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Single-nucleotide Polymorphism Scanning of Bone Morphogenetic Protein Receptor Gene and its Correlation with the Prolificacy of Plateau Tibetan Sheep

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ABSTRACT

Background: It is not clear whether there are other motifs on the BMPIR gene in plateau-type Tibetan sheep and whether there is a strong association between the mutant locus and reproductive performance.

Methods: Single-nucleotide polymorphisms (SNPs) of the *BMPRIB* gene in the Tibetan sheep populations from the Qinghai pastoral area in China were detected by Sanger sequencing and performed correlation analysis with litter size.

Result: Four SNPs (g.597 G>A, g.746 A>G, g.864 C>T and g.1113 C>A) of *BMPRIB* gene were firstly screened in Tibetan sheep. In loci g.746, relevance analysis discovered that genotypes GG and AG had 0.70 (P<0.05) and 0.79 (P<0.05) litter sizes more than the genotype AA in Tibetan sheep, respectively. In loci g.597, Tibetan sheep with genotypes AA and GA had 0.21 (P<0.05) and 0.31 (P<0.05) litter sizes more than the genotype GG, respectively. The results showed that g.746 A>G and g. 597 G>A were regarded as assisted-markers in molecular. These findings provide ideas and insights into the reproductive performance of Tibetan sheep in China.

Key words: BMPRIB, FecB, Litter size, Tibetan sheep.

INTRODUCTION

Litter size was considered as important reproductive traits of sheep. It is difficult to increase litter size using conventional breeding methods in production practice (Tian *et al*., 2009). Molecular breeding has commonly been used to improve prolificacy at home and abroad (Wang *et al*., 2013). It is generally accepted that lambing number and ovulation rate are essential for sheep reproduction. Extensive work has been carried out on genes affecting lambing numbers in different sheep breeds, culminating in the identification of major effector genes (Yang *et al*., 2020). The results indicated that the presence of the FecB gene was identified in a highly bred breed represented by the Hu and Small-tail han sheep (Wang *et al*., 2018). There is growing evidence that the FecB has been successfully introduced into sheep in many countries (Bhalavi *et al*., 2021). The earliest study on the genes related to sheep reproductive traits and the most efficient and widely used mechanism is related to the FecB gene (Yue *et al*., 2021). The FecB gene mutation has been detected in the Small-tailed han sheep (Liu *et al*., 2003, Wen *et al*., 2021) and Hu sheep (Yang *et al*., 2020) in Chinese. However, it was found that the gene was not detected in sheep breed represented by Taoset and Suffolk sheep (Hanafy *et al*., 2018). The aforementioned researches suggested that the FecB gene showed breed specificity in different sheep breeds, with a focus on expression in high fertility breeds and silencing in low fertility breeds.

Tibetan sheep are widespread in the Qinghai-Tibet Plateau (QTP) and adjacent areas. However, in the past, it was thought that Tibetan sheep were single-litter sheep breed. Our team has successfully bred a core population of multiparous Tibetan sheep to solve the key technical problem of low fecundity. The search for reliable molecular markers is now an urgent task to improve lambing numbers and develop the Tibetan sheep industry. Qiao *et al*. (2017; 2018) found FecB gene mutations are present in plateau-type Tibetan sheep. A previous finding showed that there be existed the polymorphism of FecB locus in Tibetan sheep and found that the frequency is not found to be very high (La *et al*., 2020). In the aforementioned studies, FecB gene mutation-related experiments were carried out in Tibetan sheep. However, they only conducted

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genotype and polymorphic analyses on the FecB locus. It is not clear whether there are other motifs on the *BMPRIR* gene in plateau-type Tibetan sheep and whether there is a strong association between the mutant locus and reproductive performance. Based on these questions, this thesis has carried out work on candidate loci affecting lambing numbers in Tibetan sheep using generational sequencing and association analysis models. The molecular markers obtained in this paper can provide ideas and insights into the reproductive performance of Tibetan sheep in China.

