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Associations of Single Nucleotide Polymorphisms in *BMPR-IB* Gene with Egg Production in a Synthetic Broiler Line

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ABSTRACT : Egg production traits are economically important both for egg-laying and broiler lines of chicken. In sheep, the Q249R mutation in BMPR-IB is associated with ovulation rate. The present study cloned a partial chicken *BMPR-IB* fragment which contained the corresponding ovine Q249R mutation, including partial exon 6 and exon 7 and full-length intron 6. Five nucleotide changes were identified by alignment of the fragment amplified from Jining Bairi and Zang chickens. Among these nucleotide substitutions, the C/T transition at the base position of 35 and the A/G transition at the base position of 287 were found to be highly polymorphic, and named as SNPs C35T and A287G, respectively. For the SNP C35T, 331 hens of a synthetic broiler line were genotyped by a PCR-SSCP approach and allele C was found to be dominant. For the SNP A287G, 604 birds from the synthetic broiler line, a commercial egg-laying line, as well as three Chinese indigenous chicken breeds were genotyped by a PCR-RFLP technique. The associations of these two SNPs with egg production traits in the broiler line were analyzed. The results indicated that both the C35T and the A287G SNPs were not associated with egg production at 33wks and from 33wks to 42 wks ($p>0.1$), whereas the SNP A287G was associated with egg production from 47 to 56 wks ($p<0.05$). The dominance genetic effects on this latter trait and on egg production from 33 to 42 wks were significant ($p<0.05$). (**Key Words :** Chicken, *BMPR-IB* Gene, SNP, Egg Production)

INTRODUCTION

Egg production traits are economically important both for egg-laying and broiler lines of chickens. Chinese indigenous chicken breeds, such as Zang, Wenchang and Jining Bairi, generally have a lower egg production compared to commercial egg-laying lines. Recently, breeding companies have tended to use Chinese indigenous chicken breeds as breeding materials to improve meat quality of commercial broiler lines. However, egg production of such crossbred lines is generally much lower than commercial egg-laying or broiler lines. Approaches to increasing egg production in indigenous chicken breeds, or in such crossbred broiler lines, are essential both for breeding and for egg and meat production in chickens.

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The bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily and play a key role in ovarian physiology of domestic animals (Dube et al., 1998; Shimasaki et al., 1999). A non-conservative substitution (Q249R) in the intracellular kinase domain of bone morphogenetic protein receptor IB (BMPR-IB) was found to be fully associated with the increased ovulation rate of highly prolific Booroola Merino sheep (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001). It is suggested that the Q249R mutation partially inactivates ovine BMPR-IB receptor activity, leading to a precocious differentiation of granulosa cells and of follicular maturation.

In chickens, reproduction is characterized by egg production which is fully associated with ovulation rate. BMPR-IB is expressed in the granulosa and theca of chicken ovary, with the granulosa having higher mRNA levels in all follicles than the theca (Onagbesan et al., 2003), implying an important role of chicken BMPR-IB in follicle maturation. However, the genetic effect of BMPR-IB on chicken ovulation and egg production traits remains largely unknown. In this study, we cloned a partial chicken *BMPR-IB* gene, which corresponded to the ovine *BMPR-IB* exon 6 to exon 7 fragment including the Q249R mutation

nucleotide, identified and investigated two single nucleotide polymorphisms (SNPs) in five chicken breeds (lines) and analyzed their associations with egg production traits in a synthetic broiler line.

MATERIALS AND METHODS

Chicken populations, trait measurement and DNA isolation

Four hundred and seventy-eight hens of the Luqin synthetic broiler line, which are offspring of 25 sires, were collected from Mingfa Broiler Group and used for marker-trait association analysis. These hens were reared at the same farm under similar environmental conditions. Eggs produced from the age at first laying until 33 wks, 42 wks, 47 wks and 56 wks were individually collected.

