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Mutations in *BMPR-IB* and *BMP-15* genes are associated with litter size in Small Tailed Han sheep (*Ovis aries*)[1]

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ABSTRACT: The Small Tailed Han is a prolific local sheep breed in China. The bone morphogenetic protein receptor IB (*BMPR-IB*) gene, which affects the fecundity of Booroola Merino sheep, and the bone morphogenetic protein 15 (*BMP-15*) gene, which affects the fecundity of Inverdale, Hanna, Belclare, Cambridge, and Lacaune sheep, were studied as candidate genes associated with the prolificacy of Small Tailed Han sheep. Single nucleotide polymorphisms of *BMPR-IB* and *BMP-15* genes were detected in Small Tailed Han ewes (n = 188) by PCR-RFLP. The combined effect of the 2 genes on the prolificacy of Small Tailed Han sheep was studied. The results indicated that the same *FecB* mutation (Q249R) occurred in the *BMPR-IB* gene in Small Tailed Han ewes as found in Booroola Merino ewes. The Small Tailed Han ewes with genotypes $FecB^{B}/FecB^{B}$ and $FecB^{B}/FecB^{+}$ had 1.40 ($P < 0.01$) and 1.11 ($P < 0.01$) more lambs, respectively, than those with genotype $FecB^{+}/FecB^{+}$. The same $FecX^{G}$ mutation (Q239Ter) of the *BMP-15* gene was found in Small Tailed Han ewes as in Belclare and Cambridge ewes. The Small Tailed Han ewes with the heterozygous mutant $FecX^{G}/FecX^{+}$ had 0.55 ($P < 0.01$) more lambs than those with the wild-type $FecX^{+}/FecX^{+}$. The Small Tailed Han ewes carrying mutations in both *BMPR-IB* and *BMP-15* genes had greater litter size than those with either mutation alone. In view of our results, marker-assisted selection using both *BMPR-IB* and *BMP-15* genes is warranted to increase litter size in sheep and will be of considerable economic value to sheep producers.

Key words: bone morphogenetic protein 15 gene, bone morphogenetic protein receptor IB gene, prolificacy, sheep

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INTRODUCTION

The Booroola gene (***FecB***) was the first major gene for prolificacy identified in sheep. The *FecB* locus is situated in the region of ovine chromosome 6 corresponding to the human chromosome 4q22-23 that contains the bone morphogenetic protein receptor IB (***BMPR-IB***) gene, which encodes a member of the transforming growth factor β (**TGFβ**) receptor family (Mulsant et al., 2001; Wilson et al., 2001). A nonconservative substitution (Q249R) in the *BMPR-IB* coding sequence was associated fully with the hyperprolific phenotype of Booroola ewes (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001).

Bone morphogenetic protein 15 (**BMP-15**) is a growth factor and a member of the TGFβ superfamily that is specifically expressed in oocytes. The sheep *BMP-15* gene maps to the X chromosome (Galloway et al., 2000). Bone morphogenetic protein 15 regulates granulosa cell proliferation and differentiation by promoting granulosa cell mitosis, suppressing follicle-stimulating hormone receptor expression, and stimulating kit ligand expression, all of which play a pivotal role in female fertility in mammals (Otsuka et al., 2000, 2001; Juengel et al., 2002; Otsuka and Shimasaki, 2002a,b; Moore and Shimasaki, 2005). The $FecX^{G}$ mutation (Q239Ter) in the *BMP-15* gene was associated with increased ovulation rate and sterility in Cambridge and Belclare sheep (Hanrahan et al., 2004).

The Small Tailed Han is a prolific local sheep breed in China. The mean litter size was 2.61 (Tu, 1989) and 2.65 (Wang et al., 1990) in Small Tailed Han sheep of

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Shandong Province, China. To date, there are no reports about the combined effect of the *BMPR-IB* and *BMP-15* genes on litter size in sheep.

