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Polymorphisms of Bone Morphogenetic Protein 4 (BMP4) Gene in Goats

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Abstract: Bone Morphogenetic Protein 4 (BMP4) plays a crucial role in development and productivity of mammalian. The aim of the present study was to identify and characterize polymorphisms within the coding region, the intron region and the 3'flanking region of the goat BMP4 gene in three different breeds. Three DNA fragments were amplified by Polymerase Chain Reaction (PCR) and then used for polymorphism identification by Single Stranded Conformation Polymorphism (SSCP). The fragments showing different SSCP patterns were sequenced. As a result, two new SNPs (EU104684:g.1986A>G, 2203G>A) were identified in the intron region and a short sequence with more than ten continuous and repeated CA dinucleotide were found in the 3'flanking region near the termination site of coding region and the length of repeated sequence dinucleotides CA was different in samples. Clonal sequencing analysis indicated that it was a microsatellite of dinucleotide-repeated sequence. The lengths of repeated sequence dinucleotide CA were 24, 17, 14, respectively. The PIC (Polymorphism Information Content) values in three populations were 0.3656, 0.5792, 0.3103 for Xuhuai White goat, Boer goat and Haimen goat, respectively, which indicated this goat microsatellite locus had rich polymorphism.

Key words: Goat, BMP4 gene, polymorphism, Single Nucleotide Polymorphism (SNP), microsatellite, China

INTRODUCTION

Bone Morphogenetic Proteins (BMPs) are members of the TGF-β (transforming growth factor-β) superfamily, which is a multifunctional cytokine with a two-fold function is expressed in a variety of cells (Massague, 1998). BMPs were originally identified on the basis of their ability to induce ectopic bone formation when implanted within soft tissue *in vivo* (Hogan, 1996; Bellusci *et al*., 1996; Wozney *et al*., 1988). With the deep research, its function is not limited to the formation and development of bone but it also plays roles in embryonic development, homeostasis, repair of various tissues patterning, cell differentiation and apoptosis (Nifuji *et al*., 1997).

So far, >30 members have been identified in BMP family. They were similar in structure but varied in function of different periods and different tissues. BMP4 is the most important member of BMPs. It is synthesized as a 408 amino acid precursor (pre-pro-precursor) that is proteolytically cleaved in the Golgi apparatus by pre-proconvertases that recognize the motif RRXR (Dubois *et al*., 1995), leaving a C-terminal mature protein (116 amino acids) that has seven conserved cysteine residues. The human BMP4 gene located on chromosome 14q22-q23 contained four exons and bovine on chromosome 10. BMP4 is an anabolic candidate with pleiotropic functions. During embryonic development, BMP4 plays important roles in mesoderm induction and endothelial progenitor cell differentiation, establishment of dorso-ventral polarity, ectodermal differentiation, somite formation and myogenesis induction (Winnier *et al*., 1995; Sasai and Robertis, 1997; Dale and Jones, 1999; Giudice, 2001; Wang and Ferguson, 2005). It also plays roles in determining bone mass and structure and possibly bone strength, inducing the differentiation of human ES (Embryonic Stem) cells to trophoblast and blood formation (Xu *et al*., 2002; Timothy *et al*., 2004; Jo *et al*., 2006). Furthermore, more and more reports revealed that BMP4 may play a crucial role in follicular growth and differentiation, cumulus expansion and ovulation (Onagbesan *et al*., 2003;

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Shimasaki *et al*., 2003; Glister *et al*., 2004, 2005). It is a promising candidate gene in assisted fertility and IVF protocol.

BMP4 is one of the best evolutionary conserved growth factors (Winnier *et al*., 1995), so there were few reports about BMP4 SNPs. The first polymorphic site of human gene was reported by Mangino *et al*. (1999), it was localized to nucleoted position 538 (T>C), resulting in amino acid change of Val>Ala (V147A) in their polypeptide.

Later, Semprini *et al*. (2000) and Milet *et al*. (2007) reported some other SNPs in human BMP4 gene. The polymorphisms analysis showed association with bone density in human.

