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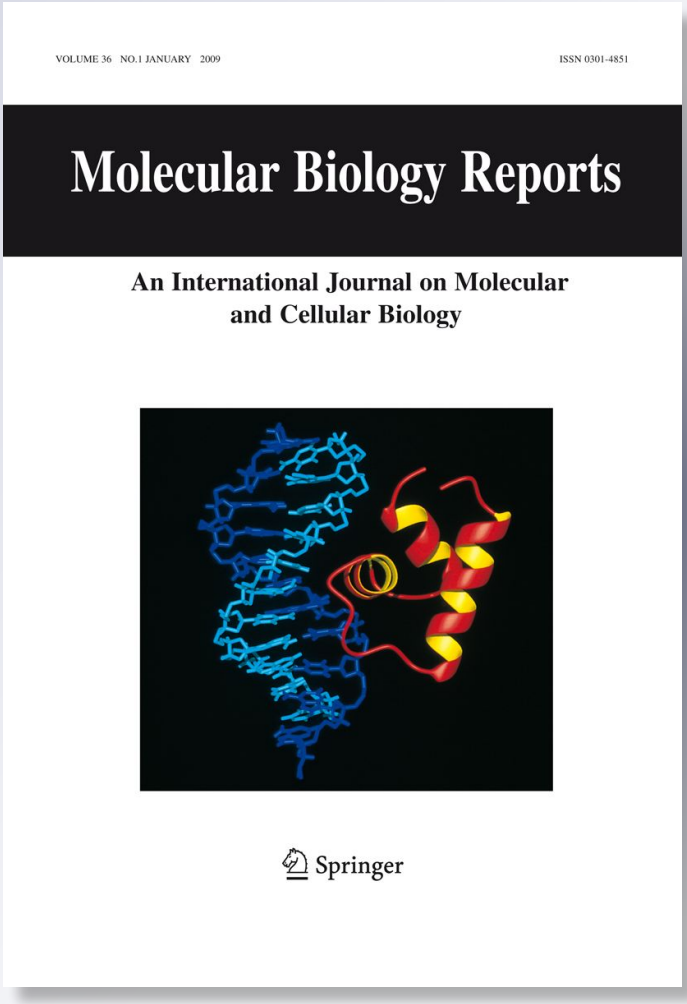

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Polymorphisms of coding region of *BMPR-IB* gene and their relationship with litter size in sheep

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Abstract The bone morphogenetic protein receptor IB (BMPR-IB) was studied as a candidate gene for the prolificacy of sheep. Nine pairs of primers (P1–P9) were designed to detect single nucleotide polymorphisms (SNPs) of exons 1–4 and 6–10 of the *BMPR-IB* gene in both high (Small Tail Han and Hu sheep) and low prolificacy breeds (Texel and Chinese Merino sheep) by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP). Only the products amplified by primers P2, P5, P6, P7, P8 and P9 displayed polymorphisms. The present study identified 22 SNPs in partial coding regions of ovine *BMPR-IB*, in which 20 SNPs were reported for the first time. In total of the 22 mutations, 18 DNA variations were originated from the Hu breed, three were found in the Small Tail Han breed (two of them were found in other sheep breeds), three in the Chinese Merino breed, and none in the Texel breed. These results preliminarily demonstrated that *BMPR-IB* is a major gene affecting the hyperprolificacy in Small Tail Han and Hu sheep, and could be used as a molecular genetic marker for early auxiliary selection for hyperprolificacy in sheep.

Keywords Bone morphogenetic protein receptor *IB* gene · PCR-SSCP · Prolificacy · Sheep

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Introduction

Ewes from the Booroola strain of Australian Merino sheep are characterized by high ovulation rate and litter size. The Booroola gene (*FecB*) is an autosomal mutation identified on the basis of segregation studies on litter size [1] and ovulation rate [2]. *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 [3, 4]. One point mutation at base 746 of the coding region (746A→G) in the highly conserved intracellular kinase signaling domain of the BMPR-IB caused an amino acid change (249Q→R) was associated fully with the hyperprolific phenotype of Booroola ewes [3–5].

