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Polymorphism of bone morphogenetic protein 4 gene and its relationship with litter size of Jining Grey goats

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Abstract Two pairs of primers (P1 and P2) were designed to detect single nucleotide polymorphisms of exon 2 and intron 2 of bone morphogenetic protein 4 (*BMP4*) gene in both high fecundity breed (Jining Grey goat) and low fecundity breeds (Boer, Angora and Inner Mongolia Cashmere goats) by single strand conformation polymorphism. Results showed that no polymorphism was detected for exon 2 (primer P1) of *BMP4* gene in four goat breeds. For intron 2 (primer P2), three genotypes (AA, AB and BB) were detected in Jining Grey and Inner Mongolia Cashmere goats, two genotypes (AB and BB) in Angora goats, and only one genotype (AA) in Boer goats. Sequencing revealed one mutation (2203G>A) of *BMP4* gene in the genotype BB in comparison to the genotype AA. The differences of litter size between AA, AB and BB genotypes were not significant ($P > 0.05$) in Jining Grey goats. A pair of primer (P3) was designed to detect polymorphism in the 3′ flanking region of *BMP4* gene that contained dinucleotide repeated sequence (CA) in the four goat breeds by microsatellite analysis. For primer P3, three genotypes (CC, CD and DD) were detected in four goat breeds. Sequencing revealed one more CA dinucleotide in genotype DD than in genotype CC. The Jining Grey does with genotype CC had 0.55 ($P < 0.05$) or 0.72 ($P < 0.05$) kids more than those with genotype CD or DD. These results preliminarily indicated that allele C of *BMP4* gene is a potential DNA marker for improving litter size in goats.

Keywords Goat · Prolificacy · Bone morphogenetic protein 4 gene · PCR-SSCP · Microsatellite

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Introduction

Bone morphogenetic proteins (BMPs) are members of the TGF-β (transforming growth factor-beta) superfamily [1]. Mouse *BMP4* gene maps to chromosome 14 and consists of four exons [2]. *BMP4* gene has an expression in mouse developing testis and epididymis [3], and also presents in bovine antral follicles [4] and rat ovarian tissues [5], especially in thecal cells [6]. BMP4 expression appeared very high in healthy follicles but barely detectable in follicles undergoing atresia, which can act directly on granulosa cells and cause important changes in FSH action [6]. BMP4 can inhibit progesterone production by granulosa cells and decrease basal granulosa cells progesterone secretion and totally abolish FSH-stimulating action both in cattle [4, 7] and sheep [8–10]. In human, BMP4 can induce the differentiation of embryonic stem cells to trophoblast [11]. So, BMP4 could have implications for reproductive function in mammals. To date, no literature revealed the relationship between *BMP4* gene and high prolificacy in goats.

The Jining Grey goat is an excellent local breed in China for its significant characteristics of sexual precocity, year-round estrus, and high prolificacy [12]. The mean litter sizes alive of Jining Grey, Inner Mongolia Cashmere, Boer and Angora goats have been reported to be 2.94 [12], 1.04

[12], 2.10 [13] and 1.31 [14], respectively. Based on the important role of *BMP4* gene in reproduction, *BMP4* gene was considered as a possible candidate gene for the prolificacy of Jining Grey goats. The objectives of the present study were firstly to detect polymorphism of exon 2 and intron 2 of *BMP4* gene in both high prolificacy breed (Jining Grey goat) and low prolificacy breeds (Inner Mongolia Cashmere, Boer and Angora goats) by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) method, secondly to detect a short sequence with more than ten continuous and repeated CA dinucleotide located in the 3′ flanking region near the termination site of coding region of *BMP4* gene by microsatellite analysis, and thirdly to investigate the association between polymorphism of *BMP4* gene and prolificacy in Jining Grey goats in which the polymorphism is segregating.

Materials and methods

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.