These results further revealed that it was an important gene in bone growth and development for mammals. As we've seen, BMP4 plays key roles in growth and development, especially in bone development and productivity.

There are more and more researches in human, mouse and bovine of BMP4 gene but research in goat is blank. As is known, the growth and reproduction traits are two crucial traits in goat breeding, so we choose it for researching. In present study, variation in the goat BMP4 gene was investigated in 414 goats from three breeds (Boer goat, Xuhuai white goat and Haimen goat), using PCR-single-strand conformational polymorphism (PCR-SSCP) and DNA sequencing analysis. We found two SNPs in intron and a novel microsatellite in 3'flanking region.

MATERIALS AND METHODS

DNA samples: Genomic DNA samples were obtained from 414 unrelated goats belonging to three breeds: Boer goat (BE, n = 200), Xuhuai white goat (XH, n = 111) and Haimen goat (HM, n = 103). They were reared in Jiangsu province of China. DNA samples were extracted from leucocytes according to Mullenbach *et al*. (1989).

PCR conditions: According to the strong homology between sheep and goat, three primer pairs, designed by the sequences of Sheep (Genebank Accession No: EE851370) and goat (Genebank Accession No: EU104684), were used for the amplification of exon 2 (P1), partial intron 2 (P2), 3'flanking region (P3) of goat BMP4 gene (Table 1).

The PCR was performed in a 25 μL reaction mixture containing 10 pmol of forward primer and the same amount of reverse primer, 200 μM dNTP (dATP, dTTP, dCTP and dGTP), 1×buffer (including 1.5 mM $MgCl_2$), 0.6 unit of Taq DNA polymerase and 50 ng goat genomic DNA.

The cycling protocol was 4 min at 94°C, 35 cycles of 94°C for 45 sec, X°C annealing for 45 sec, 72°C for 50 sec, with a final extention at 72°C for 10 min (X°C were 57, 58 and 60°C for P1, P2 and P3 primers, respectively).

SSCP and DNA sequencing: SSCP method was used to scan mutations within the amplified regions. Aliquots of 5 μL PCR products were mixed with 5 μL denaturing solution (95% formamide deionized, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), heated for 10 min at 98°C and chilled on ice immediately (Lan *et al*., 2007).

Denatured DNA was subjected to 10% PAGE (Polyacrylamide Gel electrophoresis) in 1×TBE buffer and constant voltage (150 V) for 15 h at a constant temperature of 4°C, then gels were stained with 0.1% silver nitrate (Lan *et al*., 2007).

The PCR products representing different electrophoresis patterns in different breeds were subcloned to T-vector (Promega) and sequenced in both directions in ABI PRIZM 377 DNA sequencer (PerkinElmer).

Statistical analysis: The genotypic and allelic frequencies in three goat populations were analyzed by Popgene. Population genetic indexes (e.g., gene heterozygosity, gene homozygosity, effective allele numbers and Polymorphism Information Content (PIC)) were calculated by Nei method (Nei and Roychoudhury, 1974; Nei and Li, 1979).

Table 1: The primer pair sequences and their information on BMP4 gene in goats

Name	Sequences	Annealing temperature (°C)	Sizes (bp)	Note
P1	F:5'TTTTATTATGCCAAGTCCTGC3'	57	292	Partial exon 2 (EU104684: 1123-1414nt)
	R:5'GGATACTCCAGACCGATGC3'			
P2	F:5' CTGGGGAAATGTTTGGTA3'	58	381	Partial intron 2 (EU104684: 1959-2339nt)
	R:5' GCTAAGAGTTGGGTGATGAG3'			
P3	F:5' GGAGATGGTAGTAGAGGGAT3'	60	207	Partial 3'flanking region (EE851370: 104-310nt)
	R:5' AAGTCATAAATAAGGTCAAGG3'			

RESULTS AND DISCUSSION

Partial intron 2 polymorphism: Partial intron 2 of BMP4 gene demonstrated polymorphism (namely A and B patterns) by PCR-SSCP method. The alignment between nucleotide sequences of EU104684 a nd the partial intron 2 sequencing results of three PCR products with different patterns demonstrated two mutations (EU104684:g.1986A>G, 2203G>A). Here, the genotype characterized by the same as EU104684 was designated as AA, while the mutation was designated as BB (Fig. 1).

