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Polymorphism of 5′ regulatory region of caprine *FSHR* gene and its association with litter size in Jining Grey goat

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Abstract: Single nucleotide polymorphisms of the 5′ regulatory region of the *FSHR* gene were detected in 3 goat breeds (Jining Grey, Boer, and Inner Mongolia Cashmere) by the polymerase chain reaction–single-strand conformation polymorphism method, and its effects on litter size in Jining Grey goats were also evaluated. Concerning primer P1, 2 genotypes (AA and BB) were detected in Boer goats, while only the AA genotype was identified in Jining Grey and Inner Mongolia Cashmere goats. Sequencing revealed a T→A transversion at the 26th position and an A→C transversion at the 61st position of the amplified region in genotype BB in comparison to genotype AA. Regarding primer P2, 3 genotypes (CC, CD, and DD) were identified in the 3 goat breeds mentioned above. Sequencing revealed a T→A transversion at the 70th position and a G→C transversion at the 130th position of the amplified region in genotype DD compared with CC. Does with genotype CC had 0.46 ($P < 0.05$) or 1.03 ($P < 0.05$) kids more than those with genotype CD or DD, respectively, while does with CD had 0.57 ($P < 0.05$) kids more than those with DD in Jining Grey goats. These results preliminarily showed that allele C of the *FSHR* gene was a potential marker for improving litter size in goats.

Key words: Goat, litter size, *FSHR* gene, polymerase chain reaction–single-strand conformation polymorphism

1. Introduction

Follicle-stimulating hormone (FSH), a pituitary gonadotropin, plays an essential role in mammalian reproduction, which activates a specific receptor located exclusively on Sertoli cells in the testis and on granulosa cells in the ovary (1,2). The FSH receptor (FSHR) is a member of the rhodopsin receptor family of G protein-coupled receptors comprising extended NH_2-terminal extracellular domains with numerous leucine-rich repeats that assist ligand specificity (3,4). The FSH-FSHR system relays neuronal signals from the hypothalamus to the gonads and induces feedback signals to the hypothalamus and pituitary, which keeps the endocrine balance in the reproductive axis and maintains follicle growth, development, differentiation, and maturation as well as spermatogenesis (5–8). The human *FSHR* gene is located in 2p21-p16 and consists of 10 exons. Several studies have demonstrated that variants of the *FSHR* gene were related with human reproductive abnormalities such as polycystic ovary syndrome (9), primary and secondary amenorrhea (10,11), and male infertility (12). However, the polymorphisms of the caprine *FSHR* gene and their associations with reproductive traits are almost completely unknown.

Chinese local goat breeds with different litter sizes provided the materials for us to analyze the association of the *FSHR* gene with fecundity. The Jining Grey goat is an indigenous caprine breed with the highest fecundity (average live litter size = 2.94) in Chinese goats (13). The litter size of Boer and Inner Mongolia Cashmere goats was 2.10 (14) and 1.04 (13), respectively.

One objective of the present study was to detect mutations in the 5′ regulatory region of the *FSHR* gene in a high-fecundity breed (Jining Grey goat), a middle-fecundity breed (Boer goat), and a low-fecundity breed (Inner Mongolia Cashmere goat) using the polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) method. The other objective was to investigate the association between the *FSHR* gene and fecundity in Jining Grey goats.

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2. Materials and methods

2.1. Animals and reagents

All experimental procedures were performed according to authorization granted by the Chinese Ministry of Agriculture.