The objectives of the current study were 1) to detect the single nucleotide polymorphisms of the 2 genes by PCR-RFLP and sequencing, and 2) to investigate the combined effect of the 2 genes on prolificacy of Small Tailed Han sheep.

MATERIALS AND METHODS

Animals

All experimental procedures were performed according to authorization granted by the Chinese Ministry of Agriculture. All procedures involving animals were approved by the animal care and use committee at the respective institution where the experiment was conducted. All procedures involving animals were approved and authorized by the Chinese Ministry of Agriculture.

Venous jugular blood samples (10 mL per ewe) were collected from 188 Small Tailed Han ewes lambed in 2004, along with data on litter size in the first, second, or third parity on the Jia-xiang Breeding Sheep Farm in Shandong Province, China, using acid citrate dextrose as an anticoagulant. Genomic DNA was extracted from whole blood by the phenol-chloroform method, then dissolved in TE buffer (10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0), and kept at –20°C.

The 188 ewes were chosen at random and were the progeny of 8 rams. Because the 8 rams were sold, their blood was not collected and they were not genotyped. No selection on litter size or other fertility traits was performed in the flock over previous years. Lambing seasons consisted of 3-mo groups beginning with March through May as season 1 (spring), June through August as season 2 (summer), September through November as season 3 (autumn), and December through February as season 4 (winter).

Detection of the FecB and the FecX^G Mutations

A primer pair was designed to detect single nucleotide polymorphisms in exon 6 of the *BMPR-IB* gene in prolific Small Tailed Han sheep by PCR-RFLP. Primers amplified a 140-bp band. After digestion with *Ava* II (New England Biolabs, Beverly, MA), the *BB* animals had a 110-bp band, the *B*+ animals had 140- and 110-bp bands, and the ++ animals had a 140-bp band (Wilson et al., 2001). The primer sequences were as follows:

Forward:

5′-GTCGCTATGGGGAAGTTTGGATG-3′; and

Reverse:

5′-CAAGATGTTTTCATGCCTCATCAACACGGTC-3′.

A primer pair was also designed to detect SNP of the *BMP-15* gene with *Hin*f I (Promega, Madison, WI). Primers amplified a 141-bp band (Hanrahan et al., 2004). The primer sequences were as follows:

Forward:

5′-CACTGTCTTCTTGTTACTGTATTTCAATGAGAC-3′;

and

Reverse:

5′-GATGCAATACTGCCTGCTTG-3′.

Polymerase chain reactions were carried out in a 25-μL reaction mixture containing approximately 2.5 μL of 10× PCR buffer [50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.0), 0.1% Triton X-100], 1.5 m*M* of $MgCl_2$, 200 μ*M* of each dNTP, 2 μ*M* of each primer, 50 ng of ovine genomic DNA, and 1 U of *Taq* DNA polymerase (Promega, Madison, WI). The amplification conditions for primers of the *BMPR-IB* gene were as follows: denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; with a final extension at 72°C for 5 min, on a Mastercycler 5333 (Eppendorf AG, Hamburg, Germany). The amplification conditions for primers of the *BMP-15* gene were as follows: denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 63°C for 45 s, and extension at 72°C for 1 min; with a final extension at 72°C for 10 min on a Mastercycler 5333 (Eppendorf AG).

The PCR products of 5 μL were digested separately with 10 U of *Ava* II (New England Biolabs) and 10 U of *Hin*f I (Promega) at 37°C for 4 h in a 20-μL reaction mixture. The resultant fragments were separated by electrophoresis on 3% agarose gels (Promega). The gels were visualized with ethidium bromide, photographed, and analyzed using an AlphaImager 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA).

