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Full Length Research Paper

Study on candidate gene for fecundity traits in Xingjiang Cele black sheep

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The aim of the present study is to find a potential candidate gene for high fecundity in Cele black sheep. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technology was used to detect single nuclear polymorphism (SNP) of four candidate genes (*BMPR-IB*, *BMP*15, *GDF*9, and *ESRα*) in Cele black sheep. The results showed that (i) A-G mutation was found at 746 bp in *BMPR-IB* in which the frequencies of homozygote (BB), heterozygote (B+) and wild type (++) were 0.113, 0.471, and 0.416, respectively. Significant differences were observed in litter size between ++ and B+ ($P < 0.01$) and between ++ and BB of individuals ($P < 0.05$). (ii) C-G mutation was found at exon 1 of *ESR*α in which the frequencies of homozygote, heterozygote and wild type were 0.047, 0.321 and 0.631, respectively. No significant difference was observed in litter size among the genotypes of *ESR*α ($P > 0.05$). (iii) No polymorphism was found in four mutation sites ($FecX^G$, $FecX^B$, $FecX^I$, $FecX^H$) of *BMP*15 and in one mutation site ($FecG^H$) of *GDF*9 gene. The results indicate that fecundity characteristic was positively correlated to *BMPR-IB*. However, there was no relation between fecundity characteristic and detected SNP sites of *ESRα*, *BMP*15 and *GDF*9 genes. These preliminary results showed that the *BMPR-IB* gene is either a major gene that influences the prolificacy in Cele black sheep or a molecular genetic marker in close linkage with such a gene.

Key words: Cele black sheep, fecundity candidate gene, BMPR-IB, BMP15, *GDF9*, *ESR*α.

INTRODUCTION

The prolificacy trait is a quantitative trait controlled by multiple genes. In choosing the molecular marker linked to a quantitative trait locus (QTL), the genotype can be selected directly and breeding development can be accelerated. Although sheep is a monotocous animal, some breeds show multiparous characteristics. Research on the major genes influencing fecundity in sheep will increase the yean performance and it will help understand stand the mechanism of reproduction in mammals. At present, several major genes affecting fecundity (*BMPR-IB*, *BMP*15, *GDF*9 and *ESRα*) have been discovered in different breeds of sheep that affect also the litter size.

Fec^B was confirmed to be the first major gene that affects fecundity in Booroola Merino sheep. The effect of Fec^B on ovulation and litter size was obvious. Fec^B mapped chromosome 6 in sheep and precisely mapped at the span (4q22-23) that contains *BMPR-IB* in human. It was determined that A746G mutation at exon 6 in *BMPR-IB* affected litter size in Booroola Merino ewe.

BMP15 and *GDF9* are growth factors secreted by oocyte and regulate growth and differentiation of prophase ovarian follicle. Sheep *BMP15* maps to the X chromosome. The full length coding sequence of 1179 nucleotides is contained in two exons, separated by an intron of about 5.4 kb, and encodes a prepropeptide of 393 amino acid residues. The active mature peptide is 125 amino acids long (Galloway et al., 2002). Four mutation

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Abbreviations: SNP, Single nuclear polymorphism; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **BB,** homozygote; **B+,** heterozygote; **++,** wild type.

Table 1. Primer sequence of candidate gene for prolificacy in Cele black sheep.

Gene	Site	Size	Foreword (5'-3')	Reverse (5'-3')
BMPR-IB	Fec^B	140bp	gtcgctatggggaagtttggatg	caagatgttttcatgcctcatcaacacggtc
BMP15	$FecX^G$	141bp	cactgtcttcttgttactgtatttcaatgagac	gatgcaatactgcctgcttg
BMP15	$FecX^B$	153bp	gccttcctgtgtcccttataagtatgttcccctta	ttcttgggaaacctgagctagc
BMP15	$FecX^I$	150bp	gaaagtaaccagtgttccctccacccttttct	catgattgggagaattgagacc
BMP15	$FecX^H$	240bp	tatttcaatgacactcagag	gagcaatgatccagtgatccca
GDF9	$FecG^H$	139bp	ctttagtcagctgaagtgggacaac	atggatgatgttctgcaccatggtgtgaacctga
ESR	-	115bp	ctcaacagcgtgtctccgagacc	tcggctcgttctccaggtaat

