Detection of mRNA Expression and Copy Number Variations Within the Goat FecB Gene Associated With Litter Size

Yi Bi1, Weijie Feng1, Yuxin Kang1, Ke Wang1, Yuta Yang1, Lei Qu2,3, Hong Chen1, Xianyong Lan1* and Chuanying Pan1*

1 Key Laboratory of Animal Genetics, Breeding and Reproduction of Shaanxi Provincial, College of Animal Science and Technology, Northwest A&F University, Xianyang, China, 2 Shaanxi Provincial Engineering and Technology Research Center of Cashmere Goats, Yulin University, Yulin, China, 3 Life Science Research Center, Yulin University, Yulin, China

The Booroola fecundity (FecB) gene, as the first major fecundity gene identified in Booroola sheep, has attracted careful attention. So far, previous research have uncovered the FecB mutation (Q249R) as the main mutation by virtue of which sheep exhibits multiple lambing phenomena. This mutation is now being intensively studied and widely used. However, such effect of the FecB mutation has not been applied to goats, and similar types of the FecB gene in goats still need to be studied. Thus, the current study attempted to verify potential mutations in the goat FecB gene as well as investigate their functions related to fecundity. First, FecB expression was investigated in six different goat tissues, and we found that FecB expression was highest in the mammary gland, followed by the ovary. Next, the influence of the FecB gene was analyzed from a new perspective, where five potential copy number variations (CNVs) (CNV1–5) within the FecB gene were identified for the first time, and then their effects on litter size were measured. Our results point out that CNV3 ($P = 3.44E-4$) and CNV5 ($P = 0.034$) could significantly influence the litter size of goats. Identically, the combination genotype of CNV3 and CNV5 which consisted of their dominant genotypes was also significantly associated with goat litter size ($P = 7.80E-5$). Hence, CNV3 and CNV5 could serve as potential fertility-related quantitative trait loci within the goat FecB gene.

Keywords: goat, FecB gene, mRNA expression, CNV, litter size
INTRODUCTION

The Booroola fecundity (FecB) gene, also named the bone morphogenetic protein receptor 1B (BMPR1B) gene, was first recognized as a high-prolificacy major gene in Booroola sheep (1) due to the multi-lamb phenomenon contributed by the FecB mutation (Q249R) (2, 3). Moreover, as a receptor of bone morphogenetic proteins (BMPs) (4), it is involved in the regulation of follicle-stimulating hormone (FSH) levels, which regulates the ovulation rate in animals and humans (5, 6). In detail, the diminution of the BMP signal reduced by Q249R leads to an increase in follicle-stimulating hormone (FSHR) and luteinizing hormone receptor (LHR) density and also reduces apoptosis to increase ovulation (7–9). Furthermore, the attenuation of BMP signaling can maintain the reserve of primordial follicles while promoting follicular growth and ovulation, which contributes to the overall fertility of a female (10). Subsequently, a previous study showed that the Q249R mutation can regulate the expression of BMP/SMAD signaling and significantly upregulate the expression of fecundity-related genes such as STAR, BMP6, and BMP2 (11). Moreover, it can increase ovulation by suppressing the expression of SMAD6, which acts as an inhibitor of the BMP/SMAD signaling pathway (12).

To date, the FecB gene has been deeply studied in several breeds and strains of sheep such as Hu, Chinese Merino prolific meat strain, Suffolk, Dorset, and Charolais sheep breeds (13, 14), especially the Q249R locus which was successfully applied in sheep breeding as a significant DNA marker. Although only Q249R is regarded as the major mutation related to high fertility in sheep, there are also several identified mutations within the FecB gene worth studying—for instance, g.29362047T>G. To accomplish the further experiment, first-strand cDNA was isolated from the ear tissue of 312 female goats according to the protocol of our previous study (15). In addition to single-nucleotide polymorphisms (SNPs), five insertion/deletions (indels) (4, 10, 12, 17, and 23 bp) in the BMPR1B gene were also verified in Chinese Australian White sheep. However, among them, only the 10-bp indel significantly affected the sheep litter size (16). In goat, a previous study has revealed that Smad signaling, steroidogenesis, and cell viability in granulosa cells have been altered upon modulation of the FecB gene which was similar to that documented in sheep breeds carrying the FecB mutation (17). Moreover, several research were devoted to the detection of potential mutations in goat FecB gene, but no major high-fertility-related mutation was discovered in goat until now. In detail, C94T was identified to exert a significant influence on the litter size of Liaoning cashmere goats (18). Apart from that, three novel SNPs, including G773C, A775G, and G777A, have also been uncovered in Assam hill and Markkoz goats, respectively, but their specific functions still need to be studied (19, 20). Altogether, as a major fecundity-related gene, the FecB gene as well as its function has attracted careful attention (20–25), and a number of SNPs and indels within the FecB gene have also been examined to varying degrees (26–28). However, almost all studies have focused on sheep, while no major quantitative trait locus (QTL) has yet been identified in goats. Additionally, we found that all relevant research focus on SNPs and indels without paying attention to copy number variation (CNV), which is also one of the promising DNA markers.