Statistical Analysis

Least squares analysis of variance was conducted for *BMPR-IB* genotypes, *BMP-15* genotypes, and their combined genotypes. Therefore, the following statistical model was fitted to compare differences in litter size among different genotypes:

$$y = \mu + LS + P + G1 + G2 + G1G2 + s + e,$$

where y is the phenotypic value of litter size; μ is the population mean; LS is the fixed lambing season effect (LS = 1, 2, 3, or 4); P is the fixed parity effect (*P* = 1, 2, or 3); G1 is the fixed effect for *BMPR-IB* genotypes; G2 is the fixed effect for *BMP-15* genotypes; G1G2 is the fixed interaction effect for *BMPR-IB* and *BMP-15* combined genotypes; s is the random sire effect (s = 1,

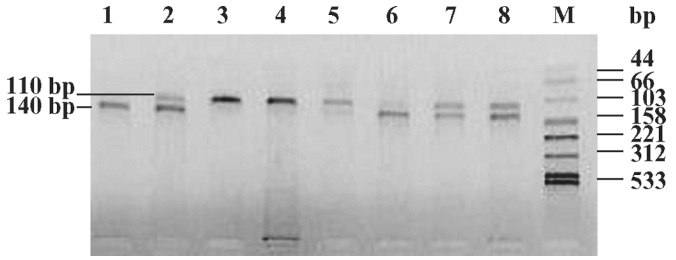

Figure 1. Image of PCR product of the *FecB* mutation of the *BMPR-IB* gene digested with *Ava* II. M = SD011 DNA marker. The wild-type allele (+) is 140 bp, and the mutant allele (*B*) is 110 bp. Lanes 2, 7, and 8 = the *B*+ genotype (heterozygote); lanes 1 and 6 = the ++ genotype (wild-type); lanes 3, 4, and 5 = the *BB* genotype (homozygous mutant).

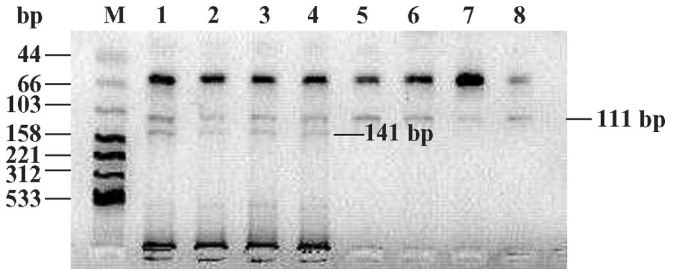

Figure 2. Image of PCR product of the $FecX^G$ mutation of the *BMP-15* gene digested with *Hin*f I. M = SD011 DNA marker. The wild-type allele (+) is 111 bp, and the mutant allele (*G*) is 141 bp. Lanes 1 to 4 = the *G*+ genotype; lanes 5 to 8 = the ++ genotype.

2, 3, 4, 5, 6, 7, or 8); and e is the random error effect of each observation. Analysis was performed using the GLM procedure (SAS Inst. Inc., Cary, NC). Mean separation procedures were performed using a least significant difference test.

RESULTS

Detection of the FecB Mutation of the BMPR-IB Gene

Three genotypes, *BB* (110 bp/110 bp), *B*+ (110 bp/140 bp), and ++ (140 bp/140 bp), were detected in Small Tailed Han sheep (Figure 1). Sequencing verified the presence (or absence) of the polymorphic *Ava* II cleavage site as assessed by agarose gel electrophoresis. The nucleotide sequence obtained from genotype *BB* was identical to the wild-type ++, except for an A→G transition at base 746 of the coding region of the *BMPR-IB* gene. This mutation results in a change in the amino acid coded from a glutamine in the wild-type to an arginine in the *BB* genotype (CAG→CGG, Q249R). The results indicated that Small Tailed Han ewes carried the same *FecB* mutation of the *BMPR-IB* gene as found in Booroola Merino ewes (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001).