sites of *BMP*15, including $FecX^I$ (Davis, 1992) of Inverdale sheep, $FecX^H$ of Hanna sheep (Davis, 1995), $FecX^G$ and $FecX^B$ of both Belclare and Cambridge sheep significantly affected the number of ovulation (Hanrahan et al., 2004). Sheep *GDF*9 has been mapped to sheep chromosome 5 (Sadighi et al. 2002). The gene spans about 2.5 kp, contains 2 exons separated by a single 1126 bp intron, and encodes a prepropeptide of 453 amino acid residues. The active mature peptide is 135 amino acid-long (Bodensteiner et al., 1999). One mutation site in *GDF*9 significantly affected litter size in ewe. The effects of *BMP*15 and *GDF*9 on litter size in ewe were an increased litter size in mutated heterozygote and infertility in mutated homozygote.

ESRα is a member of the nuclear receptor superfamily of ligand-activated transcription factor. *ESR*α-knocked out mice showed anovulation, chaotic LH regulation, and insensitivity of uterus to estrogen (Hewitt and Korach, 2003). This indicates that *ESRα* plays an important role in prolificacy.

The Cele black sheep is distributed in the Hetian district of Xinjiang Uygur Autonomous Region in China. This breed is a fine multiparous landrace that produces budge primarily. The breed is well-adapted to the arid and semi-desert environment with relatively higher ovulation and reproduction rates than other breeds. The breeding age of Cele black sheep is 1.5 – 2 years old and its gestation period is 148 – 149 days. The Cele black sheep can give birth to 8 lambs in a lifetime with an average lambing rate of 215.46% (Bai, 2007).

Up till now, no research on candidate genes influencing fecundity in Xinjiang Cele Black sheep has been reported. Therefore, we detected single nuclear polymorphism (SNP) sites of four genes related with litter size (*BMPR-IB*, *BMP*15, *GDF*9 and *ESRα*) by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Then, the correlation between genotype of these SNP sites and litter size was analyzed to determine whether these candidate genes have major effect on high fecundity in Cele black sheep.

MATERIALS AND METHODS

Experimental sheep flocks and sampling

All procedures involving animals were approved by the animal care and use committee at the respective institutions where the experiment was conducted. All procedures involving animals were approved and authorized by the Chinese Ministry of Agriculture. A total of 274 ewes were examined in this study from Cele black sheep Resource Conservative Region in Xinjiang. Blood was collected by puncturing of the jugular vein and placed in 10 ml vacuum blood collection tubes containing citrate sodium as anti-coagulant. All samples were taken back to the laboratory at 4°C. The litter size and body traits were obtained.

Main chemicals and reagents

The primers were synthesized by Shengong (Shanghai) Company Limited. Restriction enzymes *Ava* II, *Spe* I, *Xba* I, *Hinf* I and dNTP as well as DNA markers were purchased from Takara (Dalian, China). *Taq* polymerase and *BSP1286* I and *Dde* I were purchased from Promega (Madison, WI, USA).

DNA extraction

A slightly modified standard phenol chloroform procedure was used to extract DNA from blood sample. An Eppendorf biophotometer was used to assess DNA concentration and quality on the basis of absorbance of UV light at 260 and 280 nm stored at -20°C before use.

