J. Anim. Breed. Genet. ISSN 0931-2668

ORIGINAL ARTICLE

DNA polymorphism of 5′ flanking region of prolactin gene and its association with litter size in sheep

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Keywords
DNA polymorphism; litter size; prolactin gene; sheep.

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Received: 01 May 2007;
accepted: 05 June 2008

Summary

A single nucleotide polymorphism of 5′ flanking region of the prolactin gene was investigated in both high prolificacy breeds (Small Tail Han and Hu sheep) and low prolificacy breeds (Dorset and Suffolk sheep) using polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP). The results indicated that two genotypes (AA and AB) were detected in Small Tail Han sheep (n = 239), only one genotype (AA) was detected in Hu (n = 40), Dorset (n = 50) and Suffolk sheep (n = 39). The mutant homozygous genotype (BB) was not detected in four sheep breeds. In Small Tail Han sheep (n = 239), the frequency of genotypes AA and AB was 0.91 and 0.09, the frequency of the A and B alleles was 0.95 and 0.05, respectively. The fitness tests showed that the Small Tail Han sheep population was in Hardy–Weinberg equilibrium. Sequencing revealed a mutation (G → T) at the position 63 bp of the 5′ flanking region of prolactin gene in AB genotype compared with AA genotype in Small Tail Han sheep. The Small Tail Han ewes with AB genotype had 0.83 ($p < 0.05$) lambs more than those with AA genotype. These results preliminarily showed that the prolactin locus is either a major gene that influences the high prolificacy in Small Tail Han sheep or is in close linkage with such a gene.

Introduction

Prolactin is an anterior pituitary polypeptide hormone involved in many reproductive pathways and is essential for reproductive performance (Krasnow *et al.* 1990; Gellersen *et al.* 1994; Wu *et al.* 1995; Jabbour & Critchley 2001; Kruger *et al.* 2003). This action is mediated by its receptor. Prolactin receptor gene was mapped to ovine chromosome 16 (Hayes *et al.* 1996; Jenkins *et al.* 2000). Mutations in prolactin (*PRL*) gene were associated with broodiness in chickens (Jiang *et al.* 2005; Cui *et al.* 2006; Liang *et al.* 2006). The prolactin receptor (*PRLR*) locus is either a major gene that influences the high prolificacy in Small Tail Han sheep or is in close linkage with such a gene (Chu *et al.* 2007). However, the association between *PRL* gene and litter size in sheep has not been reported so far.

The Small Tail Han sheep breed that has significant characteristics of high prolificacy and year-round oestrus is an excellent local breed in China. Mean litter sizes of Small Tail Han, Hu, Dorset and Suffolk sheep have been reported to be 2.61 (Tu 1989), 2.29 (Tu 1989), 1.40 (Mohd-Yusuff *et al.*

  doi:10.1111/j.1439-0388.2008.00763.x

1992) and 1.41 (Abdulkhaliq *et al.* 1989) respectively. Based on the important role of *PRL* in reproduction, *PRL* gene was considered as a possible candidate gene for high prolificacy of sheep. The objectives of the present study were firstly to detect single nucleotide polymorphisms (SNPs) in 5′ flanking region of *PRL* gene in both two high prolificacy breeds (Small Tail Han and Hu sheep) and two low prolificacy breeds (Dorset and Suffolk sheep) by polymerase chain reaction (PCR)-single strand conformation polymorphism (PCR-SSCP), and secondly to investigate the association between *PRL* gene and prolificacy in breeds in which the polymorphism is segregating.

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 ewe) were collected from 239 Small Tail Han ewes lambed in 2005, along with data on litter size in the first, second, or third parity (Jiaxiang Sheep Breeding Farm, Shandong Province, China), 50 Dorset and 39 Suffolk ewes [HITEK Ranch (Beijing) Ltd., Co., Beijing, China] and 40 Hu ewes (Yuhang Hu Sheep Breeding Farm, Hangzhou, China) using acid citrate dextrose as an anticoagulant. Genomic DNA was extracted from whole blood by phenol–chloroform method as described by Sambrook & Russell (2001), 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 239 Small Tail Han ewes were selected at random and were the progeny of eight rams. Because the eight rams 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. Lambing seasons consisted of 3-month groups starting with March through to May as season 1 (spring), June through to August as season 2 (summer), September through to November as season 3 (autumn) and December through to February as season 4 (winter).

Primers and PCR amplification

One pair of primer provided by Hart *et al.* (1993) was used to amplify the 5′ flanking region of the ovine prolactin gene. The expected amplification fragment size was 161 bp. The primer sequences used were as follows:

- Forward: 5′-AGGTCAGAGAATTAAAGCT-3′.
- Reverse: 5′-GGAAGTGACAGTGGTTTT-3′.

Polymerase chain reaction was carried out in 25 μl volume containing approximately 2.5 μl of 10× PCR buffer (50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.0), 0.1% Triton X-100), 1.5 mmol/l of $MgCl_2$, 200 μmol/l of each dNTP, 1 μmol/l of each primer, 50 ng of genomic DNA and 2 U of *Taq* DNA polymerase. Amplification conditions were as follows: initial denaturation at 95°C for 4 min followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, extension at 72°C for 30 s with a final extension at 72°C for 7 min on Mastercycler® 5333 (Eppendorf AG, Hamburg, Germany).

