

POLYMORPHISM OF INHIBIN A GENE AND ITS RELATIONSHIP WITH LITTER SIZE IN CHINESE INDIGENOUS GOAT

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ABSTRACT

Inhibin A (INHA) is a glycoprotein hormone which has a role in regulating the synthesis and secretion of pituitary follicle-stimulating hormone. In this paper, single nucleotide polymorphisms of 5' regulatory region and exon 1 of *INHA* gene were detected in one prolific goat breed (Jining Grey goat) and five low to medium fecundity breeds (Boer, Liaoning Cashmere, Wendeng Dairy, Taihang and Inner Mongolia Cashmere goats) by PCR-SSCP and sequencing methods. Its effects on litter size in Jining Grey goats were analyzed. Concerning primer P2, two genotypes (AA and Aa) were detected in Jining Grey, Liaoning Cashmere, Wendeng Dairy and Taihang goats. Sequencing revealed a G→A transition at the 258th position of the amplified region in the genotype AA in comparison to the genotype Aa. For primer P4, two genotypes (BB and Bb) were identified in six goat breeds tested. Sequencing revealed a G→A transition at the 759th position of the amplified region in the genotype BB compared with Bb. Jining Grey goat does with genotype Bb had 0.79 (P<0.01) kids more than those with genotype BB. These results preliminarily showed that G841A of the *INHA* gene was a potential marker for improving litter size in goats.

Key words: goat; *INHA* gene; prolificacy; PCR-SSCP

INTRODUCTION

Inhibins are dimeric glycoproteins that have primarily been studied for their role in antagonism of activin-mediated release of follicle-stimulating hormone (FSH) from gonadotropes of the anterior pituitary (Cook *et al.*, 2004). Mason *et al.* had isolated two forms of inhibins, which had a common subunit α and a specific subunit β (β A or β B) (Mason *et al.*, 1985). An inhibin molecule is composed of an α -subunit and a β -subunit. And inhibin A (INHA) is the dimer of alpha and beta-A (de Kretser *et al.*, 2002). Physical mapping by fluorescence in situ hybridization (FISH) had placed *INHA* on caprine chromosome 2q41-42 (Goldammer *et al.*, 1995). There are three exons of *capra hircus* INHA gene. The transcript of goat INHA contains 1123 bp (NM_001285606), including a 1083 bp open reading frame, a 15-bp 5' UTR and a 25-bp 3' UTR.

It was reported that inhibin gene was a completely conserved gene, and some mutations might lead to the functional decrease or failure (He *et al.*, 2009), which means that a functional mutation in any of the inhibin gene would lead to a decrease in the amount of bioactive inhibin. This loss would result in an increase in the concentrations of FSH by removing the negative feedback to the pituitary, leading to premature depletion

of female follicles (Burger *et al.*, 1998; Hofmann *et al.*, 1998; Reame *et al.*, 1998). Marozzi *et al.* (2002) reported that mutations both in 5'-UTR and exon 2 of inhibin alpha gene might be associated with familial POF (premature ovarian failure) in human (Marozzi *et al.*, 2002). The Ala257Thr missense mutation in human *INHA* gene also had high statistical significance in POF, indicating that *INHA* gene was a strong candidate gene for ovarian failure (Dixit *et al.*, 2004). Additionally, many studies showed that single base substitutions in DNA sequence of *INHA* gene gave rise to fertility changes in sheep and goats. Study done by Hiendleder *et al.* showed that there was gene substitution effect of 0.08 lambs of Merinolandschafe, which indicated a QTL effect of INHA gene on litter size (Hiendleder *et al.*, 1996). Zhou *et al.* reported that the ewes with mutation homozygous or heterozygous genotypes had 1.32 or 0.77 lambs more than those with wild type in Small Tail Han Sheep, respectively (Zhou *et al.*, 2007). Also Tian *et al.* indicated that the A282G mutation in INHA promoter had significant effects on the average litter size of Small Tailed Han sheep (P < 0.05) (Tian *et al.*, 2010). In goats, it was reported that the INHA 651A/G polymorphism was a potential marker for the mean litter size of the second parity in Boer goats, which showed an overdominance effect (Wu *et al.*, 2009). Hou *et al.* identified that the polymorphism of 5' promoter region of

INHA gene could affect litter size significantly in three goat breeds (Hou *et al.*, 2012). He *et al.* reported the genotype distributions of INHA gene were significantly different between year-round estrous goat breeds and seasonal estrous goat breeds (He *et al.*, 2010). All of above results revealed the association of *INHA* to the prolificacy in goats.