PCR and PCR-RFLP reactions

The primers for detecting Fec^B mutation of *BMPR-IB* gene was cited by Wang et al. (2003). The reverse primer deliberately introduced by a point mutation would create an *Ava* II restriction site (GGACC) in PCR products from Fec^B carrier sheep, whereas PCR products from the no carriers lacked this site. $FecX^G$ (C to T nucleotide change) was detected using *Hinf* I (Hanrahan et al., 2004; Chu et al., 2005); the wild-type strand was cleaved. The $FecX^B$ (G to T nucleotide change) was detected using *Dde* I (Hanrahan et al., 2004; Chu et al., 2005); the wild-type strand was cleaved. $FecX^H$ (C to T nucleotide change) was detected using *Spe* I (Galloway et al., 2000), the mutation-type strand was cleaved. $FecX^I$ (T to A nucleotide change) was detected using *Xba* I (Galloway et al., 2000); the mutation-type strand was cleaved. $FecG^H$ (C to T nucleotide change) was detected using *Dde* I (Hanrahan et al., 2004; Chu et al., 2005); the wild-type strand was cleaved. *ESRα* (C to G nucleotide change) was detected using *BSP1286* I (Bi et al., 2005; Bai, 2007), the wild-type strand was cleaved. The primers are shown in Table 1.

PCR reactions were carried out in a total volume of 20 µL solution containing 50 ng template DNA, 50 mM KCl, 10 mM Tris-HCL, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 5% DMSO, 10 pM of each primer, and 1.5 U of *Taq* DNA polymerase. Touchdown

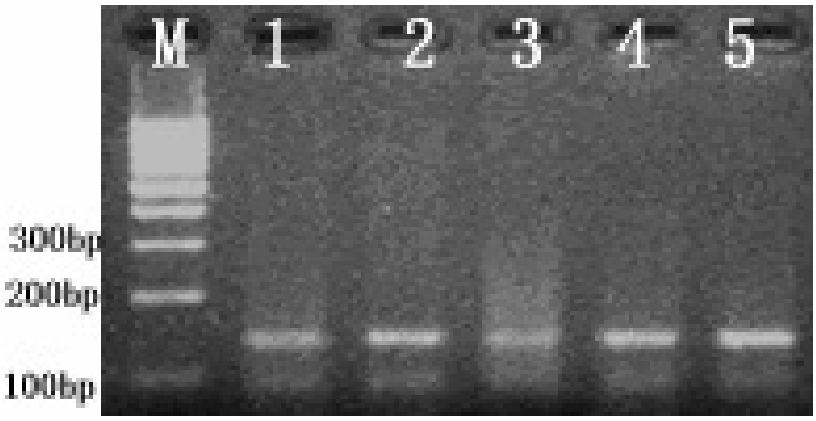

Figure 1. PCR product of *BMPR-IB*.

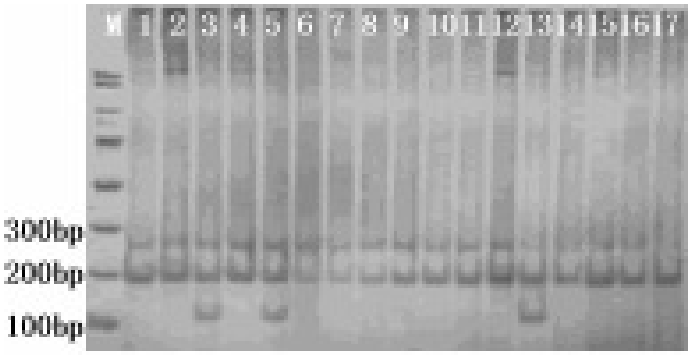

Figure 2. RFLP analysis of Fec^B mutation of *BMPR-IB* gene.

amplification was performed with the following program: 94°C for 5 min; a gradient over 14 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 40 s, with the annealing temperature subsequently decreasing by 2°C/2 cycle; 21 cycles of 94°C for 30 s, 51°C for 30 s and 72°C for 40 s; and finally, 72°C for 8 min.

In summary, the product of PCR with no purification was digested in a total of 10 µL reaction containing 1× buffer, 5 U restriction endonuclease, 300 ng PCR product at constant temperature (37°C) for 10 h in RFLP reaction. The products digested were electrophoresed on 12% neutral polyacrylamide gel, which were treated at the following conditions: 200 V, 2.5 h. Then, the gels were stained by Silver Stain (Kucharczyk Techniki Elektroforetyczne). The patterns of DNA bands were observed and photographed with ImageMaster VDS System.

