatin gene is highly conserved in gene structure among vertebrate species (Karim et al., 2000; McCroskery et al., 2003), the myostatin gene was highly polymorphic in chickens. Baron et al. (2002) identified

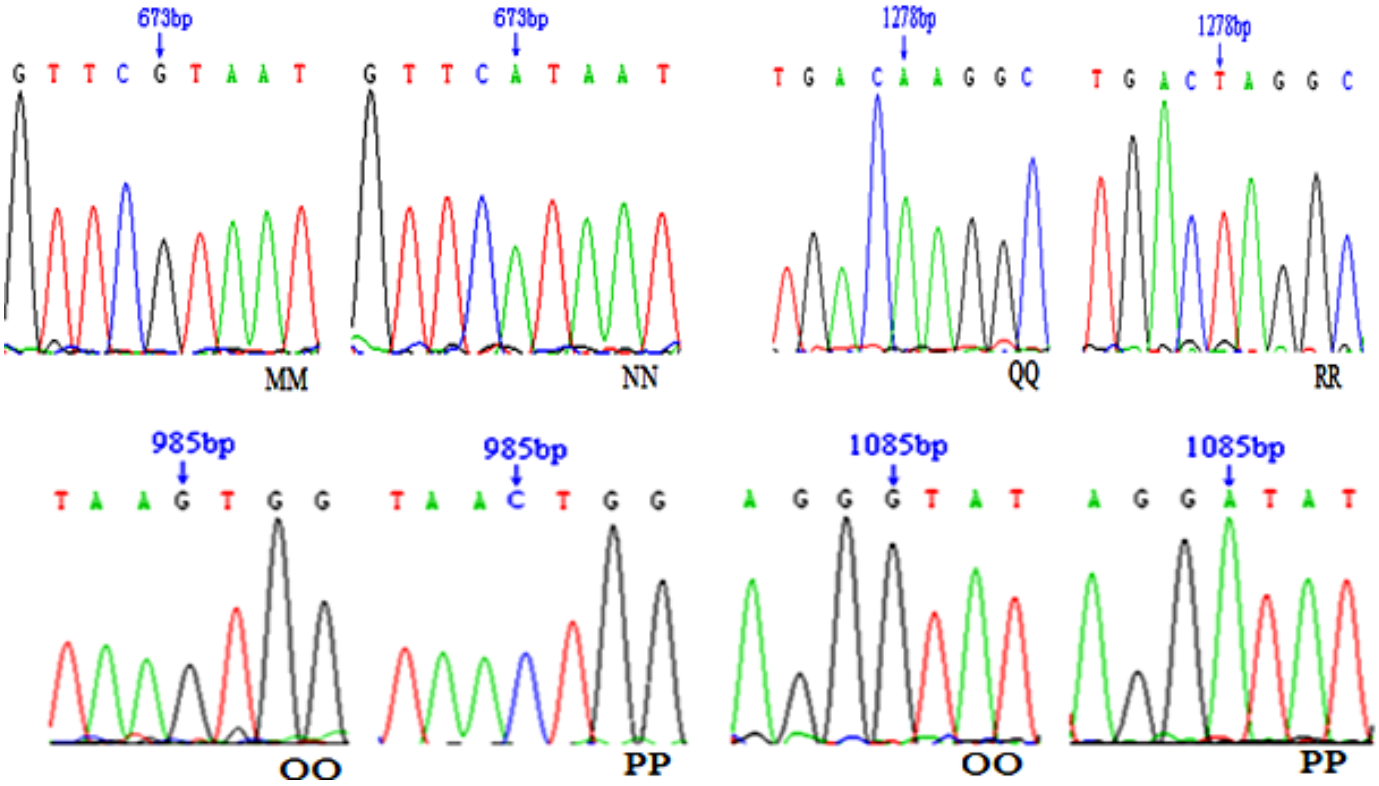

Figure 2. Sequence alignment of different genotypes of chickens.

Table 2. Genotype and allele frequencies of the 5'-upstream region of the myostatin gene in the four chicken breeds.

Breed			Bian chicken	Jinghai chicken	Youxi chicken	Arbor Acre chicken
	Number		**137**	**50**	**30**	**32**
P2	Genotype frequencies	MM	1.000 (137)	0.920 (46)	0.633 (19)	1.000 (32)
		MN	0.000 (0)	0.060 (3)	0.300 (9)	0.000 (0)
		NN	0.000 (0)	0.020 (1)	0.067 (2)	0.000 (0)
	Allele frequencies	M	1.000	0.950	0.783	1.000
		N	0.000	0.050	0.217	0.000
P4	Genotype frequencies	OO	1.000 (137)	0.920 (46)	0.667 (20)	1.000 (32)
		OP	0.000 (0)	0.080 (4)	0.300 (9)	0.000 (0)
		PP	0.000 (0)	0.000 (0)	0.033 (1)	0.000 (0)
	Allele frequencies	O	1.000	0.960	0.817	1.000
		P	0.000	0.040	0.183	0.000
P5	Genotype frequencies	QQ	0.869 (119)	0.620 (31)	0.967 (29)	0.625 (20)
		QR	0.088 (12)	0.180 (9)	0.000 (0)	0.344 (11)
		RR	0.044 (6)	0.200 (10)	0.033 (1)	0.031 (1)
	Allele frequencies	Q	0.912	0.710	0.967	0.797
		R	0.088	0.290	0.033	0.203

The numbers in the brackets are the individuals that belong to the respective genotypes.