Frequency of allele B in the analyzed populations was 0.1667, 0.0050 and 0.3010 for Xuhuai White goat, Boer goat and Haimen goat populations, respectively. For Boer population, the frequency of B allele is lower than the other two populations. The genotype distributions were in good agreement with Hardy-Weinberg equilibrium ($p>0.05$) in each breed (Table 2).

According to the classification of PIC (low polymorphism if PIC value<0.25, median polymorphism if 0.25<PIC value<0.5 and high polymorphism if PIC value>0.5), Haimen goat population belonged to a median polymorphism level, Xuhuai White goat population and Boer goat population both belonged to a low polymorphism level (Table 3). And the sequence of PIC value in the three breeds was BE<XH<HM, the results were agreed with the gene heterozygosity, effective allele numbers.

3'flanking region polymorphism: The alignment between nucleotide sequences of EE851370 and sequencing results demonstrated that there was a novel microsatellite in 3'flanking region. However, clonal sequencing analysis suggested that there existed a short sequence with more than ten continuous CA dinucleotide repeats, the CA repeat was found in the goat BMP4 gene 3'flanking region at position 157-195 (Genebank Accession No: EE851370), starting at 20 bp downstream from the termination site of coding region. A total of three alleles of the BMP4 gene were detected in the three populations, we defined them as allele A, B and C and the corresponding CA repeats were 24, 17 and 14, respectively (Fig. 2 and 3). The frequency of the alleles varied between 0.0583 and 0.7961 and B was a predominant allele in three populations. The genotype distributions were not in agreement with Hardy-Weinberg equilibrium ($p<0.05$) in three populations (Table 4).

According to the classification of PIC, Boer goat belonged to a high polymorphism level and the other two native goat breeds belonged to a median polymorphism level (Table 5).

The objective of the present study was to identify and characterize polymorphisms within the coding region, the intron region and the 3'flanking region of the goat BMP4 gene in 414 individuals from three different breeds. But we couldn't found any mutations in the coding region of BMP4 gene. It has been reported that this gene was highly evolutionary conserved (Winnier *et al*., 1995), this result was a good authentication for it.

Two mutations were detected in intron 2, the results showed that the genotype AA is a predominant genotype and A is a predominant allele at partial intron 2 locus. The frequency of allele B in Boer population was only 0.0050, much lower than the other two native goat populations and the gene heterozygosity, effective allele numbers and PIC were same as it too. The B allelic frequency and PIC values were very low in Boer goat population, it may due to selection. Boer goat was selected largely in the long-term artificial selection process, which tended to be homozygous and then fixed gradually, thus appeared the conservatism of breed (Xu *et al*., 2007). B allele may be negative in growth traits and positive in reproduction traits. As is known, Boer goat is a world famous meat breed, which has features of large size, well developed hind limb, fast growth, fine quality mutton but low productivity. However, the native breed, such as Xuhuai goat and Haimen goat, facing less selective pression usually, the body size is small, growing slowly but

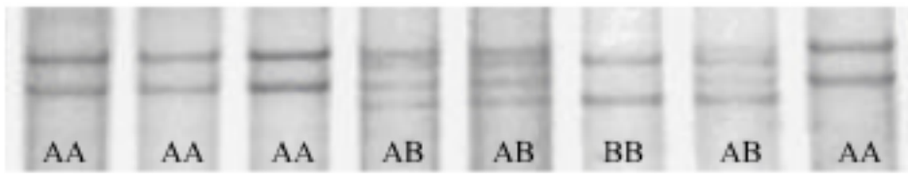

Fig. 1: DNA electrophoretic patterns on 7% PAGE after SSCP of the DNA region containing partial intron 2 of BMP4 gene in three goat breeds

Table 2: Genotype distribution and allelic frequencies in partial intron 2 of BMP4 gene in three goat breeds