The Jining Grey goat is an excellent local breed in China for its preeminent characteristics of sexual precocity, year-round estrus and high prolificacy (China, 2011). The mean litter sizes alive of Jining Grey, Liaoning Cashmere, Inner Mongolia Cashmere, Wendeng Dairy, Taihang and Boer goats have been reported to be 2.83, 1.15, 1.05, 1.94, 1.30, and 2.10 respectively (China, 2011; Malan, 2000). Based on its important role in reproduction, *INHA* gene was considered as a candidate for the prolificacy of Jining Grey goat. The objectives of the present study were firstly to detect polymorphisms of 5' flanking and exon 1 of *INHA* gene in one high prolific breed (Jining Grey goat) and five low to medium fecundity breeds (Boer, Liaoning Cashmere, Inner Mongolia Cashmere, Wendeng Dairy and Taihang goats). Secondly we were trying to investigate the association between polymorphism of *INHA* gene and prolificacy in Jining Grey goats.

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 goat doe) were collected from 6 Chinese indigenous breeds using acid citrate dextrose as an anticoagulant. The detailed information has been shown in Table 1. And 285 Jining Grey goat does kidded in 2009, along with data on litter size in the first, second, or third parity. These does were selected at random. Genomic DNA was extracted from whole blood by traditional 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 stored at -20°C.

The 285 Jining Grey goat does were the progeny of six goat bucks (n=44, 45, 47, 49, 50, 50). Because the six goat bucks 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. Four kidding seasons each consisted of 3-month groups starting with March through to May as season 1 (spring, n=74), June through to August as season 2 (summer, n=66), September through to November as season 3 (autumn, n=84) and December through to February as season 4 (winter, n=61).

Primers and PCR amplification: Four pairs of primers were selected from a previous study (He *et al.*, 2009) to amplify the 5' regulatory region and exon 1 of caprine *INHA* gene (GenBank No. EF602161.1). These primers were synthesized by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China). Primer sequence, amplified region and product size of caprine *INHA* gene are listed in Table 2.

Polymerase chain reactions were carried out in 25 μ L volume containing 1.0 μ L of 10 μ mol/L each primer, 2.5 μ L of 10 \times PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.0), 0.1% Triton X-100), 1.0-2.0 μ L of 25 mmol/L MgCl₂, 2.5 μ L of 2.5 mmol/L each dNTP, 3.0 μ L of 50 ng/ μ L caprine genomic DNA, 1.0 μ L of 2.5 U/ μ L *Taq* DNA polymerase (Promega, Madison, WI, USA), and the rest is ddH₂O. Amplification conditions were as follows: initial denaturation at 95°C for 5 min, followed by 33 cycles of denaturation at 94°C for 45 s, annealing at different temperature for 30 s by the primer (57.5°C for P1; 61°C for P2; 62°C for P3; 63°C for P4), extension at 72°C for 30 s; with a final extension at 72°C for 8 min on Mastercycler[®] 5333 (Eppendorf AG, Hamburg, Germany). The PCR products were separated by electrophoresis on 1.5% agarose gels (Promega) in parallel with a 600 bp DNA marker.