Thirty-three commercial egg-laying hens as well as 31 birds of the Jining Bairi breed were reared at the experimental station of Shandong Agricultural University. Thirty-four birds of the Wenchang breed were collected from Luoniushan Livestock Group of Hainan province. Twenty-eight birds of the Zang breed were provided by China Agricultural University. These populations were used for genotype and allele frequency analysis and, for sequencing, two Chinese indigenous chicken breeds of Jining Bairi and Zang were used.

All samples were collected by taking blood from the wing vein of chickens with acid citrate dextrose (1.32% sodium citrate, 0.48% citric acid monohydrate, 1.47% glucose) used as anticongulatant. Genomic DNA was isolated from blood samples using a DNA extraction kit (Sangon Biotechnology Company, Shanghai, China).

PCR amplification, sequencing and alignment

Primers of pBf (5′-GCTATGGGGAAGTCTGGATG-3′) and pBr (5′-TGCCTTT AATGTCTGCCGC-3′) were designed according to the chicken BMPR-IB mRNA sequence (GenBank accession No. NM_205132) to amplify the exon 6 to exon 7 fragment including intron 6. PCR was performed by mixing 1 μl (50-100 ng) of chicken genomic DNA, 2 μl of dNTPs (2.5 mM each), 1.5 μl of $MgCl_2$ (25 mM), 0.5 μl each of primers (25 μ M), 0. 2 μl of r*Taq* DNA polymerase (5 U/μl, Takara), and 2.5 μl of 10×PCR buffer in a 25 μl volume, and running on a Mastercycler gradient (Eppendorf, Germany) according to the following program: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec and final extension at 72°C for 10 min.

The PCR products were electrophoresed on 1% agarose gel, purified with Agarose Gel DNA Fragment Recovery Kit Ver. 2.0 protocols (Takara) and inserted into pMD18-T Vector (Takara) according to the provided protocols. The recombinant plasmid was transformed into competent *E. coli* DH5α cells. At least three positive clones were sequenced for each individual with an ABI 3730 sequencer and aligned with DNAMAN software version 5.2.2 to identify nucleotide substitutions.

Polymorphism, genotyping and allele frequencies

Two nucleotide changes at base positions of 35 and 287 in the exon 6 and intron 6 of chicken *BMPR-IB* (GenBank accession No. EF530593, obtained by us) were further examined for polymorphism. For the detection of C/T nucleotide substitution at the base position of 35, a PCR-SSCP approach was applied. PCR was performed with primers P35f (5′-CCATAGCAAAACAGATTCAG-3′) and P35r (5′-TCAGGA CAGTTTGGTAGATT-3′) as described above except that the annealing temperature was 52°C. SSCP was carried out by running a 12% non-denaturing polyacrylamide gel and genotyped according to Jiang et al. (2002). For the detection of A/G transition at the base position of 287, a PCR-RFLP method was used: after PCR with pBf and pBr, the product of 10 μl was digested with 10 U of restriction enzyme *Hind* III for 2 h followed by running on 2% agarose gel. After genotyping, homozygotes for each polymorphic site were subsequently sequenced and compared to confirm the genotyping result.

Individuals of the Luqin synthetic broiler line, commercial egg-laying line, Jining Bairi breed, Wenchang breed and Zang breed were genotyped for the two SNPs at base positions of 35 and 287 (SNPs C35T and A287G). Allele frequencies of the two SNPs were calculated and the tests for Hardy-Weinberg equilibrium were conducted with POPGENE version 1.32.

Marker-trait association analysis

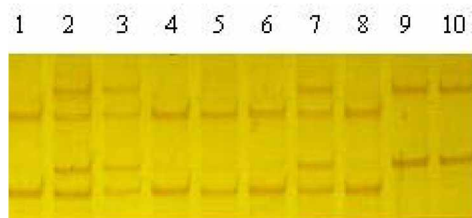
Associations of the two SNPs with egg production were analyzed in the synthetic Luqin broiler line using the GLM procedure of SAS 8.2 (SAS Institute, Inc., Cary, NC, USA). Data of egg production of E33, E34-42, and E47-56 from hens of the Luqin broiler line were analyzed using the following model: $Y_{ijk} = \mu+F_i+G_j+e_{ijk}$, where Yijk = egg production of the individual; μ= population mean of egg production; F_i = Fixed effect of the full family (i = 1, 2, ……, 25), G_j = fixed effect of the genotype j at the SNPs of chicken BMPR-IB gene (for SNP C35T, j = 1: *CC*, j = 2: *CT*, j = 3: *TT*; for SNP A287G, j = 1: *GG*, j = 2: *GA*, j = 3: *AA*), e_{ijk}= the overall error term. Type III sum of squares was used in each test. Values were considered significant at $p< 0.05$ and presented as least square means±standard errors.