The 10-mL venous jugular blood samples were collected from each of the 208 Jining Grey goat does that kidded in 2009 (first, second, or third parity; Jining Grey Goats Conservation Base, Jiaxiang County, Shandong Province, China), 41 Inner Mongolia Cashmere goat does (Inner Mongolia White Cashmere Goat Breeding Farm, Etuokeqi, Ordos City, Inner Mongolia Autonomous Region, China), and 40 Boer goat does (Qinshui Demonstration Farm, Qinshui County, Shanxi Province, China) using acid citrate dextrose as an anticoagulant. Genomic DNA was extracted from whole blood by the phenol-chloroform method (15) 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 208 Jining Grey goat does were selected at random and represented the progeny of 5 goat bucks (n = 38, 40, 41, 43, and 46). Because the 5 bucks had been sold, their blood was not collected for genotyping. Selection was not being conducted on litter size or other fertility traits in this flock over previous years. Kidding was partitioned into 4 seasons: March through May (season 1, spring, n = 54), June through August (season 2, summer, n = 49), September through November (season 3, autumn, n = 59), and December through February (season 4, winter, n = 46).

2.2. Primers and PCR amplification

Four pairs of primers were selected from a previous study carried out by Song et al. (16) (Table 1) to amplify the 5′ regulatory region of the caprine *FSHR* gene. The primers were synthesized by Shanghai Invitrogen Biotechnology Ltd. Co. (Shanghai, China). PCR was carried out in 25 μL of reaction mixture (Table 2). PCR conditions for all 4 pairs of primers were as follows: initial denaturation at 95 °C for 7 min, followed by 31 cycles of denaturation at 94 °C for 30 s, annealing for 30 s (annealing temperatures are shown in Table 1), and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. The amplification products were then identified on 1.5% agarose gels and kept at 4 °C.

Table 1. Primer sequence, amplified region, product size, and annealing temperature used in analyses of the goat *FSHR* gene.

Primer	Primer sequence (5′→3′)	Amplified region	Product size (bp)	Annealing temperature (°C)
P1	F: AAAGGCAAGGGCAATCTTC R: ACAGGCAGTGGGTTAGTGA	5′ regulatory region	205	52.7
P2	F: CTGCTGAGCTACACCATATTT R: TGTCCCTGTGGGTCACTTT		251	57.4
P3	F: CAGGGACAGTCTTACAGCGAATT R: TTGGGAGCTGGTAAGGGTCACG		259	56.3
P4	F: TACCAGCTCCCAACGCAGAC R: GGAAGGTCGGAGGGCATCT		247	57

Table 2. Amplification system of 4 primers for the caprine *FSHR* gene.

Component	Volume (μL)	Final concentration
10X PCR buffer	0.1	1X
dNTP mixture	2.0	0.1 mM
Each primer	1.0	0.2 μM
Taq polymerase	0.02	0.04 U/μL
Genomic DNA	3.0	6 ng/μL
Mg^{2+}	1.3	1.3 mM
ddH_2O	16.58	–

2.3. SSCP detection

A mixture consisting of 2.0 μL of PCR product and 7.0 μL of gel loading solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 20 mmol/L EDTA (pH 8.0), and 10% glycerol was used. The mixture was denatured at 98 °C for 10 min, then cooled on ice for 7 min and loaded on 10%–12% (12% for primers P1, P2, and P3; 10% for primer P4) neutral polyacrylamide gels (acrylamide:bisacrylamide = 39:1 for primers P1, P2, and P3; 29:1 for primer P4). Electrophoresis was performed at 165 V at 4 °C overnight. After electrophoresis, the DNA fragments in the gels were visualized by silver staining, photographed, and analyzed with the AlphaImager 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

2.4. Cloning and sequencing

PCR products of different genotypes after SSCP analysis were separated on 1.0% agarose gels and recovered using Geneclean II (Promega, Madison, WI, USA). Each DNA fragment was ligated into the pGEM-T Easy Vector (Promega) according to the manufacturer's instructions at 16 °C overnight. The recombinant plasmid was then transformed into *Escherichia coli* DH5α competent cells. Positive clones of the transformed cells were identified by restriction enzyme digestion. Three clones of each genotype were sequenced in both directions using an ABI3730 automatic sequencer (PerkinElmer Applied Biosystems, Foster City, CA, USA) by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China).