Statistical analysis

Genotypic and allelic frequencies were calculated using the PopGene software (ver. 1.31). The Hardy-Weinberg equilibrium in the mutation sites were determined by χ^2 test.

Analysis of association between the genotypes and litter size was carried out with general linear model (GLM) procedure using statistical analysis system (SAS) 9.0 software (SAS Institute Incorporation.) by the following formula

$$Y = \mu + a + e$$

Where, Y is the value of litter size, µ is the overall mean, a is the effect of the genotype and e is the random error.

RESULTS

Results of PCR-RFLP electrophoresis

As expected, the size of PCR production of *BMPR-IB* gene was 140 bp (Figure 1). After digesting with restriction endonuclease (*Ava* II), the band of homozygote (BB) or wild type (++) was 110 or 140 bp, while the band of heterozygote (B+) was 110 and 140 bp, respectively (Figure 2).

The sizes of PCR production of the four mutation sites ($FecX^H$,$FecX^I$,$FecX^G$ and $FecX^B$) in *BMP*15 gene were 240 bp (Figure 3), 150 bp (Figure 5), 141 bp (Figure 7), and 153 bp (Figure 9). The result was in accordance with expectations. After digesting with different restriction endonucleases, the four earlier mentioned mutation sites were not detected in Cele black sheep (Figures 4, 6, 8 and 10).

The size of PCR production of the mutation sites ($FecG^H$) in *GDF*9 gene was 139 bp (Figure 11). The result was according to expectation. After digesting with restriction endonucleases (*Dde* I), $FecG^H$ was not detected in

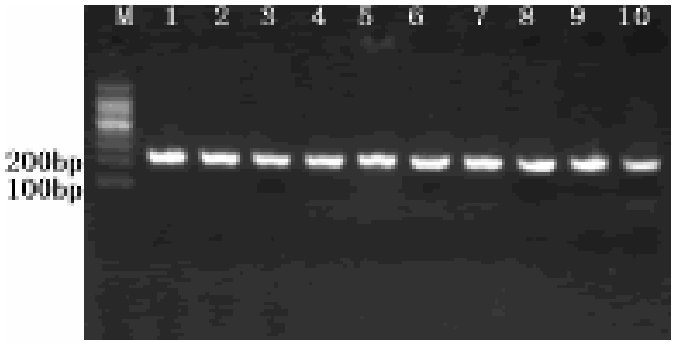

Figure 3. PCR product of FecX^{H}.

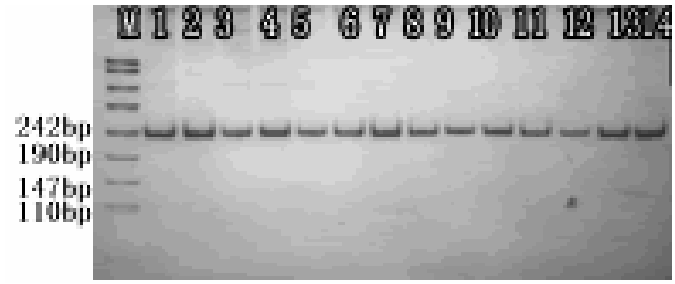

Figure 4. RFLP analysis of FecX^{H} mutation of *BMP*15 gene.

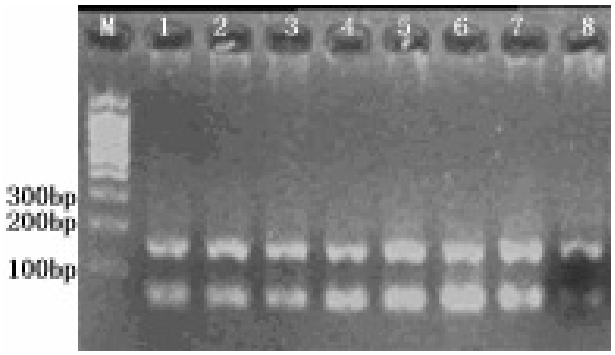

Figure 5. PCR product of FecX^{I}.