Animals and genomic DNA isolation

Venous jugular blood samples (10 ml per goat doe) were collected from 142 Jining Grey goat does kidded in 2007, along with data on litter size in the first, second, or third parity (Jining Grey goats conservation base, Jiaxiang County, Shandong Province, People's Republic of China), 40 Inner Mongolia Cashmere goat does (Inner Mongolia White Cashmere Goat Breeding Farm, Etuokeqi, Ordos City, the Inner Mongolia Autonomous Region, People's Republic of China), 40 Boer and 40 Angora goat does (Qinshui Demonstration Farm, Qinshui County, Shanxi Province, People's Republic of China) using acid citrate dextrose as an anticoagulant. Genomic DNA was extracted from whole blood by phenol–chloroform method, and then 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 142 Jining Grey goat does were selected at random and were the progeny of five goat bucks ($n = 28$, 30, 27, 26, 31). Because the five goat bucks 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. Kidding seasons consisted of 3-month groups starting with March through to May as season 1 (spring, $n = 37$), June through to August as season 2 (summer, $n = 34$), September through to November as season 3 (autumn, $n = 40$) and December through to February as season 4 (winter, $n = 31$).

Primers and PCR amplification

Three pairs of primers (P1, P2 and P3) were designed to amplify the goat *BMP4* gene based on the sheep (GenBank accession no. EE851370) and goat (GenBank accession no. EU104684) *BMP4* gene sequences. Primer sequence, amplified region and product size were listed in Table 1.

Polymerase chain reactions 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), 1.0–1.8 μl of 25 mmol/l $MgCl_2$, 2.5 μl of 2.5 mmol/l each dNTP, 3.0–3.5 μl of 50 ng/μl caprine genomic DNA, 1.0 μl of 2.5 U/μl *Taq* DNA polymerase (Promega, Madison, WI, USA), and the rest is ddH_2O.

Amplification conditions were as follows: initial denaturation at 95°C for 5 min; followed by 32 cycles of denaturation at 94°C for 30 s, annealing for 30 s (58°C for P1, 58°C for P2, 63°C for P3), extension at 72°C for 20 s; with a final extension at 72°C for 9 min on Mastercycler® 5,333 (Eppendorf AG, Hamburg, Germany).

Table 1 Primers of goat *BMP4* gene analysis

Primer	Primer sequence (5′→3′)	Amplified region	Product size (bp)
P1	F: TTTTATTATGCCAAGTCCTGC	Exon 2	292
	R: GGATACTCCAGACCGATGC	1123–1414 bp[a]	
P2	F: CTGGGGAAATGTTTGGTA	Intron 2	381
	R: GCTAAGAGTTGGGTGATGAG	1959–2339 bp[a]	
P3	F: GGAGATGGTAGTAGAGGGAT	3′ flanking	207
	R: AAGTCATAAATAAGGTCAAGG	104–310 bp[b]	

[a] According to GenBank accession no. EU104684

[b] According to GenBank accession no. EE851370

The PCR products were separated by electrophoresis on 1.5% agarose gels (Promega) in parallel with a 600 bp DNA marker.

Single strand conformation polymorphism analysis

A volume of 2 μl PCR product of P1 and P2 was transferred in an Eppendorf tube, mixed with 7 μl gel loading solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 20 mmol/l EDTA (pH 8.0) and 10% glycerol. The mixture was centrifugalized and denatured at 98°C for 10 min, then chilled on ice for 7 min and loaded on 10% neutral polyacrylamide gels (acrylamide:bisacrylamide = 39:1). Electrophoresis was performed in 1× Tris borate (pH 8.3)-EDTA buffer at 9–15 V/cm for 14–16 h at 4°C. The gels were stained with silver nitrate to identify SSCP, then photographed and analyzed using an AlphaImager™ 2,200 and 1,220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