SSCP analysis: A volume of 2 μ L PCR product was transferred in an Eppendorf tube, mixed with 7 μ L 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 12% neutral polyacrylamide gels (acrylamide:bisacrylamide=29:1). Electrophoresis was performed in 1 \times Tris borate (pH 8.3)-EDTA buffer at 9-15 V/cm for 14-16 hours at 4°C. The gels were stained with silver nitrate to identify SSCP, then photographed and analyzed using an AlphaImagerTM 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

Cloning and sequencing: After SSCP analysis, PCR products of different genotypes were separated on 1.0% agarose gels and recovered using GeneClean II kit (Promega). Each DNA fragment was ligated into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions at 16°C overnight. The ligation reactions were carried out in a 10 μ L reaction mixture containing 1 μ L of PCR product, 1 μ L of pGEM-T Easy vector (50 ng/ μ L), 1 μ L of T₄ ligase (3 U/ μ L), and 5 μ L of 2 \times ligation buffer, 2 μ L ddH₂O. Each DNA fragment was then transformed into *Escherichia coli* DH5 α competence cell. Positive clones of transformed cells were identified by restriction enzyme digestion. Two clones of each 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 i^{th} sire ($i = 1, 2, 3, 4, 5, 6$); KS_j is the fixed effect of the j^{th} kidding season ($j = 1, 2, 3, 4$); P_k is the fixed effect of the k^{th} parity ($k = 1, 2, 3$); G_l is the fixed effect of the l^{th} genotype ($l = 1, 2$), 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 and SSCP analysis: *INHA* gene of six goat breeds was amplified using four pairs of primers. PCR products were separated by running a 1.5% agarose gel electrophoresis (Figure 1). The amplified products were consistent with the target fragments and had good specificities, which could be directly analyzed by SSCP.

The PCR products amplified by primers P1 and P3 had no polymorphism (data not shown), and the PCR products amplified by primers P2 and P4 displayed polymorphisms (Figure 2 and Figure 3).

Sequencing of different genotypes: In this study, sequence differences between the goat breeds were

produced by two mutations (G258A and G759A) (Figure 4 and Figure 5). The G841A mutation existed in the exon 1 of *INHA* gene, and did not cause amino acid change. Sequence alignment indicated that the homology of 884 bp nucleotides of 5'-flanking and exon 1 of caprine *INHA* gene of this study and bovine gene (BTU16237) was 94.3%, and the homology of exon 1 and deduced amino acid sequence was 97% and 94% respectively.

Allele and genotype frequencies of INHA gene in different goat breeds: As shown in Table 3, for primer P2, polymorphisms were found in Jining Grey, Liaoning Cashmere, Wendeng Dairy and Taihang goats. There were only two genotypes (Aa and predominant AA) existing in these four breeds. Concerning primer P4, genotypes BB and Bb were detected in all of goat breeds tested. The genotype distribution for locus P4 showed conspicuous differences between high fecundity breed (Jining Grey goat) and other five breeds with low to medium fecundity ($P < 0.01$, data not shown).

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 *INHA* gene ($P > 0.05$). For primer P4, the litter size in Jining Grey goats was significantly influenced by sire, kidding season and parity ($P < 0.05$), and was significantly influenced by *INHA* gene ($P < 0.01$). The least squares mean and standard error for litter size of different *INHA* genotypes in Jining Grey goats were given in Table 4. For primer P2, the differences of the litter size between AA and Aa genotypes were not significant in Jining Grey goats ($P > 0.05$). For primer P4, the Jining Grey goat does with genotype Bb had 0.79 ($P < 0.01$) kids more than those with genotype BB.

Table 1. Information list of samples used in this study

Breed	Sample size	Sampling location
Jining Grey goat	285	Jining Grey Goats Conservation Base, Jiaxiang County, Shandong Province, P. R. China
Wendeng Dairy goat	40	Wendeng City, Shandong Province, P. R. China
Inner Mongolia Cashmere goat	30	Inner Mongolia White Cashmere Goat Breeding Farm, Etuokeqi, Ordos City, the Inner Mongolia Autonomous Region, P. R. China
Liaoning Cashmere goat	40	Liaoning Cashmere Goat Breeding Center, Liaoyang City, Liaoning Province, P. R. China
Taihang goat	30	Wuzhi County, Henan Province, P. R. China
Boergoat	36	Qinshui Demonstration Farm, Qinshui County, Shanxi Province, P. R. China

Table 2. Primer sequence, amplified region and product size of caprine *INH A* gene.