RESULTS

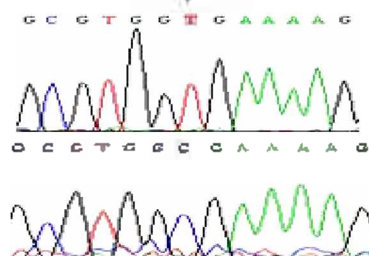
Chicken *BMPR-IB* exon 6 to exon 7 sequence and analysis

In order to obtain the partial sequence of chicken

Figure 1. Amplification of chicken *BMPR-IB* gene exon 6 to exon 7. M, DL 2000 DNA marker (2,000, 1,000, 750, 500, 250 and 100 bp, respectively). Lanes 1-6, samples.

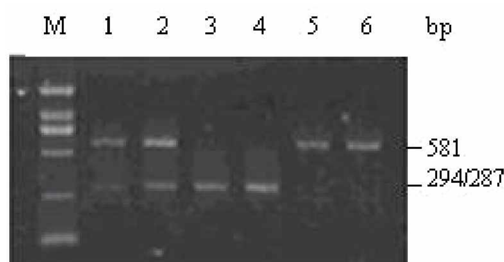


(a)

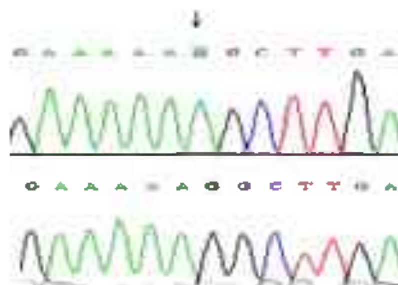


(b)

Figure 2. Genotyping by PCR-SSCP (a) and sequencing (b) results of the SNP C35T in chicken *BMPR-IB* gene exon 6. (a) Lanes 1,4-6,8, *CC*; Lanes 2, 3, 7, *CT*; Lanes 9 and 10, *TT*. (b) upper, *TT*; lower, *CC*; Arrow indicates C/T substitution.



(a)



(b)

Figure 3. Genotyping (a) by PCR-RFLP and sequencing (b) of the SNP A287G in chicken *BMPR-IB* gene intron 6. (a) M, DL 2,000 DNA marker (2,000, 1,000, 750, 500, 250 and 100 bp, respectively). Lanes 1, 2, *GA*; Lanes 3, 4, *AA*; Lanes 5, 6, *GG*. (b) upper, *AA*; lower, *GG*; Arrow indicates A/G substitution.

BMPR-IB gene exon 6 to exon 7, we selected primers from exon 6 and exon 7 respectively. The boundaries of exon 6 and 7 were decided after alignment of chicken *BMPR-IB* mRNA with mouse *BMPR-IB* genomic structure (GenBank accession No. NW 000203). The predicted fragment contains the site corresponding to the ovine Q249R mutation.

A DNA fragment of 581 bp was specifically amplified (Figure 1), sequenced and deposited to GenBank (GenBank accession No. EF530593 for both Zang and Jining Bairi chicken). Alignment of the nucleotide sequences of Jining Bairi chicken and Zang chicken revealed five nucleotide changes: T/C at 35, C/T at 166, G/C at 224, A/G at 287, and G/A at 303. The T/C transition was located at exon 6 and the others at intron 6. The substitution of T/C at 35 was a silent mutation, as the mutated nucleotide does not alter the amino acid (Gly) that it encodes. The Q249R mutation in Booroola Merino sheep was not detected in chickens.