Detection of the $FecX^G$ Mutation of the BMP-15 Gene

Two genotypes, ++ (111 bp/111 bp) and *G*+ (141 bp/111 bp), were detected in Small Tailed Han sheep (Figure 2). Forward and reverse sequencing identified the *Hin*f I RFLP polymorphism as a C/T single nucleotide change at position 718 of the *BMP-15* gene. The nucleotide sequence obtained from genotype *G*+ was identical to the wild-type ++, except for a C→T change at nucleotide 718 of the *BMP-15* gene. This mutation introduces a premature stop codon in place of glutamic acid at amino acid residue 239 of the unprocessed protein, which presumably results in complete loss of BMP-15 function (CAG→TAG, Q239Ter). The results indicated that Small Tailed Han ewes carried the same $FecX^G$ mutation of the *BMP-15* gene as found in Belclare and Cambridge ewes (Hanrahan et al., 2004).

Allelic and Genotypic Frequencies in Small Tailed Han Sheep

Allelic and genotypic frequencies of the *FecB* mutation of the *BMPR-IB* gene and the $FecX^G$ mutation of the *BMP-15* gene are presented in Table 1. For the *BMPR-IB* gene, frequencies of genotypes *BB*, *B*+, and ++ were 0.52, 0.42, and 0.06, respectively. For the *BMP-15* gene, frequencies of genotypes *G*+, ++, and *GG* were 0.60, 0.40, and 0.00, respectively.

Combined genotypic frequencies of the *FecB* and $FecX^G$ mutations are presented in Table 2. Combined genotype *BBG*+ had the highest frequency (0.29), whereas ++*G*+ and ++++ had the lowest frequency (0.03). It should be noted that these allelic and genotypic frequencies are based on a sample of fertile ewes, rather than the population as a whole, because these polymorphisms are associated with infertility, and because one of them is sex-linked. In addition, the use of only 8 sires limits the ability to make inferences about the entire population based on these sample data.

Effects of BMPR-IB and BMP-15 Genes on Litter Size in Small Tailed Han Sheep

Litter size in Small Tailed Han sheep was significantly influenced by *BMPR-IB* and *BMP-15* genotypes ($P = 0.0012$ and 0.0025, respectively). In addition, a highly significant interaction was observed between the *BMPR-IB* and *BMP-15* genotypes ($P = 0.0063$).

The least squares means and SE for litter size of different genotypes in Small Tailed Han sheep are given in Table 3. The Small Tailed Han ewes with genotypes *BB* and *B*+ had 1.40 ($P < 0.01$) and 1.11 ($P < 0.01$) more lambs, respectively, than those with genotype ++. The Small Tailed Han ewes with the heterozygous mutant *G*+ genotype had 0.55 ($P < 0.01$) more lambs than those with the wild-type ++ genotype. The Small Tailed Han

Table 1. Allelic and genotypic frequencies of the *FecB* mutation of the *BMPR-IB* gene and the *FecX*^{G} mutation of the *BMP-15* gene in Small Tailed Han ewes[1]

Gene	No. of ewes	Allelic frequency		Genotypic frequency		
BMPR-IB	188	+	*B*	++	*B*+	*BB*
		0.27	0.73	0.06(12)[2]	0.42(78)	0.52(98)
BMP-15	188	+	*G*	++	*G*+	*GG*
		0.70	0.30	0.40(76)	0.60(112)	0.00(0)

[1]B = *FecB* mutation; G = *FecX*^{G} mutation; + = wild-type.
[2]Numbers in parentheses are numbers of individuals that belong to the respective genotypes.

ewes carrying mutations in both *BMPR-IB* and *BMP-15* genes had greater litter size than those with either mutation alone. The effect of the *BMPR-IB* gene mutation was greater than that of the *BMP-15* gene mutation on litter size in Small Tailed Han ewes.

DISCUSSION

The *FecB* mutation is present in Booroola Merino (Australia; Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001), Garole (India; Davis et al., 2002), Javanese (Indonesia; Davis et al., 2002), Small Tailed Han (China; Liu et al., 2003; Wang et al., 2003a; Jia et al., 2005; Yan et al., 2005; Davis et al., 2006), and Hu (China) sheep (Wang et al., 2003a, b, 2005; Yan et al., 2005; Davis et al., 2006; Guan et al., 2006). This study also showed that the *FecB* mutation is present in Small Tailed Han sheep (China). Therefore, these 5 ovine breeds may share a common ancestor.