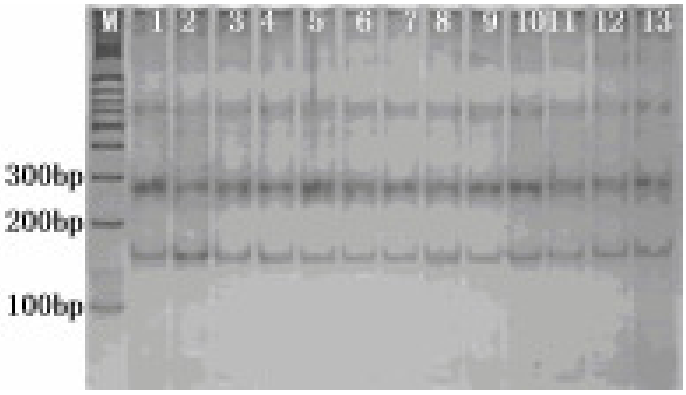

Figure 6. RFLP analysis of FecX^{I} mutation of *BMP*15 gene.

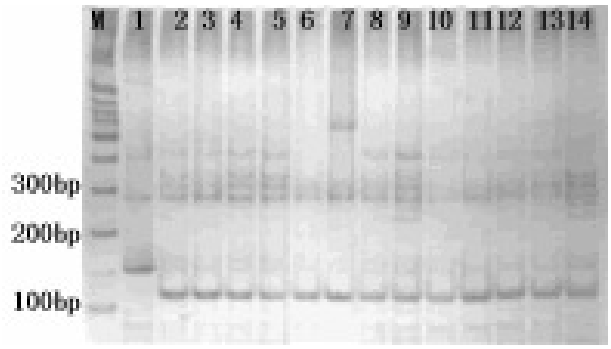

Figure 8. RFLP analysis of $FecX^{G}$ mutation of *BMP*15 gene.

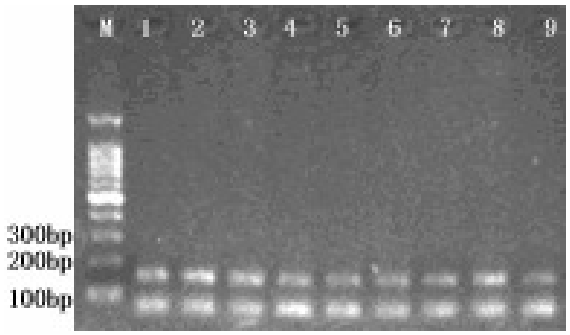

Figure 9. PCR product of $FecX^{B}$.

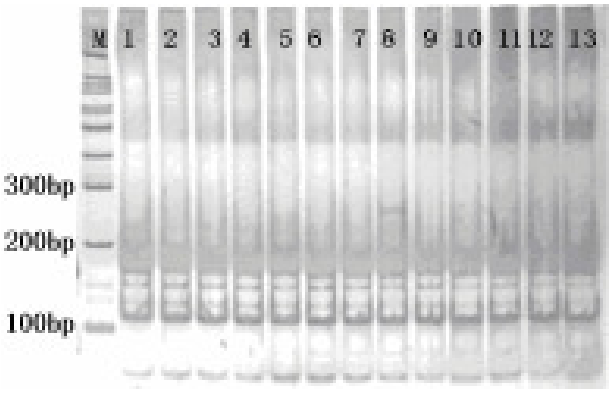

Figure 10. RFLP analysis of $FecX^{B}$ mutation of *BMP*15 gene.

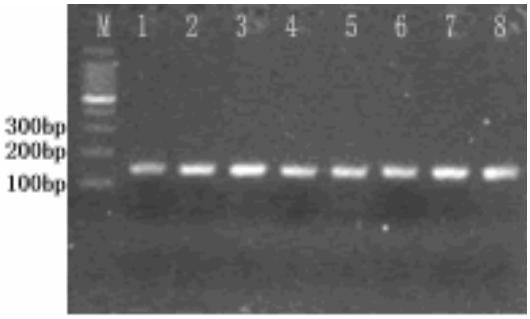

Figure 11. PCR product of $FecG^{H}$.