MATERIALS AND METHODS

Experimental animals

The 120 experimental sheep used in this study were from the 4 Tibetan sheep ranches in Haibei Prefecture, Qinghai, China, including the Ebao and MoLe Ranch in Qilian County, ShaLiuHe Ranch in Gangcha County and Xihai Ranch in Haiyan County. The geographical detail of the sampling sites is shown in Fig 1. All sheep were subjected to venous blood collection. Detailed information, such as the ewe's identification number, ranch information and litter size, were recorded. In this experiment, the number of Tibetan lambing ewes included single lambs and double lambs, with 69 single-lambing ewes and 51 double-lambing ewes.

Identification of candidate loci

Extraction of genomic DNA from the blood of Tibetan sheep was carried out according to the instructions and stored at -20°C. Based on the *BMPRIR* sequence in sheep found on NCBI, the primers targeting the positions 597, 746, 864 and 1113 of the *BMPRIB* gene were designed using Primer Premier 5.0 and DNAMAN. The primers for position 746 were considered based on the published work "Technical Regulations for Molecular Detection of FecB, the Major Gene of Multiparous Sheep" (Chu *et al*., 2009). Detailed information on primers is presented in Table 1. The main reason for designing primers for the other three loci in this study was that many details on SNPs were obtained by previous studies through resequencing, which comprised these three loci of the *BMPRIB* gene. The synthesis of the primers for this experiment was done by Beijing Huada Gene Co., Ltd. The total volume of the polymerase chain reaction (PCR) system was 20 μL, including 1 μL of DNA template, 10.5 μL of 2 × PCR DNA polymerase mixture, 7.5 μL of ddH_2O, 0.5 μL of downstream primer and 0.5 μL of upstream primer. The pre-denaturation of the PCR program was performed at 94°C for 5 min, followed by denaturation at 94°C for 30 s, annealing at the corresponding annealing temperature for 30 s and extension at 72°C for 30 s for a total of 34 cycles and further followed by a final extension at 72°C for 10 min and then storage at 4°C. The agarose gel electrophoresis was conducted for the PCR products and the qualified products were sent to Sangon Biotech (Shanghai) Co., Ltd. for Sanger sequencing. DNAstar was used for genotyping the four mutation sites in the experimental sheep.

Data processing and statistical analysis

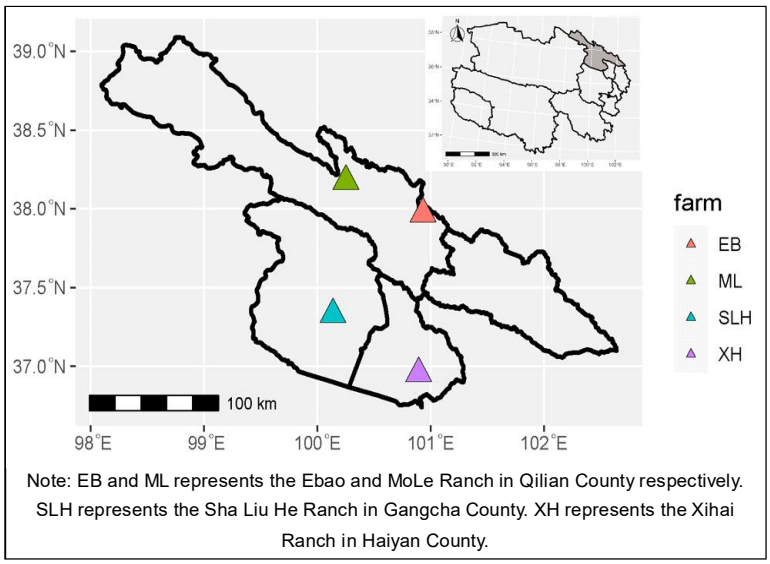
Gene frequencies, effective allele number (Ne), genotype frequencies, homozygosity (Ho), PIC and heterozygosity (He) were determined for each locus using Microsoft Excel 2013. Observed heterozygosity and expected heterozygosity were calculated using Plink software (Version 7.1) and POPGENE software (Version 3.2). Correlation statistical analysis between the mutant locus and lambing number traits in Tibetan sheep was conducted with the following model:

$$Y_{ijh} = \mu + G_i + F_i + E_{ijh}$$

Where

Y_{ijh}= The observed phenotype value.