The Small Tail Han and Hu sheep are excellent local breeds in China for their significant characteristics of hyperprolificacy and year-round estrus. The mean litter sizes alive of Small Tail Han, Hu, Chinese Merino and Texel sheep have been reported to be 2.61 [6], 2.29 [6], 1.23 [6] and 1.41 [7], respectively. Based on the crucial role of *BMPR-IB* gene in the regulation of terminal folliculogenesis and the control of ovulation rate, *BMPR-IB* was considered as a possible candidate gene for prolificacy of

sheep. The objectives of the present study were firstly to detect single nucleotide polymorphisms (SNPs) of exons 1–4 and 6–10 of *BMPR-IB* gene in high (Small Tail Han and Hu sheep) and low prolificacy breeds (Texel and Chinese Merino sheep) by PCR-SSCP, and secondly to investigate the association between *BMPR-IB* gene and prolificacy in Small Tail Han sheep in which the polymorphisms are segregating.

Materials and methods

Animals

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 140 Small Tail Han ewes lambed in 2006, along with data on litter size in the first, second, or third parity (Jiaxiang Sheep Breeding Farm, Shandong Province, China), 40 Chinese Merino ewes (Ziniquan Breeding Sheep Farm, Shihezi City, Xinjiang Uygur Autonomous Region, China), 40 Texel ewes (HITEK Ranch [Beijing] Ltd. Co., Beijing, China) and 38 Hu ewes (Yuhang Hu Sheep Breeding Farm, Hangzhou, China) using acid citrate dextrose as an anticoagulant. Genomic DNA was extracted by phenol–chloroform method, and dissolved in TE buffer (10 mmol/l Tris–HCl [pH 8.0], 1 mmol/l EDTA [pH 8.0]) and kept at −20°C.

The 140 Small Tail Han ewes were selected at random and they were the progeny of five rams. Because the five rams had been sold, their blood was not collected for genotyping. No selection on litter size or other fertility traits was performed in the flock over previous years. Lambing seasons consisted of 3 month groups starting with March through to May as season 1 (spring), June through to August as 2 (summer), September through to November as 3 (autumn) and December through to February as 4 (winter).

Primers and PCR amplification

Nine pairs of primers were designed according to mRNA sequence (GenBank accession AF357007) of ovine *BMPR-IB*. Exons 1–4 and 6–10 of *BMPR-IB* were amplified. Primer sequence, amplified region, product size and annealing temperature were listed in Table 1.

PCR were carried out in 25 μl volume containing approximately 1.0 μl of 10 μmol/l each primer, 2.5 μl of 10× PCR buffer (50 mmol/l KCl, 10 mmol/l Tris–HCl [pH 8.0], 0.1% Triton X-100), 1.5–1.8 μl of 25 mmol/l $MgCl_2$, 2.5 μl of 2.5 mmol/l each dNTP, 3.0 μl of 50 ng/μl genomic DNA, 1.0 μl of 2.5 U/μl *Taq* DNA polymerase

Table 1 Primer sequence, amplified region, product size and annealing temperature

Primer	Primer sequence (5′→3′)	Product size (bp)	Amplified region	Annealing temperature (°C)
P1	F: AAGCAAACTTCCTTGATAACAT	163	Exon 1 (137–299)	54.6
	R: CTGCAAATATTGTTGACCGA			
P2	F: GCAGCACAGATGGATATTGTTT	106	Exon 2 (296–401)	58.4
	R: CGACACTGAAAATCTGAGCCT			
P3	F: GGACACTCCCATTCCTCATC	104	Exon 3 (402–505)	56.8
	R: CTCTGTTTTTCAGTGGAGGAAG			
P4	F: GAGATTTTGTTGACGGACCTA	103	Exon 4 (503–605)	57.5
	R: TACCTGAAGTAACAGAATAAAATGA			
P5	F: GGTCCAGAGGACAATAGCAA	196	Exon 6 (741–936)	60.2
	R: GCCCAAGATGTTTTCATGC			
P6	F: GCTTCATTGCTGCAGATAT	299	Exon 7 (935–1233)	59.5
	R: CCTAATAAACTTAACAGCCAA			
P7	F: TATTAGTGACACGAATGAAGT	188	Exon 8 (1227–1414)	61.1
	R: CTATACCTCCTGACACACAT			
P8	F: GTCAGGAGGTATAGTGGAAGAATATC	137	Exon 9 (1401–1537)	55.8
	R: CGTCACTGCTCCACCGGTT			
P9	F: GACGAGTGTCTCAGGCAGATG	133	Exon 10 (1534–1666)	59.5
	R: CTCAGAGCTTAATGTCCTGGGA			

F stands for forward primer, *R* stands for reverse primer

(Promega, Madison, WI, USA), and the rest is ddH_2O. Amplification conditions were as follows: initial denaturation at 94°C for 8 min; followed by 32 cycles of denaturation at 94°C for 30 s, annealing for 30 s (annealing temperature in Table 1), extension at 72°C for 30 s; with a final extension at 72°C for 10 min on Mastercycler® 5333 (Eppendorf AG, Hamburg, Germany).