Figure 12. RFLP analysis of $FecG^{H}$ mutation of *GDF*9.

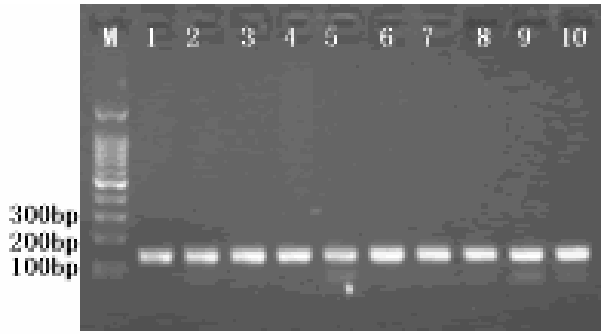

Figure 13. PCR product of *ESR*.

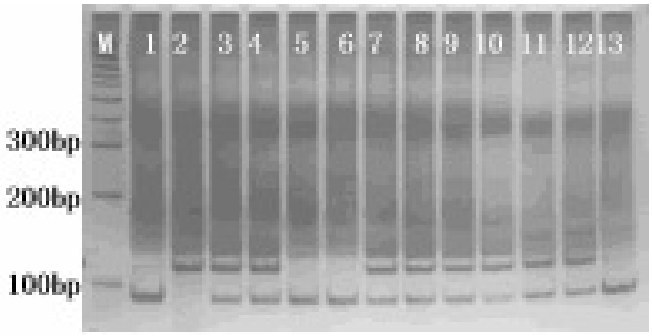

Figure 14. RFLP analysis of *ESR* gene

Cele black sheep (Figure 12).

The size of production of *ESRα* was 115 bp (Figure 13), and the result was as expected. After digesting with restriction endonuclease (*BSP1286* I), the band of ++ or EE was 92 bp or 115 bp while the band of E+ was 115 and 92 bp (Figure 14), respectively.

The allele and genotype frequencies of *BMPR-IB* and *ESRα* in Cele black Sheep

The allele and genotype frequencies of *BMPR-IB* and *ESRα* in Cele black sheep are shown in Table 2. Wild types of *BMPR-IB* and *ESRα* were dominant genotypes. The frequencies of allele + in *BMPR-IB* and *ESRα* were 0.652 and 0.792, respectively. The result of the Hardy-Weinberg equilibrium for the two loci in Cele black sheep indicates that two SNP sites were in Hardy-Weinberg equilibrium ($P > 0.05$).

Effects of *BMPR-IB* genotypes or *ESRα* genotypes on litter size of Cele Black Sheep

The correlations between *BMPR-IB* genotype or *ESRα* genotype and litter size were analyzed statistically (Table

Table 2. Genotype and allele frequency in Cele black sheep.

Gene	Genotype frequency (Number)			Allele frequency	
	Homozygote (BB or EE)	Heterozygote (B+ or E+)	Wild type (++)	B or E	+
BMPR-IB	0.113 (31)	0.471(129)	0.416(114)	0.348	0.652
ESR	0.047 (13)	0.321(88)	0.631(173)	0.208	0.792

Table 3. Least squares mean for litter size of different *BMPR-IB* and *ESR* genotypes in Cele black sheep.

Gene	Genotype	No.	Least squares mean
BMPR-IB	++	11	$1.622^{A} \pm 0.096$
	B+	129	$2.17^{B} \pm 0.091$
	BB	31	$2.230^{B} \pm 0.148$
ESR	++	173	2.005 ± 0.067
	E+	88	1.953 ± 0.086
	EE	13	2.072 ± 0.212

Least squares means with different superscripts within the same column differ (P < 0.01).

3). The results showed that both B+ and BB genotypes increased litter size as compared with ++ (P < 0.01). No significant difference was observed between B+ and BB in litter size (P > 0.05). This indicates that the mutation of *BMPR-IB* significantly affected litter size in Cele Black sheep. There was no difference in litter size among the three genotypes of *ESRα* gene (P > 0.05).