Table 3. Associations between the genotypes and body weight carcass traits for P5 in the Bian female chicken.

Age	QQ (119)	QR (12)	RR (6)
Hatch	35.15 ± 0.36	36.83 ± 1.12	37.33 ± 1.59
6 weeks	425.67 ± 5.29	452.50 ± 16.65	467.00 ± 23.54
8 weeks	582.91 ± 6.52^{b}	635.58 ± 20.52^{a}	641.00 ± 29.02^{ab}
10 weeks	746.24 ± 8.79^{b}	822.42 ± 27.69^{a}	748.50 ± 39.17^{ab}
12 weeks	907.52 ± 10.36^{B}	1010.67 ± 32.63^{A}	993.50 ± 46.14^{AB}
14 weeks	1072.61 ± 12.77^{b}	1172.25 ± 40.20^{a}	1204.83 ± 56.85^{a}
16 weeks	1183.69 ± 14.53^{Bb}	1298.75 ± 45.76^{ABa}	1382.50 ± 64.71^{Aa}
18 weeks	1269.61 ± 15.81^{Bb}	1387.75 ± 49.79^{ABa}	1488.17 ± 70.42^{Aa}

Values are presented by the least squares means ± standard errors. Means within a line with different lowercase superscripts differ significantly ($P < 0.05$); means within a line with different capital superscripts differ very significantly ($P < 0.01$).

seven single nucleotide polymorphisms (SNPs) and one deletion in the exon 2 of the myostatin gene in broiler and/or layer chicken lines. Ye et al. (2007) identified thirteen SNPs in the exons 1, 2 and 3 and the introns 1 and 2 of the myostatin gene in three elite commercial broiler lines. In the present study, four mutations (G673A, G985C, G1085A, and A1278T) were detected in the Jinghai and Youxi chickens, while merely one mutation (A1278T) was detected in the Bian and Arbor Acre chickens in the 5'-upstream region of the myostatin gene. It showed that the Bian and Arbor Acre chickens were more conserved than the Jinghai and Youxi chickens in the amplified region. Gu et al. (2002) also scanned the 5'-upstream region of the myostatin gene and detected five SNPs in different chicken lines. When comparing with the chicken DNA sequence of the myostatin gene (GenBank Accession No. AF346599), it showed that the five SNPs found by Gu et al. (2002) were G1336A, T1346C, G1473A, A1491G, and C1503T. In this study, mutation G1336A was not found in the amplified region (Position in AF346599:351-1359), but four novel mutations were found, of which three (G985C, G1085A, and A1278T) were included in the amplified regions by Gu et al. (2002).

Myostatin gene and its relationship with some economic traits

Polymorphisms for the myostatin gene have been used in marker-assisted selection in cattle, but not in chickens. Casas et al. (1998) showed that calves with two copies of the inactive allele of the myostatin gene were more likely to die before weaning, that calves with one copy of the inactive allele had leaner and more muscled carcasses than animals without inactive alleles, and that calves with zero copy of the inactive allele were the highest in fat content. Two polymorphic sites (G2100A and G2109A) in the exon 1 of the myostatin gene in Wenling native chicken were studied and the two mutations had important genetic effects on carcass traits (Zhu et al., 2007). Ye et al. (2007) found out that myostatin SNPs had significant associations with growth and mortality traits in broiler chickens. Gu et al. (2003) reported a polymorphic site in the 5'-upstream region of the myostatin gene, which was associated with hatch weight in F_2 chickens from a cross of broiler and Silky chicken, while in the present study, we found out that the differences between the QQ, QR and RR genotypes on hatch weight were not significant ($P > 0.05$). However, association analysis showed that Bian chickens of the QR genotype had higher body weight than those of the QQ genotype ($P < 0.05$ or $P < 0.01$) from 8 to 12 weeks of age. Bian chickens of the QR genotype also had higher body weight than those of the RR genotype from 10 to 12 weeks of age, although, the difference was not significant ($P > 0.05$). It seems that there exist some interaction effects during these periods. The findings from the mutation A1278T in the Bian chicken were very interesting. Bian chickens of the QR and RR genotypes had significantly higher body weight than those of the QQ genotype ($P < 0.05$ or $P < 0.01$) from 14 to 18 weeks of age. Allele R was the additive gene on growth traits. Bian chickens had a copy of the mutant allele which had increased body weight by 99.64 (at 14 weeks old) to 118.14 g (18 weeks) when compared with the wild type. The homozygotes carried two copies of the mutant allele which had increased body weight by 132.22 (14 weeks) to 218.56 g (18 weeks) when compared with the wild type. The analysis showed that a transcription factor binding site (V-Maf) was formed when allele A changed to T at this locus. Maf recognition elements in the DNA sequence can bind with the protein of Maf superfamily and regulate the transcription of the corresponding gene (Yun et al., 2008). Thus, we speculate that the mutation may function as a negative regulator by depressing the expression of the myostatin gene on transcription level in the Bian chicken, although, expression level studies will be needed to confirm this hypothesis.

Conclusion

In this research, four novel mutations (G673A, G985C,

G1085A, and A1278T) were detected in the 5'-upstream region of the myostatin gene. These results suggest that the myostatin gene may have certain effects on growth traits in the Bian chicken. The novel mutation (A1278T) could be a useful molecular marker for planning improvements in the growth traits of the Bian chicken.

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