Single strand conformation polymorphism detection and cloning and sequencing

These steps followed the method of Yan et al. [8].

Statistical analysis

The following fixed effects model was employed for analysis of litter size in Small Tail Han ewes and least squares mean was used for multiple comparisons in litter size among different genotypes.

$$y_{ijklm} = \mu + S_i + LS_j + P_k + G_l + e_{ijklm},$$

where y_{ijklm} is the phenotypic value of litter size; μ is the population mean; S_i is the fixed effect of the ith sire ($i = 1, 2, 3, 4, 5$); LS_j is the fixed effect of the jth lambing season ($j = 1, 2, 3, 4$); P_k is the fixed effect of the kth parity ($k = 1, 2, 3$); G_l is the fixed effect of the lth genotype ($l = 1, 2, 3$) and e_{ijklm} is the random residual effect of each observation. Analysis was performed using the general linear model procedure of SAS (ver 8.1) (SAS Institute Inc., Cary, NC, USA). Mean separation procedures were performed using a least significant difference test.

Table 2 Polymorphic sequence variations in *BMPR-IB* gene in four sheep breeds

Primer	Genotype	Variant		Amino acid change
P1	*AA→CC*	A1	G192A	ATG,Met→ATA, Ile
	AA→DD	B1	T195C	None
P5	*++→BB*	C1	A746G	CAG, Gln→CGG, Arg
P6	*GG→HH*	D1	T864C	None
	GG→KK	E1	T852C	None
		E2	C891T	None
		E3	C910T	None
		E4	G922T	GCA, Ala→TCA, Ser
		E5	C936G	None
		E6	C963T	None
		E7	G1017C	None
	GG→LL	F1	G810A	None
		F2	T864C	None
		F3	T1043C	ATA, Ile→ACA, Thr
P7	*MM→MN*	G1	C1113A	None
		G2	C1119T	None
		G3	T1147C	None
		G4	T1170C	None
		G5	T1206C	None
	MM→PP	H1	C1113A	None
		H2	T1147C	None
		H3	T1170C	None
		H4	T1206C	None
P8	*QQ→RR*	J1	C1311T	None
P9	*UU→ZZ*	K1	A1467G	None
		K2	C1470A	None
		K3	T1473G	None

Results

Only the PCR products amplified by primers P2, P5, P6, P7, P8 and P9 displayed polymorphisms. Four genotypes (AA, AC, CC and DD) were detected for primer P2, three (++, B+ and BB) for primer P5, five (GG, GH, HH, KK and LL) for primer P6, three (MM, MN and PP) for primer P7, two (QQ and RR) for primer P8, and two (UU and ZZ) for primer P9.

Polymorphic sequence variations in *BMPR-IB* in four sheep breeds were showed in Table 2. The 22 SNPs were identified in partial coding regions of ovine *BMPR-IB* gene in the present study, in which 20 SNPs were reported for the first time. In total of the 22 mutations, 18 base variations were originated from the Hu breed, 3 were found in the Small Tail Han breed (two of them were found in other sheep breeds), 3 in the Chinese Merino breed, and none in the Texel breed.

Except for genotypes MM, MN and PP, amino acid sequences of genotypes AA, ++, GG, QQ and UU were the same as that of ovine *BMPR-IB* gene published in GenBank (AF357007).

The frequencies of 19 genotypes and 16 alleles in four sheep breeds were presented in Table 3. As for primers P2, P5, P6, P7, P8 and P9, the genotypes and alleles were 4, 3, 5, 3, 2, 2 and 3, 2, 4, 3, 2, 2, respectively. These results indicated that the polymorphisms in coding region of *BMPR-IB* gene were rather abundant among these sheep breeds.

The test result of difference for *BMPR-IB* genotype distribution of primer P5 in four sheep breeds was summarized in Table 4. There was highly significant difference ($P < 0.001$) in the *BMPR-IB* genotype distribution between high (Small Tail Han and Hu sheep) and low prolificacy breeds (Texel and Chinese Merino sheep). And in either group, no significant difference ($P > 0.05$) was monitored.