μ= The average value.



Note: EB and ML represents the Ebao and MoLe Ranch in Qilian County respectively. SLH represents the Sha Liu He Ranch in Gangcha County. XH represents the Xihai Ranch in Haiyan County.

Fig 1: Geographical distribution of experimental samples.

G_i= Representatives genotype effect size.
F_i= Representatives ranch effect values.

It is assumed that E_{ijh}= Independent and follow. The N(0, σ2) distribution. The *lm* (y~G+F) model in R was called and performed one-way ANOVA in this analysis. All four SNP sites were tested for significance using the aforementioned model.

RESULTS AND DISCUSSION

SNPs identified by sequencing

Using the four pairs of well-designed primers, combinatorial pooled sequencing and DNAstar analysis, four important SNP loci in the *BMPRIB* gene were preliminarily identified, located at g.597, g.746, g.864 and g.1113 in the coding sequence region of *BMPRIB*, respectively (Fig 2). The sequencing results revealed that g.746 was a known locus and the other three loci were the first to be identified in Tibetan sheep.

Genetic polymorphisms and diversity of *BMPRIB* gene

After the determination of SNP loci, all the remaining individuals were sent to Sanger sequencing for the genotyping of the four loci. Based on the genotyping results, the allelic frequency and genotype frequency from four locus in the Tibetan sheep populations are provided in Table 2, 3, respectively. Allele A was found to be dominant at both the g.597 and g.746 loci in the population to be tested. Based on the four loci we also calculated their PIC size with the aim of measuring their polymorphic richness. The g.597 G>A and g.746 A>G loci were moderately polymorphic in the population to be tested, while the other two loci belonged to the poor genetic diversity in the population to be tested.

The x^2 test indicated that the two locus, including g.597 and g.746 were in Hardy-Weinberg equilibrium, except for other two locus (P>0.05).

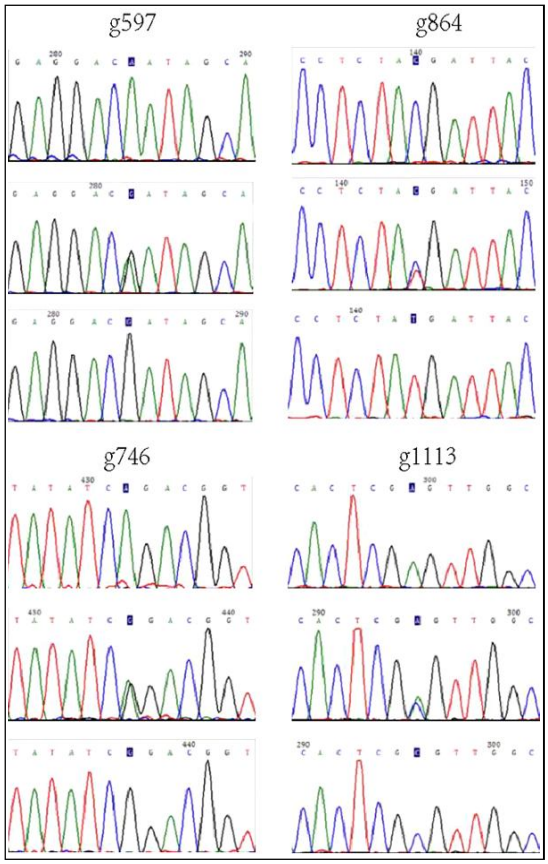


Fig 2: *BMPRIB* gene sequencing chromatograms.

Table 1: List of primers.