Primer	Sequence (5'→3')	Amplified region	Location ¹⁾ (bp)
P1	F:GACACAGCTGGAGAACAAGAG R:AGGGAGACAGAGCAAGCCAG	5'regulatoryregion	34-221 (188bp)
P2	F:CTGGCTTGCTCTGTCTCCC R:CTGAGCCTTATCTCCCCTC		202-427 (226bp)
P3	F:GAGTGGGAGATAAGGCTCAG R:GAAGCCACATAGCTCCCCTG		428-656 (229bp)
P4	F:CAGGGGAGACTATGTGGCTTC R:CTGCAGCCGAAAAAGGATG		Exon1 (272bp)

¹⁾Location corresponding to GenBank U16237.

Table 3. Allele and genotype frequencies of *INH A* gene in six goat breeds

Breed	Number	Primer P2					Primer P4				
		Allele frequency		Genotype frequency			Allele frequency		Genotype frequency		
		<i>A</i>	<i>a</i>	<i>AA</i>	<i>Aa</i>	<i>aa</i>	<i>B</i>	<i>B</i>	<i>BB</i>	<i>Bb</i>	<i>bb</i>
Jining Greygoat	285	0.95	0.05	0.91 (258)	0.09 (27)	0	0.54	0.46	0.08 (24)	0.92 (261)	0 (0)
Boer goat	36	1.00	0.00	1.00 (36)	0.00 (0)	0 (0)	0.94	0.06	0.89 (32)	0.11 (4)	0 (0)
Liaoning Cashmere goat	40	0.92	0.08	0.85 (34)	0.15 (6)	0 (0)	0.88	0.12	0.75 (30)	0.25 (10)	0 (0)
Wendeng Dairy goat	40	0.96	0.04	0.93 (37)	0.07 (3)	0 (0)	0.98	0.02	0.95 (38)	0.05 (2)	0 (0)
Taihang goat	30	0.93	0.07	0.87 (26)	0.13 (4)	0 (0)	0.95	0.05	0.90 (27)	0.10 (3)	0 (0)
Inner Mongolia Cashmere goat	30	1.00	0.00	1.00 (30)	0.00 (0)	0 (0)	0.93	0.07	0.87 (26)	0.13 (4)	0 (0)

Note: the numbers in the brackets are the genotype individuals.

Table 4. Least squares mean and standard error for litter size of different *INHA* genotypes in Jining Grey goats

Primer	Genotype	Number	Littersize
P2	AA	258	2.76 ^a ±0.11
	Aa	27	2.51 ^a ±0.20
P4	BB	24	2.01 ^b ±0.19
	Bb	261	2.80 ^a ±0.09

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 ($P<0.01$).

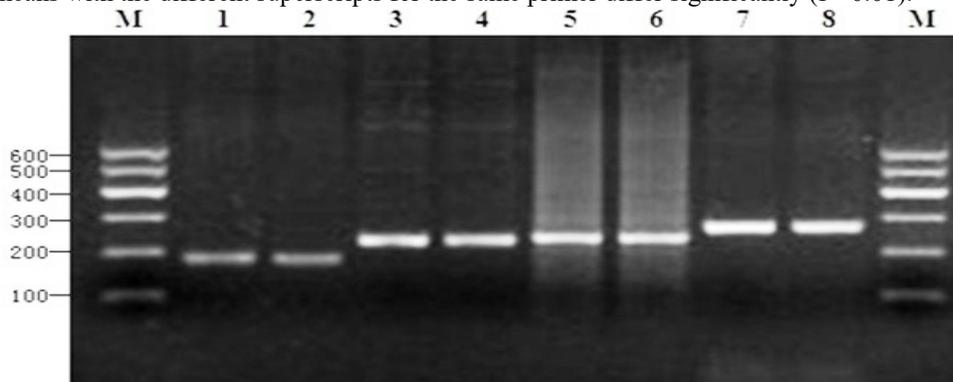


Figure 1. PCR products of four pairs of primers. 1-2: primer P1; 3-4:primer P2; 5-6:primer P3; 7-8:primerP4; M:600bp DNA ladder