Litter size was significantly influenced by sire, lambing season and parity ($P < 0.05$, $P < 0.05$, and $P < 0.05$, respectively). The least squares means (LSM) and standard

Table 3 Allele and genotype frequencies of *BMPR-IB* gene in four sheep breeds

Breed			Small Tail Han sheep	Hu sheep	Texel sheep	Chinese Merino sheep
Number			136	38	40	36
Primer P2	Genotype frequency	AA	0.750 (102)	0.737 (28)	1.000 (40)	1.000 (36)
		AC	0.228 (31)	0.000 (0)	0.000 (0)	0.000 (0)
		CC	0.022 (3)	0.000 (0)	0.000 (0)	0.000 (0)
		DD	0.000 (0)	0.263 (10)	0.000 (0)	0.000 (0)
	Allele frequency	A	0.864	0.737	1.000	1.000
		C	0.136	0.000	0.000	0.000
		D	0.000	0.263	0.000	0.000
Number			140	35	36	38
Primer P5	Genotype frequency	++	0.107 (15)	0.000 (0)	1.000 (36)	1.000 (38)
		B+	0.336 (47)	0.314 (11)	0.000 (0)	0.000 (0)
		BB	0.557 (78)	0.686 (24)	0.000 (0)	0.000 (0)
	Allele frequency	+	0.275	0.157	1.000	1.000
		B	0.725	0.843	0.000	0.000
Number			138	36	40	40
Primer P6	Genotype frequency	GG	0.616 (85)	0.361 (13)	1.000 (40)	0.650 (26)
		GH	0.297 (41)	0.000 (0)	0.000 (0)	0.000 (0)
		HH	0.087 (12)	0.000 (0)	0.000 (0)	0.000 (0)
		KK	0.000 (0)	0.639 (23)	0.000 (0)	0.000 (0)
		LL	0.000 (0)	0.000 (0)	0.000 (0)	0.350 (14)
	Allele frequency	G	0.764	0.361	1.000	0.650
		H	0.236	0.000	0.000	0.000
		K	0.000	0.639	0.000	0.000
		L	0.000	0.000	0.000	0.350
Number			138	38	40	36
Primer P7	Genotype frequency	MM	1.000 (138)	0.474 (18)	1.000 (40)	1.000 (36)
		MN	0.000 (0)	0.342 (13)	0.000 (0)	0.000 (0)
		PP	0.000 (0)	0.184 (7)	0.000 (0)	0.000 (0)
	Allele frequency	M	1.000	0.645	1.000	1.000
		N	0.000	0.171	0.000	0.000
		P	0.000	0.184	0.000	0.000
Number			140	35	36	38
Primer P8	Genotype frequency	QQ	1.000 (140)	0.657 (23)	1.000 (36)	1.000 (38)
		RR	0.000 (0)	0.343 (12)	0.000 (0)	0.000 (0)
	Allele frequency	Q	1.000	0.657	1.000	1.000
		R	0.000	0.343	0.000	0.000
Number			136	35	36	38
Primer P9	Genotype frequency	UU	1.000 (136)	0.600 (21)	1.000 (36)	1.000 (38)
		ZZ	0.000 (0)	0.400 (14)	0.000 (0)	0.000 (0)
	Allele frequency	U	1.000	0.600	1.000	1.000
		Z	0.000	0.400	0.000	0.000

The numbers in the parentheses are the individuals that belong to the respective genotypes

errors for litter size of different genotypes of *BMPR-IB* in Small Tail Han sheep were given in Table 5. For primer P2, the differences of the LSM for litter size between AA, AC and CC were not significant ($P > 0.05$). For primer P5, the ewes with genotype BB or B+ had 1.51 ($P < 0.001$) or 1.02 ($P < 0.001$) lambs more than those with genotype ++; the ewes with genotype BB had 0.49 ($P < 0.01$) lambs more than those with genotype B+. For primer P6,

Table 4 Test of difference for *BMPR-IB* genotype distribution of primer P5 in four sheep breeds

Primer	Breed	Hu sheep	Texel sheep	Chinese Merino sheep
P5	Small Tail Han sheep	4.583	110.924***	113.949***
	Hu sheep		71.000***	73.000***
	Texel sheep			0.000

df = (2–1) (3–1) = 2, χ^2 (df = 2, 0.05) = 5.99, χ^2 (df = 2, 0.001) = 13.82, *** $P < 0.001$

Table 5 Least squares mean and standard error for litter size of different genotypes of *BMPR-IB* gene in Small Tail Han sheep