SSCP detection

A volume of 2 μl PCR product was transferred into an Eppendorf tube, mixed with 5 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 centrifuged and denatured at 98°C for 10 min, then chilled on ice for 5 min and loaded on 15% neutral polyacrylamide gels (acrylamide:bisacrylamide = 37.5:1). Electrophoresis was performed in 1× Tris borate (pH 8.3)–EDTA buffer at 9–15 V/cm at 4°C overnight. After electrophoresis, the DNA fragments in the gels were visualized by silver staining, photographed and analysed by an AlphaImager™ 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% agarose gels and recovered using Geneclean II kit (Promega, Madison, WI, USA). The ligation reaction was conducted as per the instructions of the manufacturer (Promega). Each DNA fragment was then transformed into *Escherichia coli* DH5α 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 twice. 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 Ltd. Co., (Shanghai, China).

Statistical analysis

The following fixed effects model was employed for analysis of litter size in Small Tail Han ewes and least squares mean was used for multiple comparison in litter size among different genotypes.

$$y_{ijklm} = \mu + S_i + \mathrm{LS}_j + P_k + G_l + e_{ijklm},$$

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, 7, 8), LS_j is the fixed effect of the j^{th} lambing 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

Genomic DNA of four sheep breeds was amplified using one pair of primers for *PRL* gene. PCR products were detected by running a 2% agarose gel electrophoresis. The amplified product was consistent with the target fragment and had good specificity, which could be directly analysed by SSCP.

SSCP analysis

Two genotypes (AA and AB) were detected in prolific Small Tail Han sheep (Figure 1). Only one genotype (AA) was detected in other three sheep breeds. The mutant homozygous genotype (BB) was not detected in four sheep breeds.

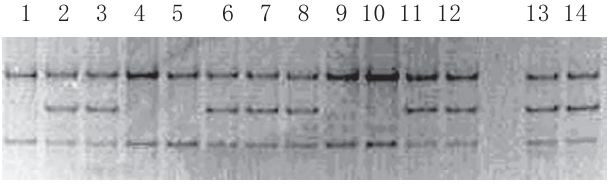

Figure 1 SSCP analysis of PCR amplification of prolactin gene in Small Tail Han sheep. Lanes 2, 3, 6–8, 11–14, AB genotype; lanes 1, 4, 5, 9, 10, AA genotype.

Sequencing of different genotypes

So far, owing to lack of the DNA sequence of the ovine prolactin gene, the ovine sequence obtained in the present study was compared with the bovine DNA for prolactin 5′ flanking regulatory region (GenBank accession number X16641, Wolf *et al.* 1990). Sequencing revealed that AB genotype had one point mutation (a G → T change at the position 63 bp in the amplification fragment) compared with AA genotype in Small Tail Han sheep.

Allele and genotype frequencies of the prolactin gene in four sheep breeds

In Small Tail Han (n = 239), Hu (n = 40), Dorset (n = 50) and Suffolk sheep (n = 39), the frequency of genotype AA was 0.91, 1, 1 and 1, respectively, and the frequency of genotype AB was 0.09, 0, 0 and 0 respectively. The frequency of the A and B alleles was 0.95 and 0.05 in prolific Small Tail Han sheep and 1 and 0 in other three sheep breeds respectively. The tests (χ^2 = 0.56, p = 0.757) showed that the Small Tail Han sheep population was in Hardy–Weinberg equilibrium, consistent with the Small Tail Han sheep 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.

Allele and genotype frequencies of the prolactin gene within sire families in Small Tail Han sheep

The 239 Small Tail Han ewes were selected at random and were the progeny of eight rams. Allele and genotype frequencies of prolactin gene within sire family in Small Tail Han sheep were listed in Table 1. Based on genotypic distribution and statisti-

Table 1 Allele and genotype frequencies of prolactin gene within sire family in Small Tail Han sheep

	Allele frequency		Genotype frequency	
Sire family	A	B	AA	AB
No.1 ram	0.95	0.05	0.90(27)	0.10(3)
No.2 ram	0.95	0.05	0.90(27)	0.10(3)
No.3 ram	0.95	0.05	0.90(27)	0.10(3)
No.4 ram	0.95	0.05	0.90(27)	0.10(3)
No.5 ram	0.95	0.05	0.90(27)	0.10(3)
No.6 ram	0.95	0.05	0.90(27)	0.10(3)
No.7 ram	0.97	0.03	0.93(27)	0.07(2)
No.8 ram	0.97	0.03	0.93(28)	0.07(2)

The numbers in parentheses are the individuals genotyped.

Table 2 Analysis of variance for litter size in Small Tail Han sheep

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*-value
Sire	12.551	7	1.793	2.750**
Lambing season	5.208	3	1.736	2.663*
Parity	3.990	2	1.995	3.060*
Genotype	2.549	1	2.549	3.909*
Residual	146.709	225	0.652	

*$p < 0.05$, **$p < 0.01$.