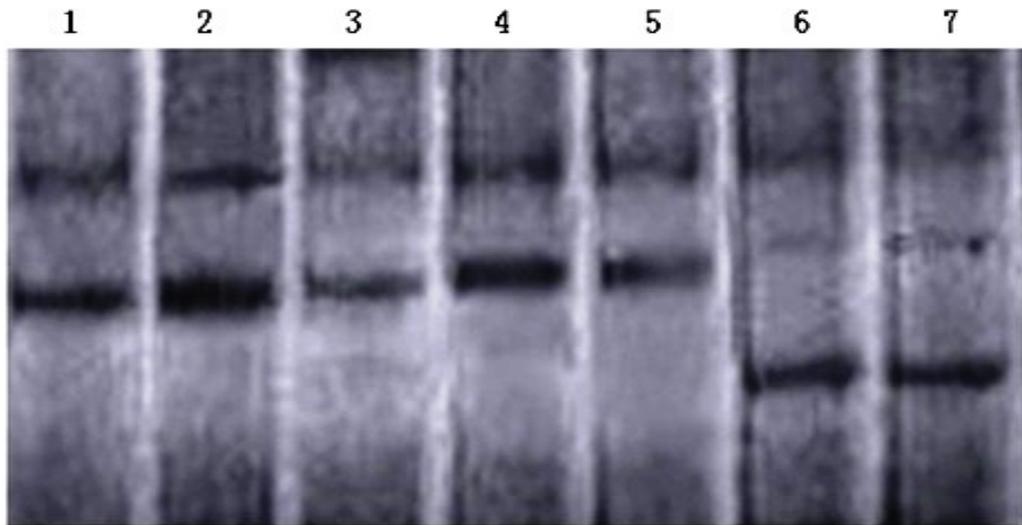


Figure 2. SSCP analysis on PCR products of primer P2 of *INHA* gene. 6, 7:Aa genotype; 1-5: AA genotype

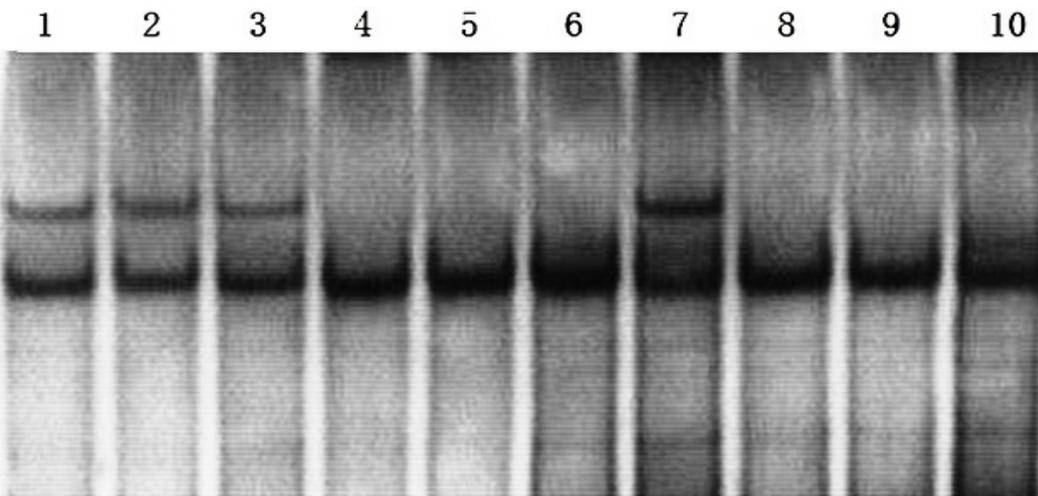


Figure 3. SSCP analysis on PCR products of primer P4 of *INHA* gene.1-3, 7: Bb genotype; 4-6, 8-10:BB genotype