DISCUSSION

The effects on fecundity of major genes in sheep have been the focus of this research. Mutations of the four genes (*BMPR-IB*, *BMP*15, *GDF*9 and *ESRα*) affected litter size in different sheep breeds. Therefore, these are major genes influencing fecundity in sheep. Cele black sheep mainly produce budge and have high litter size (Bai, 2007). At present, no report is available relating to the effect on fecundity of major gene in Cele black sheep. We detected SNP sites in *BMPR-IB*, *BMP*15, *GDF*9 and *ESRα* genes which could affect litter size. The correlation between the SNP sites and litter size in Cele black sheep was analyzed as well.

The Fec^{B} mutation was discovered in Booroola sheep initially. Consequently, it was reported that Fec^{B} was derived from a mutation of A746G in *BMPR-IB* gene. A 746G mutation led to the change of Q249R. The impaired gene function by the mutation lowered inhibition of ligand to progesterone secreted by granular cell, improved the differentiation of granular cell and maturation of ovarian follicle, and increased ovulation. A 746G polymorphism in *BMPR-IB* was detected in Garole sheep (Davis et al., 2002; Polley et al., 2009), Kendrapada sheep (Kumar et al., 2008), Japanese sheep (Davis et al., 2002), Small Tailed Han sheep (Liu et al. 2003; Wang et al. 2003; Yan et al. 2005), Hu sheep (Wang et al. 2003), and Duo Lang sheep (Zhong, 2005). Subsequently, polymorphism influenced litter size in the above-mentioned breeds. The mutation in *BMPR-IB* gene in Cele black sheep and allele B also significantly affected litter size (P<0.05). These preliminary results showed that the *BMPR-IB* gene is either a major gene that influences the prolificacy in Cele black sheep or a molecular genetic marker in close linkage with such gene.

As proteins regulate oocyte secretion, the change of important sites in *BMP*15 and *GDF*9 can significantly affect ovulation in ewe. C/T mutation at E+67 bp of *BMP*15 in $FecX^{H}$-carried ewe led to the change of Glu at amino acid 23 into termination codon (Galloway et al., 2000). T/A mutation at E+92 bp of *BMP*15 in $FecX^{I}$-carried ewe led to the change of Val at amino acid 31 into Asp (Galloway et al., 2000). C/T mutation at E2+718 bp of *BMP*15 in $FecX^{G}$-carried ewe led to the change of Glu at amino acid 239 into termination codon. G/T mutation at E2+1100 bp of *BMP*15 in $FecX^{B}$-carried ewe led to the change of Ser at amino acid 99 into Ile. C/T mutation at E2+1184 bp of *GDF*9 in $FecG^{H}$-carried ewe led to the change of Ser at amino acid 77 into Phe. Mutation of four sites in *BMP*15 and mutation of one site in *GDF*9 had similar effects on ovulation in ewe. The effects increased litter size in mutated heterozygote and infertility in mutated homozygote (Hanrahan et al., 2004). In this study, we used RFLP approach to detect the above-mentioned five mutation sites. No polymorphism existed among these sites in Cele black sheep suggesting that the molecular mechanism affecting the multiparous performance in Cele black sheep was different from those of Inverdale sheep, Hanna sheep, Belclare sheep and Cambridge sheep.

ESRα gene was reported to be a major gene that affected litter size in swine (Rothschild et al. 1994). Therefore, *ESRα* has been used as a molecule marker in assisting breeding (Rothschild et al. 1996; Short et al. 1997; Southwood et al. 1998). Chu et al. (2005) reported that litter size of Small Tailed Han sheep with genotypes AB and BB was higher than those with genotype AA (P < 0.05). This showed that the *ESRα* is a potential candidate gene that influences prolificacy in Small Tailed Han sheep (Bi, 2005). We showed that the same SNP site of *ESRα* gene exists in Cele Black sheep, although no significant

difference was observed among genotypes ($P > 0.05$). This observation suggests that the effect of this SNP site on litter size in Cele black sheep was different from that of the Small Tailed Han sheep.

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