Genotype and allele frequencies

The T/C substitution at the base position of 35 was checked by the PCR-SSCP approach and three genotypes of *TT*, *TC* and *CC* were clearly revealed (Figure 2a) and confirmed after sequencing (Figure 2b). The A/G transition at the base position of 287 was genotyped by PCR-RFLP with the result shown in Figure 3a; three genotypes of *AA*, *AG* and *GG* were resolved which were also confirmed by sequencing (Figure 3b). As these two nucleotide substitutions were highly polymorphic, they were named as SNPs C35T and A287G respectively.

Genotype and allele frequencies were calculated after genotyping the populations of five chicken breeds (lines) and listed in Table 1. For the SNP C35T, allele *C* is predominantly higher than allele *T* in the Luqin synthetic broiler line and the population was in a Hardy-Weinberg equilibrium ($p = 0.2337$). For the SNP A287G, in addition to the Luqin synthetic broiler line, four more breeds (line) including a commercial egg laying line and three Chinese indigenous chicken breeds, i.e. Jining Bairi, Wenchang and Zang were investigated. The allele frequency of *A* was higher than that of *G* in the Luqin broiler line, commercial egg-laying line, Jining Bairi and Wenchang chickens; however, in Zang chickens, the allele frequency of *G* was higher than that of *A*. All four populations except for the commercial egg-laying line were in a Hardy-Weinberg equilibrium ($p>0.05$).

Associations of polymorphisms with egg production

Associations of the two SNPs with egg production were

Table 1. Genotype and allele frequencies at the SNPs C35T and A287G of chicken *BMPR-IB* gene in five chicken populations

SNPs	Breed	Genotype frequency (n)			Allele frequency	
		11	12	22	1	2
C35T	Luqin broiler line	0.499 (165)	0.435 (144)	0.066 (22)	0.716	0.284
A287G	Luqin broiler line	0.245 (117)	0.536 (256)	0.219 (105)	0.513	0.487
	Commercial egg-laying line	0.333 (11)	0.667 (22)	0.000 (0)	0.667	0.333
	Jining Bairi	0.323 (10)	0.387 (12)	0.290 (9)	0.517	0.483
	Wenchang	0.412 (14)	0.471 (16)	0.117 (4)	0.648	0.352
	Zang	0.107 (3)	0.607 (17)	0.286 (8)	0.411	0.589

For the SNP C35T, 1 represents allele *C*, 2 represents allele *T*, and for the SNP A287G, 1 represents allele *A* and 2 represents allele *G*, respectively.

Table 2. Least squares means±standard errors of egg production according to the genotypes at the SNP C35T of chicken *BMPR-IB* gene

Trait	Genotype			Genetic effect	
	CC	*CT*	*TT*	Additive	Dominance
E33	42.29±1.17 (n = 156)	42.44±1.29 (n = 135)	44.12±3.34 (n = 22)	-0.91±1.77	0.77±2.19
E34-42	39.63±1.08 (n = 109)	39.24±1.14 (n = 98)	38.56±2.74 (n = 16)	0.53±1.47	0.15±1.86

E33: egg production from the age at first laying until 33 wks; E34-42: egg production from 33 wks to 42 wks.

Table 3. Least squares means ± standard errors of egg production according to the genotypes at the SNP A287G of chicken *BMPR- I B* gene

Trait	Genotype			Genetic effect	
	GG	*GA*	*AA*	Additive	Dominance
E33	43.91±1.86 (n = 53)	41.21±1.15 (n = 150)	44.23±1.67 (n = 61)	-0.16±1.29	-2.86±1.72
E34-42	41.18±1.63 (n = 42)	38.16±1.05 (n = 122)	41.47±1.42 (n = 51)	-0.14±1.16	-3.16*±1.54
E47-56	35.42^a±1.53 (n = 27)	38.45^b±0.89 (n = 69)	34.86^a±1.61 (n = 45)	0.28±1.06	3.14*±1.49

E33: egg production from the age at first laying until 33 wks; E34-42: egg production from 33 wks to 42 wks; E47-56: egg production from 47 wks to 56 wks.