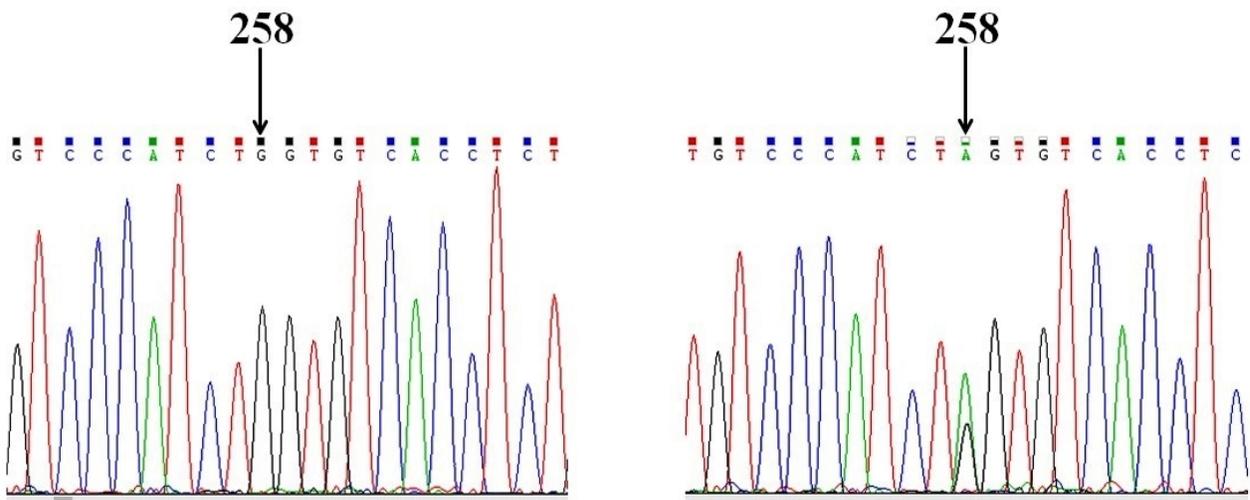


Figure 4. Sequence comparison of AA and Aa genotypes at 258 bp of *INHA* gene in goats

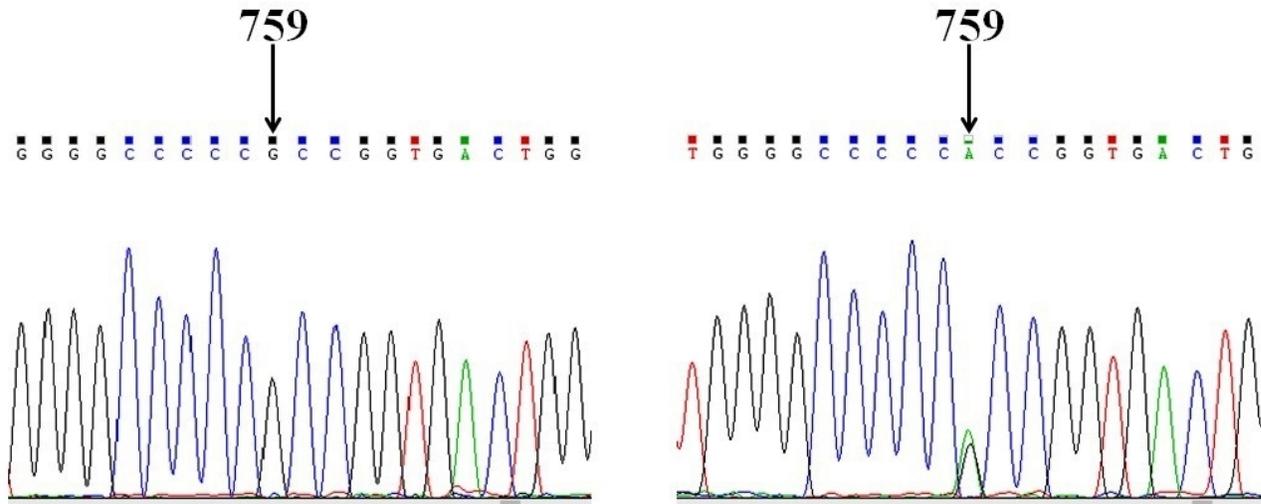


Figure 5. Sequence comparison of BB and Bb genotypes at 759 bp of *INHA* gene in goats