2.5. Statistical analysis

The litter size of Jining Grey goat does was analyzed using the following fixed effects model. The least squares means were used for multiple comparisons in litter size among the different genotypes.

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

where y_{ijklm} is the phenotypic value of litter size; m 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 8.1 (SAS Institute Inc., Cary, NC, USA). Mean separation procedures were conducted using a least significant difference test.

3. Results

3.1. PCR amplification

PCR products of the caprine *FSHR* gene amplified by 4 pairs of primers were consistent with the target fragments and had a good specificity, which could be directly analyzed by SSCP.

3.2. SSCP analysis

Only the products amplified by primers P1 and P2 displayed polymorphisms. Two genotypes (AA and BB; Figure 1) for primer P1 and 3 genotypes (CC, CD, and DD; Figure 2) for primer P2 were identified by PCR-SSCP.

3.3. Sequencing of different genotypes

Four different homozygous genotypes were cloned and sequenced. As for primer P1, 2 mutations (T26A and A61C) at the 26th and 61st positions of the amplified region were analyzed using program MEGA 5 (17) while comparing genotype BB with AA (Figure 3). Regarding primer P2, sequencing results revealed 2 mutations (T70A and G130C) at the 70th and 130th positions of the amplified fragment in genotype DD compared with CC (Figure 4).

3.4. Allele and genotype frequencies of the caprine *FSHR* gene

As shown in Table 3, for primer P1, polymorphisms were found only in the Boer goats and genotype BB was just detected in one doe, but all individuals of the Jining Grey and Inner Mongolia Cashmere goats belonged to the AA genotype. Concerning primer P2, 3 genotypes were detected in the 3 goat breeds. The genotype distribution showed conspicuous differences between the high-fecundity breed (Jining Grey goats) and the middle- and

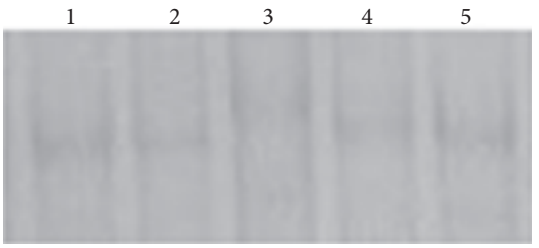


Figure 1. SSCP analysis of PCR products by primer P1 in Boer goats. Lanes 1, 2, 4, and 5: AA genotype; lane 3: BB genotype.

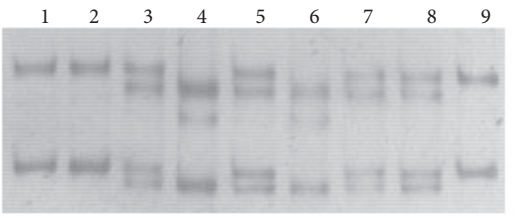
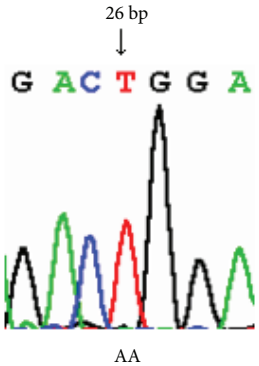
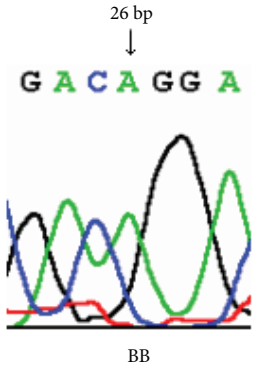
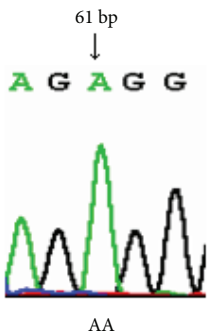


Figure 2. SSCP analysis of PCR products by primer P2 in different goat breeds. Lanes 1, 2, and 9: CC genotype; lanes 4 and 6: DD genotype; lanes 3, 5, 7, and 8: CD genotype.