Primer name	Primer sequence	Amplicon length (bp)	Annealing temperature (°C)
P597	F: 5′-ATAACTTGTCTCACCAGT-3′	599	51
	R: 5′-ATTTCATAGCGGATCTAG-3′		
P746	F: 5′-GTCGCTATGGGGAAGTTTGGATG-3′	519	58
	R: 5′-CAAGATGTTTTCATGCCTCATCAACACGGTC-3′		
P864	F: 5′-AAAGGACGATAGTTGAAAG-3′	240	50
	R: 5′-CCACTGACTGCGGAATAG-3′		
P1113	F: 5′-CGGTAAGTGTGACATTGA-3′	495	54
	R: 5′-AGCAGCTCCAGCAGCATC-3′		

Table 2: Genotype distribution and allele frequency of four SNP loci of the *BMPRIB* gene in Tibetan sheep.

Locus	Number of ewes	Genotype distribution			Allele frequency		HWE (χ^2)	*P* value
g.597	120	GG (0.392)	GA (0.200)	AA (0.408)	G (0.492)	A (0.508)	3.180	0.097
g.746	120	GG (0.150)	GA (0.383)	AA (0.467)	G (0.342)	A (0.658)	2.620	0.105
g.864	120	TT (0.117)	TC (0.300)	CC (0.583)	T (0.267)	C (0.733)	6.500	0.011
g.1113	120	AA (0.283)	AC (0.267)	CC (0.450)	A (0.417)	C (0.583)	9.441	0.008

Note: HWE= Hardy-weinberg equilibrium.

Correlation of mutant sites and lambing numbers

In this experiment, the number of Tibetan lambing ewes included single lambs and double lambs, with 69 single-lambing ewes and 51 double-lambing ewes. The animals came from four pastures in Haibei Prefecture. The results of correlation analysis revealed that the presence of two loci, including g.746 and g.597, were important for lambing numbers in Tibetan sheep (Table 4). In loci g.746, relevance analysis discovered that genotypes GG and AG had 0.70 ($P<0.05$) and 0.79 ($P<0.05$) litter sizes more than the genotype AA in Tibetan sheep, respectively. In loci g.597, Tibetan sheep with genotypes AA and GA had 0.21 ($P<0.05$) and 0.31 ($P<0.05$) litter sizes more than the genotype GG, respectively.

Previously, our team also used molecular biology techniques to detect FecB gene mutations in the multiparous population of Tibetan sheep were present (Qiao *et al*., 2017, Qiao *et al*., 2018). However, only one major-effect locus was studied and no correlation analysis with litter size has been conducted yet. Therefore, whether a significant correlation exists between FecB mutations and litter size traits in Tibetan sheep remains elusive. Hence, systematically explaining whether the *BMPRIB* gene in the Tibetan sheep populations had other mutation sites or whether the correlations between mutations and litter size were statistically significant was important. Hence, SNP scanning of the *BMPRIB* gene was conducted. Besides, the association analyses between mutation sites and litter sizes were performed to find other causative mutations responsible for reproduction. The mutation sites found in this study provided useful clues and a theoretical basis for improving the genetic mechanism of how the FecB gene determined sheep litter sizes and for effectively promoting the expansion of the multiparous sheep population.