In sheep, 5 different point mutations [*FecX*^{I} (Inverdale), Galloway et al., 2000; *FecX*^{H} (Hanna) Galloway et al., 2000; *FecX*^{L} (Lacaune), Bodin et al., 2003; *FecX*^{G} (Belclare and Cambridge), Hanrahan et al., 2004; and *FecX*^{B} (Belclare), Hanrahan et al., 2004] have been identified in the *BMP-15* gene, each having a major effect on ovulation rate. Ewes heterozygous for any one of these *BMP-15* mutations have increased ovulation rates, whereas homozygous ewes are sterile due to a failure of normal ovarian follicular development (Davis et al., 1992; Braw-Tal et al., 1993; Galloway et al., 2000; Bodin et al., 2003; Hanrahan et al., 2004). Crossing *FecX*^{I} and *FecX*^{H} sheep produces *FecX*^{I}/*FecX*^{H} infertile females, which are phenotypically indistinguishable from *FecX*^{I}/*FecX*^{I} females (Davis et al., 1995). Belclare heterozygous *FecX*^{G}/*FecX*^{B} ewes were also sterile (Hanrahan et al., 2004). None of Small Tailed Han and Hu sheep carried the *FecX*^{B} or *FecX*^{H} mutation (Liu et al., 2003; Chu et al., 2005a,b). None of Garole, Javanese, Small Tailed Han, or Hu sheep had the *FecX*^{I} mutation (Davis et al., 2002; Liu et al., 2003; Chu et al., 2005a; Wang et al., 2005; Davis et al., 2006). The prolific Hu sheep did not have the *FecX*^{G} mutation (Chu et al., 2005b). The current study and Chu et al. (2005b) indicated that Small Tailed Han sheep had the same *FecX*^{G} mutation (C718T) of the *BMP-15* gene as Belclare and Cambridge ewes. It is unknown whether prolific Small Tailed Han and Hu sheep carry the *FecX*^{L} mutation. To our knowledge, this is the first case in which the same *FecX*^{G} mutation has been found in apparently unrelated breeds (Belclare/Cambridge and Small Tailed Han). Regarding the *FecX*^{G} mutation, it would be very interesting to compare the local haplotype around the mutation, as well as to compare genetic distances between Belclare/Cambridge and Small Tailed Han sheep, using other markers. This would help to determine whether mutant animals in these 2 breeds derive from a unique mutational event or whether the mutation occurred twice independently.

No *GG* ewes were detected among the 188 Small Tailed Han ewes in this study. Hanrahan et al. (2004) reported that *GG* ewes were sterile. Ewes of this genotype were not detected in 83 Belclare ewes, whereas 12 *GG* ewes were detected in 129 Cambridge ewes (Hanrahan et al., 2004). Possible reasons no *GG* ewes were observed in this study include: (i) *GG* ewes exist in the Small Tailed Han breed, but the method used to select ewes for this study (only ewes with litter records were used) excluded all infertile *GG* ewes, and (ii) *GG* ewes do not exist in the Small Tailed Han breed. If *GG* ewes exist in Small Tailed Han sheep, 60% of fertile Small Tailed Han ewes are *G*+, and matings are at random, infertility must be associated with *GG* ewes throughout this breed. Because there are no reports of infertility among Small Tailed Han ewes to date, we hypothesize

Table 2. Combined genotypic frequencies of the *FecB* and *FecX*^{G} mutations in Small Tailed Han ewes[1]

	Combined genotype					
	BBG+	*BB*++	*B*+*G*+	*B*+++	++*G*+	++++
Genotypic frequency	0.29(54)[2]	0.23(44)	0.28(52)	0.14(26)	0.03(6)	0.03(6)

[1]B = *FecB* mutation; G = *FecX*^{G} mutation; + = wild-type.
[2]Numbers in parentheses are numbers of individuals that belong to the respective genotypes.