Microsatellite analysis

A volume of 5 μl PCR product of P3 was mixed with 0.5 μl gel loading solution (bromchlorphenol blue loading buffer). The mixture was loaded on 12% neutral polyacrylamide gels (acrylamide:bisacrylamide = 38:2). The DNA marker is pBR322/*Msp*I for a standard. Electrophoresis was performed in 1× Tris borate (pH 8.3)-EDTA buffer at 9–15 V/cm for 12 h at 4°C. The gels were stained with silver nitrate to identify genotype, then photographed and analyzed using an AlphaImager™ 2,200 and 1,220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

Cloning and sequencing

PCR products of different homozygous genotypes were separated on 1.0% agarose gels and recovered using Geneclean II kit (Promega). The DNA fragments were ligated into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions at 4°C overnight. The ligation reactions were carried out in a 5 μl reaction mixture containing 1.5 μl of PCR product, 0.5 μl of pGEM-T Easy vector (50 ng/μl), 0.5 μl of T4 ligase (3 U/μl), and 2.5 μl of 2× ligation buffer. The recombinant plasmid was then transformed into *Escherichia coli* DH5α competent cell. Positive clones of transformed cells were identified by restriction enzyme digestion. Two clones of each homozygous genotype were selected and sequenced. Each clone was sequenced for thrice. The target DNA fragments in recombinant plasmids were sequenced from both directions using an ABI3730 automatic sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China).

Statistical analysis

The following fixed effects model was employed for analysis of litter size in Jining Grey goat does and least squares mean was used for multiple comparison in litter size among different genotypes.

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

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 ($i = 1, 2, 3, 4, 5$); KS_j is the fixed effect of the jth kidding 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.

Results

PCR amplification of goat *BMP4* gene

Genomic DNA of four goat breeds was successfully amplified using three pairs of primers for *BMP4* gene. The PCR products were separated on 1.5% agarose gels (Fig. 1). The results showed that amplification fragment sizes were consistent with the target ones and had a good specificity, which could be directly analyzed by SSCP or microsatellite.

Single strand conformation polymorphism analysis

There was no polymorphism in PCR products amplified by primer P1. The PCR products amplified by primer P2

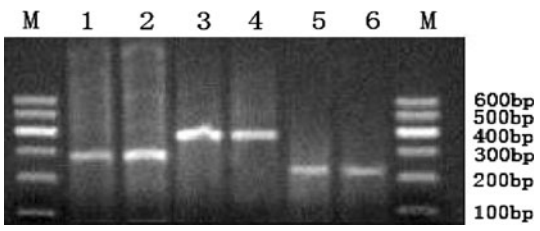

Fig. 1 PCR products of three pairs of primers. *1–2* PCR products of P1; *3–4* PCR products of P2; *5–6* PCR products of P3; M. 600 bp DNA marker

displayed polymorphisms. Three genotypes (AA, AB and BB) were detected (Fig. 2).

BMP4 gene 3′ flanking region microsatellite analysis

The PCR products amplified by primer P3 displayed microsatellite polymorphisms. Three genotypes (CC, CD and DD) were detected.

Sequencing of different genotypes

For primer P2, sequencing revealed one single nucleotide mutation 2203G>A (according to EU104684) of intron 2 of *BMP4* gene in BB genotype compared with AA genotype (Fig. 3).

For primer P3, sequencing analysis suggested that there existed a short sequence with more than ten continuous CA dinucleotide repeats, the CA repeats were found in the goat *BMP4* gene 3′ flanking region at position 157–195 (according to EE851370), starting at 20 bp downstream from the termination site of coding region. The CA repeats were 19 and 20 in CC and DD genotypes (Fig. 4).

Allele and genotype frequencies of *BMP4* gene in four goat breeds

Allele and genotype frequencies of *BMP4* gene in four goat breeds were presented in Table 2.

For primer P2, χ^2 test ($\chi^2 = 0.02$, $P = 0.9897$) showed that the Jining Grey goat population was in Hardy–Weinberg equilibrium, consistent with the Jining Grey goats tested being a random sample from a large random-mating population, in which genotype frequencies had not been distorted by recent selection, mutation, or migration. For primer P3, χ^2 test ($\chi^2 = 30.83$, $P = 0.0000$) showed that the Jining Grey goat population was not in Hardy–Weinberg equilibrium, in which genotype frequencies had been distorted by recent selection, mutation, or migration.