Exactly as expected, the results in this experiment revealed that the presence of two loci, including g.746 and g.597, were important for lambing numbers in Tibetan sheep. In terms of g.746 locus, two genotypes, including GG and AG exhibit excellent lambing traits comparison with genotype AA. Previous evidence confirmed that ovarian glandular activity were controlled by the *BMPRIB* gene and the g.746 locus was identified as a key locus for fertility and ovulation promotion, leading to the designation of the locus as FecB (Yang *et al*., 2020). Interestingly, it was found that FecB mutations tend to be expressed in high-prolific sheep breeds, such as Hu sheep and Small-tailed han sheep and are absent in low-prolific sheep breeds (Guan *et al*., 2006, Chu *et al*., 2007). In previous studies, it was found that B allele of g.746 locus was dominant in the most high-prolific breeds (Hanafy *et al*., 2018). The Tibetan sheep was considered a low-prolific breed in the past and the FecB gene was absent. Differentiate from previous results, the G allele frequency and A allele frequency were 0.342 and 0.658 in the Tibetan population to be tested in this study, respectively. Although the B allele of the FecB locus was not dominant, it was demonstrated that FecB mutations existed in the Tibetan population to be tested. In this analysis, the litter sizes of the g.746 homozygote and heterozygote sheep were significantly higher than that of the wild type. Most studies showed that the FecB gene mutation in most sheep populations was the “major locus” responsible for increased fecundity (Yang *et al*., 2020, Wen *et al*., 2021). Another study found that individuals carrying genotype BB and B+ in this locus tends to have more lambing numbers compared to wild type genotype groups in Hu and Altay sheep (Li *et al*., 2012). The aforementioned findings suggested that the results of our study were similar to the pattern found in Hu and Altay breeds, further supporting and verifying the findings that the FecB gene was the major gene-determining sheep litter size.

In the case of the *BMPRIB* gene, research continues to identify more loci associated with lambing numbers, such

Table 3: Heterozygosis analysis of four SNP loci of *BMPRIB* gene in Tibetan sheep.

Locus	Number of ewes	Ho	O (HET)	E (HET)	Ne	PIC value
g.597	120	0.800	0.200	0.500	1.250	0.275
g.746	120	0.617	0.383	0.450	1.622	0.253
g.864	120	0.700	0.300	0.391	1.429	0.164
g.1113	120	0.733	0.267	0.486	1.364	0.012

Note: Ho= Homozygosity, O(HET)= Observed heterozygosity, E(HET)= Expected heterozygosity, Ne= Effective allele number, PIC= Polymorphic information content.

Table 4: Association analysis between the litter sizes and four loci of *BMPRIB* gene in Tibetan sheep.

Locus	Genotype (mean±SD)			*P* value
g.597	AA (1.489±0.505)^{a}	GA (1.583±0.504)^{a}	GG (1.277±0.452)^{b}	0.003671
g.746	AA (1.018±0.134)^{b}	AG (1.804±0.401)^{a}	GG (1.722±0.461)^{a}	0.000002
g.864	CC (1.583±0.483)^{a}	CT (1.583±0.500)^{a}	TT (1.357±0.497)^{a}	0.056
g.1113	CC (1.444±0.504)^{a}	AC (1.375±0.492)^{a}	AA (1.441±0.504)^{a}	0.229

Note: The *P* value was set to 0.05 for this test. Same letter indicates no significant difference and different letters indicate a significant difference.

as in Hu sheep and Luzhong mutton sheep (Yang *et al*., 2020, Di *et al*., 2021). Excitingly, other novel loci for the *BMPRIB* gene on Tibetan sheep have also been identified on the work we have carried out. In the case of the g.597 locus, it presents an excellent value for moderate polymorphism utilization in the population to be tested and has to be potential candidate loci. Additionally, two genotypes, including AA and GA exhibit excellent lambing traits comparison with genotype AA. Therefore, g.597 locus has the potential to be a powerful molecular marker for molecular breeding of Tibetan sheep.

CONCLUSION

In the present study, four mutation sites were identified in the Tibetan sheep populations by sequencing. Four SNPs (g.597G >A, g.746 A>G, g.864 C>T and g.1113 C > A) were screened in *BMPRIB* gene. In loci g.746, relevance analysis discovered that genotypes GG and AG had 0.70 ($P<0.05$) and 0.79 ($P<0.05$) litter sizes more than the genotype AA in Tibetan sheep, respectively. In loci g.597, Tibetan sheep with genotypes AA and GA had 0.21 ($P<0.05$) and 0.31 ($P<0.05$) litter sizes more than the genotype GG, respectively. The results showed that g.746 A>G and g. 597 G>A were regarded as assisted-markers in molecular. These findings provide ideas and insights into the reproductive performance of Tibetan sheep in China.

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Conflict of interest

The authors declare that there is no conflicts of interest.

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