[a, b] Means within a row without common subscripts differ (p<0.05); * p<0.05.

analyzed. For the SNP C35T, least squares means of E33 and E34-42 of hens with genotypes *CC*, *CT* and *TT* did not differ significantly (p>0.5) (Table 2). For the SNP A287G, differences among least squares means of E33 and E33-42 of hens with genotypes *GG*, *GA* and *AA* did not reach significance (p>0.1) (Table 3); however, the genetic effect of this SNP on E47-56 was significant: individuals with genotype *GA* produce more eggs than both *GG* and *AA* genotypes (p = 0.02<0.05). The dominance effect of the SNP G287A on egg production traits of E47-56 and E33-42 was also significant (p<0.05).

DISCUSSION

In livestock, some DNA markers have been found to be associated with variations in reproduction traits, such as polymorphisms in the estrogen receptor gene with litter size in pigs and Q249R mutation in the *BMPR-IB* gene with ovulation rate in sheep. In chicken, Cui et al. (2005) found that a polymorphism in the promoter region of the chicken prolactin receptor gene was associated with egg production. Choi et al. (2006) identified two SNPs in the uncoupling protein gene which are associated with daily percent lay. Ding et al. (2008) showed that the expression of *apoB* and *PURH* genes in liver changed after selection for egg production. In order to identify more DNA markers that are associated with egg production in chickens, the present study selected chicken *BMPR-IB* as a candidate gene to characterize its polymorphisms and analyze their associations with egg production.

Chicken *BMPR-IB* mRNA sequences were first identified by Sumitomo et al. (1993) and Lim et al. (2005). Based on this sequence and mouse *BMPR-IB* sequence, the present study cloned and sequenced chicken *BMPR-IB* exon 6 to exon 7, including intron 6, and identified five nucleotide differences between Jining Bari and Zang chickens. However, the Q249R mutation, which is the causal mutation of prolificacy in Booroola Merino sheep, was not detected in chickens.

The *BMPR-IB* fragment obtained by us had a 99% (576/581) homology with chicken genomic sequence deposited in Genbank (GenBank accession No. NM_205132). The *BMPR-IB* exon 6 sequence of chicken and quail (*Coturnix coturnix*) were identical except for the

C/T site and has a high identity ranging from 87% to 91% with mammals. At the base position of 35, nucleotide C is most conserved, however, in the rat, it is replaced by A and in the mouse, by T. In the current study, the allele frequency of C (0.716) was much higher than T (0.284), showing that the C allele is more common, consistent with nucleotide replacement during evolution. The C/T substitution does not alter the amino acid that it encodes, and the difference among species may indicate codon preference.

Among the five nucleotide substitutions, two SNPs of C35T and A287G were confirmed. For the SNP A287G, the frequency of allele *G* was higher than that of allele *A* in Zang chckens, which was different from the other four breeds (lines). The difference may lie in the Zang chicken being distributed in plateau regions. Our association results revealed no association of SNP C35T with egg production in a synthetic broiler line, while the SNP A287G was found to be significantly associated with E47-56, and the dominance effect was also significant. Lim et al. (2005) reported that BMP signaling, including BMPR-IB, is involved in chick diencephalic development, and the expression level of BMPR-IB decreases in the theca of chicken ovary from F1 to F3 follicles. Onagbesan et al. (2003) suggested that BMPR-IB is possibly involved in follicular differentiation and maintenance of the follicular hierarchy. Therefore, the expression level or the activity of BMPR-IB in the granulosa and/or theca of chicken ovary may be associated with oocyte maturation. As the SNP A287G is located in intron 6, it is assumed to be a closely linked marker to causal mutations with chicken egg production.

In conclusion, the present study obtained the chicken *BMPR-IB* exon 6 to exon 7 sequences, identified two polymorphic sites and analyzed their associations with egg production. The association results indicated that the SNP A287G has a significant effect on egg production from 47 to 56 wks in a synthetic broiler line, the mechanism of which requires further investigation.

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