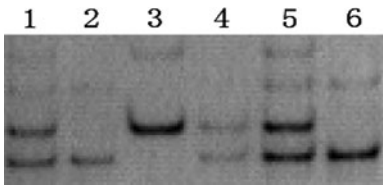

Fig. 2 SSCP analysis of PCR amplification using primer P2 in different goat breeds. *2, 6* AA genotype; *1, 4, 5* AB genotype; *3* BB genotype

Influence of fixed effects on litter size in Jining Grey goats

For primer P2, the litter size in Jining Grey goats was significantly influenced by sire, kidding season and parity ($P < 0.05$), and was not significantly influenced by BMP4 genotype ($P > 0.05$). For primer P3, the litter size in Jining Grey goats was significantly influenced by sire, kidding season, parity, and BMP4 genotype ($P < 0.05$). The least squares mean and standard error for litter size of different BMP4 genotypes in Jining Grey goats were given in Table 3.

For primer P2, the differences of the litter size between AA, AB and BB genotypes were not significant in Jining Grey goats ($P > 0.05$).

For primer P3, the Jining Grey goat does with genotype CC had 0.55 ($P < 0.05$) or 0.72 ($P < 0.05$) kids more than those with genotype CD or DD, the does with genotype CD had 0.17 ($P > 0.05$) kids more than those with genotype DD.

Discussion

Polymorphisms of *BMP4* gene

The first polymorphic site (147A>V) of *BMP4* gene was found by *Hph*I enzyme digestion which presented with similar frequency in Caucasian, Hispanic, and African populations [15]. Ramesh Babu et al. [16] detected a major haplotype defined by G-C-T allele in SNPs −5826G>A, 3564C>T and 6007C>T, respectively of *BMP4* gene in postmenopausal women. Chu et al. [18] found a SNP (305C>A) in exon 3 of *BMP4* gene of Small Tail Han, Chinese Merino, Corriedale and South African Mutton Merino sheep, which resulted in an amino acid change of alanine→aspartic acid. Capasso et al. [17] detected −3445T>G of *BMP4* in Southern Italian population with cutaneous melanoma.

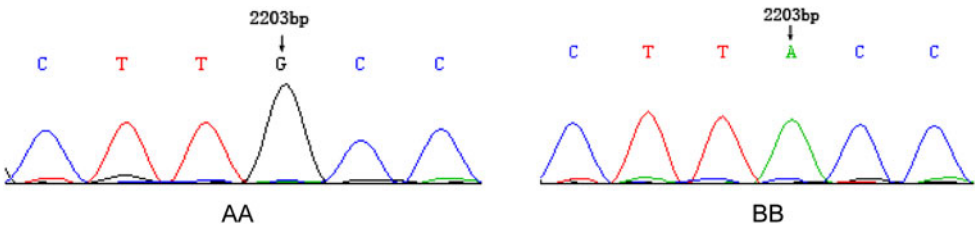

Fig. 3 Sequence comparison of AA and BB genotypes of primer P2 of goat *BMP4* gene

CC :

1 GGAGATGGTA GTAGAGGGAT GTGGGTGCCG CTGAGATCAG GCCTTCCTTG

GGGACATACA

61 CACACACACA CACACACACA CACACACACA CACACATCCC ATCCACTACT

CACCCACACA

121 CTACACAGAC TGCTTCCTTA TAGCTGGACT TTTATCTTAA AAAAAAAAAG

GAAAAAAAAT

181 CTAAACATTC ACCTTGACCA TATTTATGAC TT

(212 bp)

DD :