Primer	Genotype	Number	Litter size
Primer P2	*AA*	102	$2.34^{A} \pm 0.11$
	AC	31	$2.27^{A} \pm 0.16$
	CC	3	$2.18^{A} \pm 0.20$
Primer P5	++	15	$1.14^{C} \pm 0.16$
	B+	47	$2.16^{B} \pm 0.14$
	BB	78	$2.65^{A} \pm 0.11$
Primer P6	*GG*	85	$2.42^{A} \pm 0.13$
	GH	41	$2.21^{A} \pm 0.18$
	HH	12	$1.98^{A} \pm 0.21$

Least squares means with the same superscript for the same pair of primer have no significant difference ($P > 0.05$). Least squares means with the different superscripts within the same pair of primer differ significantly ($P < 0.01$ or $P < 0.001$)

the differences of the LSM for litter size between GG, GH and HH were not significant ($P > 0.05$).

Discussion

Polymorphisms of ovine *BMPR-IB* gene

The *FecB* mutation (Q249R) is present in Booroola Merino (Australia) [3–5], Garole (India) [9], Javanese (Indonesia) [9], Small Tail Han (China) [8, 10, 11, 14–17], Hu (China) [8, 11–14, 18], and Kendrapada sheep (India) [19]. This study also showed that the *FecB* mutation is present in Small Tail Han and Hu sheep (China). Therefore, these six ovine breeds may share a common ancestor.

Souza et al. (2001) found two point mutations (A746G and C1113A) by screening 20 Scottish Blackface Merino cross ewes of Booroola genotypes in the kinase domain of BMPR-IB [5]. The present study also identified these two point mutations. A746G mutation was in both Small Tail Han and Hu sheep, not in Texel or Chinese Merino sheep. C1113A mutation was only in Hu sheep, not in other three sheep breeds.

The present study identified 22 SNPs in exons 1–4 and 6–10 of ovine *BMPR-IB*. Among them, four SNPs (A746G, G922T, T1043C, G192A) led to the change of amino acids (Table 2). According to the known structure of protein kinase genes, the first SNP located between β_2 and β_3 sheets in nucleotide (ATP)-binding lobe, and the others located in substrate-binding lobe. Specifically, the latter three SNPs situated, respectively, in the subdomain 3, 6, 8 [20] of kinase domain of *BMPR-IB*.

In two high (Small Tail Han and Hu sheep) and six low fecundity breeds (Dorset, Texel, German Mutton Merino, South African Mutton Merino, Chinese Merino and Corriedale sheep), 17 of 21 SNPs in Inhibin β_A gene were from the Hu sheep breed [21]. In the current study, 18 of 22 SNPs were from the Hu sheep breed. The Hu sheep breed is a special local breed in China. Chinese Hu sheep are used mainly for lambskin production and may be found throughout the Taihu Lake area that covers Jiangsu province, Zhejiang province and the vicinity of Shanghai. This sheep breed is famous for its beautiful lambskin, sexual precocity, year-round estrus, and hyperprolificacy [6, 22].This is why so many variations in the Hu sheep breed deserve further study. For primers P2, P6, P8 and P9, no heterozygotes were reported in the Hu sheep breed. Possible reasons include: (1) Heterozygotes exist in the Hu sheep breed, but infertile heterozygotes were probably rapidly removed from the population, or heterozygotes suffered from distinct developmental and reproductive defects, they suffered from the embryonic death, or they were sifted out before arriving at reproductive age. (2) Heterozygotes do not exist in the Hu sheep breed. To verify this hypothesis, a more comprehensive sample should be reported in the Hu sheep.

Effect of BMPR-IB mutation on sheep litter size

The effect of *FecB* gene is additive for ovulation rate (the number of ova shed at each ovulatory cycle) and partially dominant for litter size. One copy increases ovulation rate by 1.3–1.6 and two copies by 2.7–3.0; litter size is increased by 0.9–1.2 in ewes carrying a single copy and 1.1–1.7 in ewes with two copies [1, 2].

In the BB or B+ ewes, Q249R substitution would impair the inhibitory effect of BMPR-IB on granulosa cell steroidogenesis, leading to their advanced differentiation and an advanced maturation of follicles [3]. This A746G mutation could cause the phenotype characteristic of the

Booroola animals which is the precocious development of a large number of small antral follicles resulting in increased ovulation rate [5]. The results in the present study showed that the mean litter size of *BB, B+ and ++* genotypes was 2.65, 2.16 and 1.14 in Small Tail Han ewes, respectively. The fact that the *FecB* mutation is present in prolific Small Tail Han and Hu sheep will promote us to develop breeding strategies to maximize the benefits of increased prolificacy in these breeds and their crosses.

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