Table 3 Least squares mean and standard error for litter size of different prolactin genotypes in Small Tail Han sheep

Genotype	Sample number	Litter size
AA	217	$1.97^{b} \pm 0.13$
AB	22	$2.80^{a} \pm 0.18$

[a,b]Least squares means with the different superscripts differ significantly ($p < 0.05$).

cal analysis, the eight rams should be AA genotype at the prolactin locus tested at the probability level of more than 95%. Therefore, the eight rams are unlikely to carry the allele B.

Influence of fixed effects on litter size in Small Tail Han sheep

The results of variance analysis for litter size in Small Tail Han sheep are summarized in Table 2. Litter size was significantly influenced by sire, lambing season, parity, and *PRL* genotype ($p < 0.01$, $p < 0.05$, $p < 0.05$ and $p < 0.05$ respectively).

The least squares mean and standard error for litter size of different *PRL* genotypes in Small Tail Han sheep are given in Table 3. The heterozygous AB ewes had 0.83 ($p < 0.05$) lambs more than the homozygous AA ewes.

Discussion

Polymorphisms of prolactin gene

Two alleles of the 857-bp segment of the bovine prolactin gene consisting of 235-bp 5′ flanking sequence including the promoter, exon I (83 bp) and 539-bp intron A were detected by PCR-SSCP (Zhang *et al.* 1994). Similar mutations have been reported in several species. A *Hae* III PCR-RFLP polymorphism was identified in the second intron of the ovine *PRL* gene (Vincent & Rothschild 1997). The SNPs were identified in the human *PRL* gene (Stevens *et al.* 2001; Mellai *et al.* 2003). Nucleotide sequence polymorphism was identified within exon 4 of the bovine prolactin gene (Brym *et al.* 2005). A 24-bp indel (insertion or deletion) in the proximal part of the chicken *PRL* promoter was detected (Cui *et al.* 2005, 2006; Jiang *et al.* 2005; Liang *et al.* 2006). Six SNPs (C-2402T, C-2161G, T-2101G, C-2062G, T-2054A, G-2040A) in the promoter region of the *PRL* gene were identified in 177 individuals from White Leghorn, Yangshan, Taihe Silkies, White Rock and Nongdahe chicken breeds (Cui *et al.* 2006). The frequencies of C-2402, C-2161, C-2062, G-2040 and insertion allele of 24-bp indel were 1.0 in the White Leghorn chickens (the White Leghorn is an excellent layer breed without broodiness and produces more than 300 eggs per year), whereas those frequencies were close to zero in the Yangshan chickens (the Yangshan is a Chinese native breed with strong broodiness and produces less than 70 eggs per year) (Cui *et al.* 2006). Four SNPs (C-2425T, T-2215C, G-2063A, A-1967G) and a polyA length polymorphism in 5′ flanking region of the *PRL* gene were detected in several populations of Chinese native Yuehuang, Taihe Silkies and imported White Leghorn layer chickens (Liang *et al.* 2006).

In the present study, PCR-SSCP polymorphism was identified in the *PRL* gene in Small Tail Han sheep. To our knowledge, it is the first defined polymorphism in 5′ flanking region of the ovine *PRL* gene. Because the eight rams do not carry the allele B, no homozygous BB ewes were detected in Small Tail Han sheep. Whether the allele B is not segregating in any of the rams is an interesting question. Extensive sampling of more rams and DNA analysis would be required to verify this hypothesis. Such a study would have important implications for the sheep industry.

Effect of prolactin gene on reproductive performance

Prolactin is generally accepted as crucial to the onset and maintenance of broodiness in avian species. The chickens with ID genotype (I = insertion allele, D = deletion allele) of the 24-bp indel of the *PRL* promoter region had a significantly higher incidence of broodiness ($p < 0.01$) than those with II or DD genotype (Jiang *et al.* 2005; Cui *et al.* 2006; Liang *et al.* 2006). The chickens with ID genotype, which are of the highest incidence of broodiness, had the highest PRL mRNA level (Liang *et al.* 2006). These results preliminarily showed that the 24-bp indel locus of the *PRL* gene is associated with broodiness in chickens.

The present study preliminarily showed that the prolactin locus is either a major gene that influences the high prolificacy in Small Tail Han sheep or is in close linkage with such a gene. The joint effects of both *PRLR* and *PRL* genes in prolific Small Tail Han sheep deserve further study. This is a very interesting biological question. This *PRL* gene test has the potential to be a powerful tool when used in conjunction with traditional selection methods for some sheep. Before using these results for marker-assisted selection, additional data are needed to confirm the significant effects seen in the Small Tail Han ewes.

Acknowledgements

This work was supported by National Science and Technology Basic Conditions Platform Construction Program of China (No. 2004DKA30450), by National Key Basic Research and Development Program of China (No.2006CB102105) and by special fund for basic scientific research of Institute of Animal Science, Chinese Academy of Agricultural Sciences (ywf-rc-1 and ywf-td-1).

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