DISCUSSION

Polymorphisms of *INHA* gene: Shelling *et al.* determined a 769G→A transition in the *INHA* gene of human by using SSCP and DNA sequencing, which resulted in a non-conservative amino acid change, with a substitution from alanine to threonine (Shelling *et al.*, 2000). Three mutations (-252C>A, -124A>G, -16C>T) in the promoter and a TG repeat at -300th site of human *INHA* gene were detected (Harris *et al.*, 2005). Twelve nucleotide mutations were detected in Boer, Matou and Nubi goats in common, of which the 911T>C and 946A>C mutations resulted in corresponding change of amino acid residues (299V/A and 311T/P) (Wu *et al.*, 2009). Hua (2009) determined 11 of the 12 mutations found by Wu *et al.* (2009) in Boer and Haimen goats (Hua, 2009; Hua *et al.*, 2007). In the present study, three novel mutations were detected, in which two mutations (C228T and G250A) located in 5'-flanking region and one mutation (G841A) in exon 1 of caprine *INHA* gene.

Effect of *INHA* gene on reproductive performance: Abundant studies on human indicated that *INHA* gene was a strong candidate for POF (Dixit *et al.*, 2004; Harris *et al.*, 2005; Marozzi *et al.*, 2002). The G769A mutation in human *INHA* gene should be one of the important causes increasing the likelihood of POF, which led to Ala257Thr missense mutation in *INHA* with high statistical significance in POF (nine out of 80, 11.2%) (Fisher's exact test, $P=0.0005$) (Chand *et al.*, 2010; Dixit *et al.*, 2004). Study on Korean women demonstrated that the *INHA* -16C/T and -124A/G polymorphisms are possible genetic factors associated with the prevalence of POF although contrary to a case study (Chand *et al.*, 2010; Kim *et al.*, 2011). In sheep, the mutations of *INHA* gene were also related to the reproductive performance like litter size (Hiendleder *et al.*, 1996; Zhou *et al.*, 2007).

In Boer, Matou and Nubi goats, the 651A/G in exon 2 was suggested as a useful marker for litter size of the second parity, showing an overdominance effect (Wu *et al.*, 2009). Hua (2009) found that -446C>T, 651A>G and 946A>C were associated with litter size significantly in Nubi, Matou, Boer and Matou goats respectively (Hua, 2009). The 567G>A in exon 2 was genotyped by PCR-RFLP method and association studies indicated its effect on litter size in Boer and Haimen goats (Wu *et al.*, 2009). In exon 2, another missense mutation G-A (R-H) existed at 125th locus showing obvious effect on litter size in Dazu Black and Nanjiang Yellow goats (Zhao *et al.*, 2012). In addition, -506->G, -446C>T, -393G>T, P1-C80G and /126G insertion were reported to be molecular markers linked with litter size in goats, even with average milk production in the first four parities, but exon 1 was conserved with no polymorphisms (Hou *et al.*, 2012; Wang *et al.*, 2008; Wang *et al.*, 2010; Wu *et al.*, 2009). Goat *INHA* gene was shown significant effect on fecundity at the molecular genetic level by these studies.

In the present study, the mutations (G258A) in the 5'-flanking region of goat *INHA* gene might not be the genetic markers for reproductive trait since they had no significant effect on litter size in Jining Grey goat. Nucleotide mutations in 5'-flanking region did not necessarily affect *INHA* gene expression and function in goats (Zi *et al.*, 2008). However, G759A mutation in the exon 1 of goat *INHA* gene made an obvious difference in the allelic and genotypic frequency between the high fecundity goat breed and any one of the medium or low fecundity goat breeds. We had known that *INHA* is a highly conserved gene, and any mutation maybe lead to the functional decrease or failure. Although the G841A was a synonymous mutation, not resulting in change of amino acid residue, it had remarkable effect on litter size in Jining Grey goat. Individuals with Bb genotype showed good reproductive performance. The G841A

silent mutation may affect mRNA splicing and/or stability, even the kinetics of protein translation, thus giving rise to the functional change of *INHA* gene (Komar, 2007).

In conclusion, two mutations in goat *INHA* gene had been detected and the G759A mutation was associated with high litter size in Jining Grey goat. Genotype Bb can be regarded as a candidate marker of high prolificacy in goats. For lack of functional researches, further studies should be done so as to validate the effects of G759A mutation. This paper preliminarily provides reference for marker assisted selection for reproductive performance in goats.

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