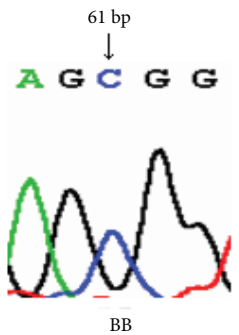
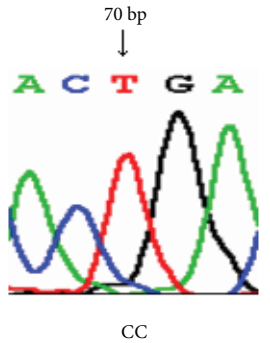
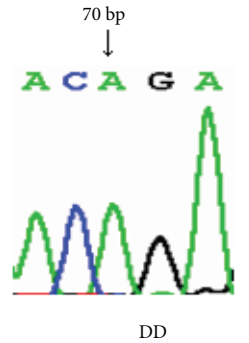
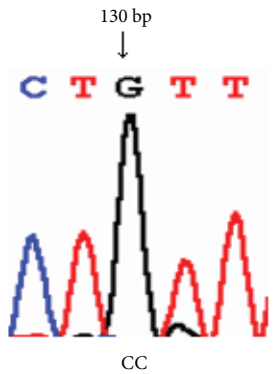


Figure 3. Sequencing analysis between genotypes AA and BB.







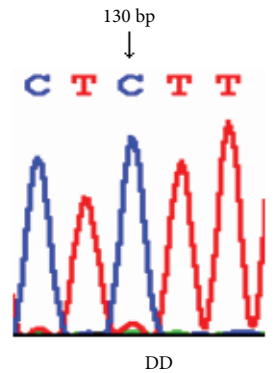


Figure 4. Sequencing analysis between genotypes CC and DD.

Table 3. Allele frequency and genotype frequency of the *FSHR* gene in 3 goat breeds.

	Breed		Jining Grey goat	Inner Mongolia Cashmere goat	Boer goat
	Number		208	41	40
P1	Genotype frequency	AA	1.000 (208)	1.000 (41)	0.975 (39)
		BB	0.000 (0)	0.000 (0)	0.025 (1)
	Allele frequency	A	1.000	1.000	0.975
		B	0.000	0.000	0.025
	Number		208	41	40
P2	Genotype frequency	CC	0.462 (96)	0.195 (8)	0.225 (9)
		CD	0.317 (66)	0.390 (16)	0.250 (10)
		DD	0.221 (46)	0.415 (17)	0.525 (21)
	Allele frequency	C	0.620	0.390	0.350
		D	0.380	0.610	0.650

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

low-fecundity breeds (Boer and Inner Mongolia Cashmere goats) (P < 0.01, data not shown).

3.5. Influence of fixed effects on litter size in Jining Grey goats

The litter size of Jining Grey goats was significantly influenced by the sire, kidding season, parity, and genotype of the *FSHR* gene (all P < 0.05). The least squares mean and standard error for litter size of different *FSHR* genotypes in Jining Grey goats are given in Table 4. Does with genotype CC had 0.46 (P < 0.05) or 1.03 (P < 0.05) kids more than those with genotype CD or DD, respectively, while does with CD had 0.57 (P < 0.05) kids more than those with DD in Jining Grey goats.

4. Discussion

4.1. Polymorphisms of *FSHR* gene

Marson et al. (18) identified an *Alu*I-RFLP polymorphism in exon 10 of the *FSHR* gene of a *Bos taurus* × *Bos indicus* beef composite population consisting of genotypes GG (193/63/50 bp), GC (243/193/63/50 bp), and CC (243/63 bp), respectively. Two common mutations within exon 10, which resulted in a Thr307Ala change in the hinge region and Ser680Asn change in the intracellular domain of the FSHR protein, were identified in both sheep and humans (19–22). In general, Thr(307) is almost in linkage disequilibrium with Asn(680), and Ala(307) almost with Ser(680).