	Genotype frequencies (numbers)			Allelic frequencies		
Breeds	AA	AB	BB	A	B	χ^2 (HWE)*
XH	0.7117 (79)	0.2433 (27)	0.0450 (5)	0.8333	0.1667	1.8702
BE	0.9900 (198)	0.0100 (2)	0.0000 (0)	0.9950	0.0050	0.0025
HM	0.4951 (51)	0.4078 (42)	0.9701 (10)	0.6990	0.3010	0.1324

χ^2 (HWE)* = Hardy-Weinberg Equilibrium χ^2 value. Its p-value was above $\alpha = 0.05$

toleranceing coarse food grain and worse conditions and wool is excellent, especially high productivity. This fact just coincided with the results that Boer goat with the lowest B allelic frequency. But whether the growth and reproduction traits of goats were associated with the BMP4 polymorphism, needed to be further defined by association studies in more populations so as to delineate the effect on it.

In present study, a novel microsatellite was found. The microsatellite was identified basing on clonal sequencing analysis, it was a short sequence with more than ten continuous and repeated CA dinucleotides, tightly linked with BMP4, located in the 3'flanking region only 20 nucleotides downstream from the termination site of coding region. The remarkable characteristic of this new microsatellite had less allele number, only three. There were two possible reasons for this result. One is that microsatellite production was related to nonsister chromatid unequal exchanges (Jiang, 1998), the microsatellite in the study was near with BMP4, which tightly linked to it in the process of meiosis. So it reduced the probability of unequal exchanges greatly and leaded to the low allele number. The other is that the populations used in this study were little and therefore the effect of random variation is less acute (Huang *et al*., 2005).

The functional significance of the microsatellite can not be ignored because numerous microsatellite and minisatellite DNAs have been proposed as hotspots for recombination (Jeffreys *et al*., 1998; Templeton *et al*., 2000). Some microsatellite sequences may influence recombination directly by their effects on DNA structure. It has been proposed that GT, CA, CT, GA, GC or AT repeat-binding proteins could participate in recombination processes by inducing Z-conformation or other alternative secondary DNA structure (Biet *et al*., 1999; Karlin *et al*., 1998). Additionally the microsatellite may be in linkage disequilibrium with variation in other regions of the gene with functional or structural significance.

Herein, we identified two silent SNPs and one novel microsatellite which might be meaningful for searching more goat genetic markers, constructing goat gene map and accumulating genetic data and so on. However, these results should be considered as preliminary ones, further investigations will be essential for detecting the polymorphism of this gene in a broad variety of goat breeds and populations. It will be urgently needed to apply these SNPs and microsatellite as markers in association studies to determine whether genetic variation at the goat BMP4 locus has any quantitative effect on growth and reproduction traits.

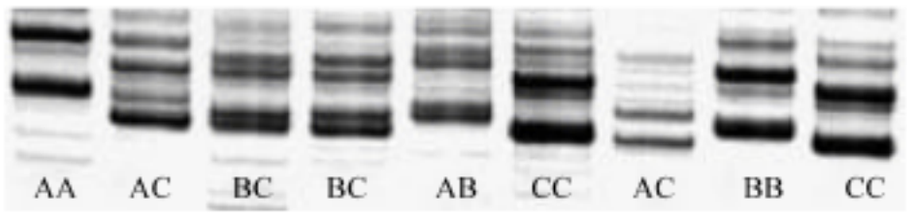

Fig. 2: DNA electrophoretic patterns on 12% PAGE after SSCP of the DNA region containing 3'flanking region of BMP4 gene in three goat breeds.