1 GGAGATGGTA GTAGAGGGAT GTGGGTGCCG CTGAGATCAG GCCTTCCTTG

GGGACATACA

61 CACACACACA CACACACACA CACACACACA CACACACATC CCATCCACTA

CTCACCCACA

121 CACTACACAG ACTGCTTCCT TATAGCTGGA CTTTTATCTT AAAAAAAAAA

AGGAAAAAAA

181 ATCTAAACAT TCACCTTGAC CATATTTATG ACTT

(214 bp)

Fig. 4 Sequence comparison of CC and DD genotypes of primer P3 of goat *BMP4* gene The underlined sequences represent CA repeat dinucleotide

In the present study, no polymorphism was detected in exon 2 of *BMP4* gene in goat populations tested. One SNP (2203G>A) was found in intron 2 and one microsatellite polymorphism existed in a repeated CA dinucleotide which located in the 3′ flanking region near the termination site of coding region of *BMP4* in four goat breeds.

Table 3 Least squares mean and standard error for litter size of different BMP4 genotypes in Jining Grey goats

Primer	Genotype	Number	Litter size
P2	AA	68	$2.90^{a} \pm 0.14$
	AB	60	$2.78^{a} \pm 0.17$
	BB	14	$2.55^{a} \pm 0.20$
P3	CC	106	$2.97^{a} \pm 0.12$
	CD	22	$2.42^{b} \pm 0.16$
	DD	14	$2.25^{b} \pm 0.18$

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

Relationship of *BMP4* gene with reproductive performance

BMP4 is crucial in spermatogenesis and in maintaining epididymal integrity. *BMP4* heterozygous male mice show compromised fertility due to degeneration of germ cells, reduced sperm counts and decreased sperm motility and those with homozygous mutants die during embryogenesis [3]. Mouse embryos with *BMP4* knockout contained no primordial germ cells and also lacked other extraembryonic mesoderm derived tissues such as the allantois [19].

Intrinsic ovarian BMPs system is involved in determining FSH activity and sensitivity in the granulosa cells during follicle growth and development. The interaction of physiological concentrations of BMP4 with its receptor can cause marked stimulatory and inhibitory effects on FSH-induced steroidogenesis [6]. When ewe pituitary cell cultures were treated with BMP4 in a concentration gradient for 48 h, FSH release was decreased in a dose-dependent manner [9]. BMP4 can also inhibit progesterone production

Table 2 Genotype and allele frequencies of P2, P3 of *BMP4* gene in four goat breeds

Breed			Jining Grey goat	Inner Mongolia Cashmere goat	Boer goat	Angora goat
Number			142	40	40	40
P2	Genotype frequency	AA	0.48 (68)	0.25 (10)	1.00 (40)	0 (0)
		AB	0.42 (60)	0.53 (21)	0 (0)	0.68 (27)
		BB	0.10 (14)	0.22 (9)	0 (0)	0.32 (13)
	Allele frequency	A	0.69	0.51	1.00	0.34
		B	0.31	0.49	0	0.66
P3	Genotype frequency	CC	0.75 (106)	0.68 (27)	0.38 (15)	0.30 (12)
		CD	0.15 (22)	0.07 (3)	0.22 (9)	0.45 (18)
		DD	0.10 (14)	0.25 (10)	0.40(16)	0.25 (10)
	Allele frequency	C	0.82	0.71	0.49	0.52
		D	0.18	0.29	0.51	0.48

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

by granulosa cells in a FSH-induced manner in cattle [4, 7] and sheep [8–10].

Chu et al. [18] detected one mutation (305C>A) in exon 3 of *BMP4* gene of Small Tail Han, Chinese Merino, Corriedale and South African Mutton Merino sheep. The Small Tail Han ewes with genotype BB had 0.61 ($P < 0.05$) or 1.01 ($P < 0.05$) lambs more than those with genotype AB or AA. This mutation had an additive effect for litter size in Small Tail Han sheep.

In the present study, the C allele of the microsatellite located in the 3′ flanking region of *BMP4* gene was a potentially effective DNA marker which would improve goat litter size. However, the conclusion was only preliminary; it was worth increasing the number of goat breeds, and expanding the number of samples to make in-depth study.

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