In Yunling Black and Boer goats, 4 mutations were identified in the coding region of the *FSHR* gene, which were A486C (Arg162Ser), C1042G (Pro348Ala), T1930A (Phe644Ile), and T2036C (Thr679Ile), respectively (23). In our previous study on the 5′ flanking region of the sheep *FSHR* gene, 2 novel SNPs (g. -681T > C and g. -629C > T) were detected in Hu sheep and 3 novel SNPs (g. -200G > A, g. -197G > A, and g.-98T > C) were found in Small Tail Han sheep (24). Moreover, several polymorphic sites in the 5′ regulatory region of the caprine *FSHR* gene were identified. The -739G > T mutation was detected in the *FSHR* gene of Boer goats by PCR-SSCP (16), while -93C > A, -80G > C, -63 C > A, -56C > G, and -55T > C were found in the *FSHR* gene of Xiangdong Black, Nanjiang Brown, and Guizhou Black goats by PCR-SSCP (25). In our study, in the 5′ regulatory region of the *FSHR* gene, 2 novel mutations (T26A and A61C) were identified in Boer goats and another 2 novel mutations (T70A and G130C) were identified in Jining Grey, Inner Mongolia Cashmere, and Boer goats.

4.2. Effect of *FSHR* gene on reproductive performance

Marson et al. (18) used the probability of pregnancy at first breeding to evaluate the effect of its polymorphism on sexual precocity in a *Bos taurus* × *Bos indicus* beef composite population and found that heterozygous heifers with genotype GC showed a higher pregnancy rate (66%), but no significant effects were observed (P = 0.8831). Cui et al. (23) reported that the FSHR mRNA and protein expression levels were positively correlated with fecundity in Yunling Black goats. Reduced FSHR levels may be associated with the fewer observed oocytes and, consequently, fewer follicles. In terms of Ser680Asn mutation in the human *FSHR* gene, women homozygous for the Ser(680) variant had higher follicular FSH levels and longer follicular phase lengths, which indicated a lower sensitivity to FSH, and higher recombinant FSH doses were needed for Ser/Ser homozygous women undergoing in vitro fertilization treatment (20,21). In addition, the haplotype of the homozygous Ala307-Ser680 variant seems to be associated with significantly higher basal serum FSH levels and a higher amount of FSH required for ovarian stimulation in women undergoing assisted reproduction (19). All these points suggest that the *FSHR* genotypes could influence the ovarian response to FSH stimulation.

Concerning polymorphisms of the caprine *FSHR* gene, 5 mutations mentioned above had significant effect on the litter size in Guizhou Black goats (P < 0.05), and the litter size of does with genotype BB was significantly higher than that of does with genotypes AA and AB. However, these mutations of *FSHR* had no significant effect on the litter size in either Xiangdong Black or Nanjiang Brown goat does (P > 0.05) (25). In our results, allele C in the 5′ regulatory region of the caprine *FSHR* gene was associated with high fecundity in Jining Grey goats.

Table 4. Least squares means and standard errors for litter size of different genotypes of *FSHR* gene in Jining Grey goats.

Primer	Genotype	Number of samples	Litter size
P2	CC	96	2.98 ± 0.14a
	CD	66	2.52 ± 0.18b
	DD	46	1.95 ± 0.21c

Least squares means followed by different letters significantly differ at P < 0.05.

In conclusion, 4 novel mutations in the 5′ regulatory region of the caprine *FSHR* gene were detected. The genotype distribution for T70A and G130C loci was evidently different among high-, middle-, and low-fecundity goat breeds and allele C of the *FSHR* gene was associated with high fecundity in Jining Grey goats. The present study indicated that the caprine *FSHR* gene may influence the reproductive performance, but our results are preliminary. Further association studies with larger numbers of samples and function research on these polymorphisms will be required.

Acknowledgments

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