Table 3. Least squares means and SE for litter size of different *BMPR-IB* and *BMP-15* genotypes in Small Tailed Han sheep

Genotype[1]	No. of ewes	Litter size
BMPR-IB genotypes		
BB	98	$2.65^{A} \pm 0.10$
B+	78	$2.36^{A} \pm 0.12$
++	12	$1.25^{B} \pm 0.17$
BMP-15 genotypes		
G+	112	$2.61^{A} \pm 0.09$
++	76	$2.06^{B} \pm 0.13$
Combined genotypes		
BBG+	54	$2.80^{Aa} \pm 0.08$
B+G+	52	$2.55^{ABa} \pm 0.09$
BB++	44	$2.23^{Bb} \pm 0.11$
B+++	26	$1.98^{BCb} \pm 0.12$
++G+	6	$1.40^{Cc} \pm 0.14$
++++	6	$1.10^{Dd} \pm 0.13$

[A–D]Least squares means with different superscripts within each of the 3 sets (*BMPR-IB*, *BMP-15*, or combined genotypes) differ at $P < 0.01$.

[a–d]Least squares means with different superscripts within each of the 3 sets (*BMPR-IB*, *BMP-15*, or combined genotypes) differ at $P < 0.05$.

[1]B = *FecB* mutation; G = $FecX^{G}$ mutation; + = wild-type.

that there were no *GG* ewes in the Small Tailed Han breed, as was the case in Belclare sheep. Mating of *G+* rams and *G+* ewes, extensive sampling, and DNA analysis would be required to verify this hypothesis. Such a study would have important implications for the sheep industry.

The BMP-15 binds to BMPR-IB and to BMPR-II (Moore et al., 2003). The interaction between the Booroola and Inverdale mutations appears to be multiplicative in that animals that are heterozygous for both the Booroola mutation and the Inverdale mutation have ovulation rates greater than the increase expected for an additive effect alone (Davis et al., 1999). The Small Tailed Han ewes carrying mutations in both *BMPR-IB* and *BMP-15* genes had greater litter size than those with either mutation alone in the current study. To our knowledge, the Small Tailed Han sheep is the third sheep breed where mutations in 2 different genes have been shown to segregate, after the Belclare/Cambridge (GDF-9 and BMP-15; Hanrahan et al., 2004) and the Lacaune sheep (FecL and BMP-15; Bodin et al., 2003), and the first where the effect of the different genotypes on litter size was determined. Because of a low number of ++ animals at the *FecB* locus, it is difficult to determine whether the combined effect of both mutations is additive or synergistic. This topic is worthy of further study. The present results have important implications for the sheep industry because Small Tailed Han flocks will have ewes of widely different levels of prolificacy and ewes homozygous for $FecX^{G}$ will be sterile.

The regulatory mechanism of BMPR-IB and BMP-15 in prolific Small Tailed Han and Hu sheep deserves further study. Ongoing investigations into the basis of the prolific phenotype of Small Tailed Han and Hu ewes are likely to reveal further insights into the events controlling follicle and oocyte development.

IMPLICATIONS

The Small Tailed Han is a prolific local sheep breed in China. The Small Tailed Han ewes in this study carried the same *FecB* mutation (Q249R) of the bone morphogenetic protein receptor IB (*BMPR-IB*) gene as found in Booroola Merino ewes and the same $FecX^{G}$ mutation (Q239Ter) of the bone morphogenetic protein 15 (*BMP-15*) gene as found in Belclare and Cambridge ewes. Moreover, Small Tailed Han ewes carrying mutations in both *BMPR-IB* and *BMP-15* genes had greater litter size than those with either mutation alone. In view of our results, marker-assisted selection using both *BMPR-IB* and *BMP-15* genes is warranted to increase litter size in sheep and will be of considerable economic value to sheep producers.

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