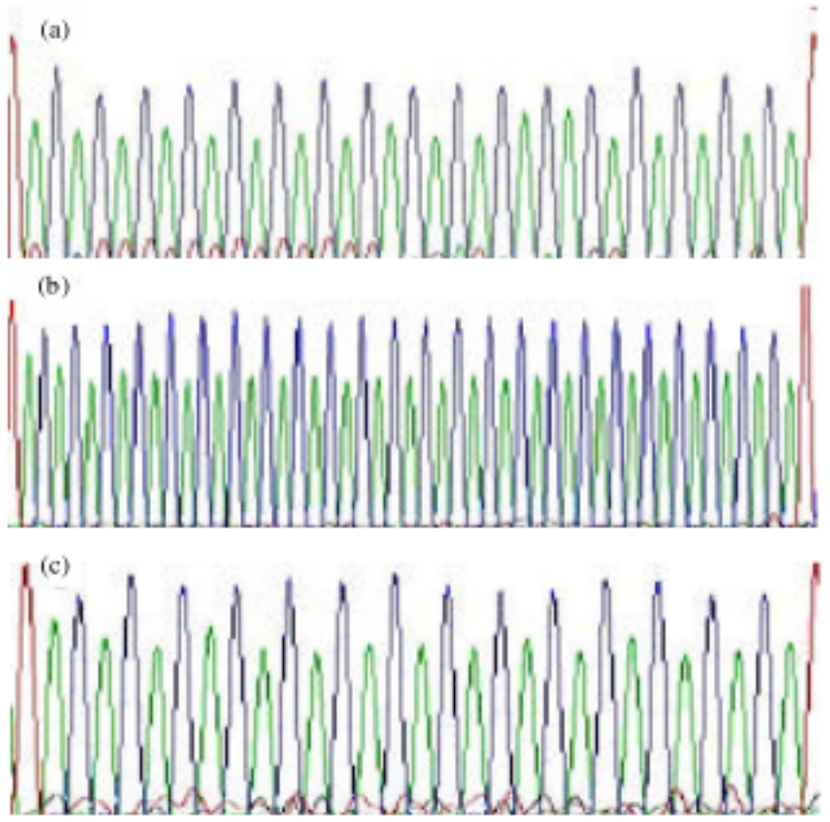

Fig. 3: Clonal sequencing results of microsatellite (CA) n site in 3'flanking region of BMP4 gene. a: clonal sequencing results from the BB genotype, showing $(CA)_{17}$; b: clonal sequencing results from the AA genotype, showing $(CA)_{24}$; c: clonal sequencing results from the CC genotype, showing $(CA)_{14}$

Table 3: Population genetic indexes in partial intron 2 of BMP4 gene in three goat breeds

Breeds	Gene homozygosity	Gene heterozygosity	Effective allele numbers	PIC
XH	0.7222	0.2778	1.3846	0.2392
BE	0.9901	0.0099	1.0100	0.0099
HM	0.5792	0.4208	1.7264	0.3323

Table 4: Genotypic and allelic frequencies in 3'flanking region of BMP4 gene in three goat breeds

	Genotype frequencies (numbers)						Allelic frequencies			
Breeds	AA	BB	CC	AB	AC	BC	A	B	C	χ^2 (HWE)*
XH	0.0991 (11)	0.6757 (75)	0.0270 (3)	0.0541 (6)	0.0450 (5)	0.0991 (11)	0.1486	0.7523	0.0991	54.2736
BE	0.1600 (32)	0.2200 (44)	0.0500 (10)	0.1350 (27)	0.1550 (31)	0.2800 (56)	0.3050	0.4275	0.2675	27.2382
HM	0.0971 (10)	0.7282 (75)	0.0097 (1)	0.0680 (7)	0.0290 (3)	0.0680 (7)	0.1456	0.7961	0.0583	45.7763

χ^2 (HWE)* = Hardy-Weinberg Equilibrium χ^2 value. Its p-values was above $\alpha = 0.05$

Table 5: Population genetic indexes in 3'flanking region of BMP4 gene in three goat breeds

Breeds	Gene homozygosity	Gene heterozygosity	Effective allele numbers	PIC
XH	0.5978	0.4022	1.6728	0.3656
BE	0.3473	0.6527	2.8790	0.5792
HM	0.6584	0.3416	1.5188	0.3103

CONCLUSION

Through SSCP analysis, no polymorphism was found on exon 2 of BMP4 but partial intron 2 amplified with P2 primer and partial 3'flanking region amplified with P3 primer showed polymorphisms. two new SNPs (EU104684:g.1986A>G, 2203G>A) were identified in the intron region and a short sequence with more than ten continuous and repeated CA dinucleotide were found in the 3'flanking region near the termination site of coding region and the length of repeated sequence dinucleotides CA was different in samples.

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