Therefore, in this work, we aimed to investigate potential CNVs within the BMPR1B gene and, for the first time, measure their effects on goat litter size, thereby trying to provide information for DNA editing and microarray as well as laying the theoretical foundation for improving goat fecundity.

MATERIALS AND METHODS

Animals, Total RNA, and Genomic DNA Isolation

In order to obtain RNA samples for further experiments, a total of six different tissues (corpus luteum, large follicle, skeletal muscle, uterus, ovary, and mammary gland) of the goat were collected (n = 3). Total RNA extraction was carried out using TRIzol total RNA extraction reagent (Takara, Dalian, China), following the instructions of the manufacturer. Then, 1% agarose gel electrophoresis in 6× loading buffer was used to evaluate the integrity of total RNA. RNA was then quantified and qualitatively analyzed using a Nanodrop 2000 Spectrophotometer. Qualified RNA samples were stored at −80°C. To accomplish the further experiment, first-strand cDNA was synthesized by Prime Script™ RT Reagent Kit (Takara, Dalian, China). After that, these cDNAs were conserved at −20°C for subsequent experiments.

Shaanbei white cashmere (SBWC) female goats (2–3 years) that were healthy and under the same feeding and management conditions were used as the DNA sample in this work. All genomic DNAs were isolated from the ear tissue of 312 female goats according to the protocol of our previous study (29) and then stored at −40°C. In addition, all litter size and growth trait records of these goats were provided by the staff from farm records.

| Loci | Primer sequence (5' to 3') | Tm (°C) | Length | Location |
|------|-----------------------------|---------|--------|----------|
| CNV1-F | TGGAAAACGAGCCAGGGAAGGAA | 57.97   | 141 bp | Intron 1 |
| CNV1-R | TAACCTCTCATACCTTTTCCTCC | 57.43   |        |          |
| CNV2-F | AGAGGCTGAGGCTGCTAATGTTT | 57.08   | 158 bp | Intron 1 |
| CNV2-R | GACTGCTCATTTGTGGTTGGAAG | 59.73   |        |          |
| CNV3-F | CAGATTTCAGCCCTTTGCGGGG    | 59.83   | 111 bp | Intron 2 |
| CNV3-R | TTGGGCACTGCAAAAGAAAG      | 59.60   |        |          |
| CNV4-F | CAGTCTATCCGCTGACTAGCAGCA | 59.18   | 165 bp | Intron 1 |
| CNV4-R | TGCCATTTAGTGGTGAGGCAA    | 59.93   |        |          |
| CNV5-F | CGAAGGTAAAACGAACTAGACAGACA | 58.60   | 200 bp | Intron 2 |
| CNV5-R | ACGAGCATGACCAGGGGAAAGG   | 58.90   |        |          |
| MC1R-F | GGCCCTGAAGGGGAAATCACA   | 61.27   | 126 bp |          |
| MC1R-R | AGTGGGCTCTGGAGATGGGAGAC | 60.33   |        |          |
| FecB1-F | GTTGTACAGAGGTATAGTGAGAAAG | 59.29  | 93 bp  |          |
| FecB1-R | AAGAAGCTGATCTCTTCATGCGGG  | 59.86   |        |          |
| GAPDH-F | AAAGTGCGACATCGCTGCCCCAT | 60.04   | 116 bp |          |
| GAPDH-R | CCGTCTTCTGCTGCTGACTTG  | 59.97   |        |          |
Figures and Tables:

**Figure 1** | Gene structure of the goat FecB gene.

**Figure 2** | The mRNA expression profile of goat FecB gene, using the corpus luteum and large follicle as references (selecting three individuals for each tissue with three parallel repeats). *P < 0.05; **P < 0.01.

**Table 2** | Information on the copy number variations within the goat FecB gene.

| Loci  | Chromas | Start      | End        | Length |
|-------|---------|------------|------------|--------|
| CNV1  | 6       | 30065201   | 30067200   | 2,000  |
| CNV2  | 6       | 30086001   | 30088400   | 2,400  |
| CNV3  | 6       | 30116801   | 30118800   | 2,000  |
| CNV4  | 6       | 30121201   | 30123200   | 2,000  |
| CNV5  | 6       | 30180401   | 30182000   | 1,600  |

**Table 3** | Typical frequencies of copy number variations within FecB gene in Shaanbei white cashmere goats.

| Loci | Sizes | Typic frequencies |
|------|-------|-------------------|
|      |       | Loss   | Median | Gain  |
| CNV1 | n = 112 | 0 (n = 0) | 0.196 (n = 22) | 0.804 (n = 90) |
| CNV2 | n = 312 | 0.147 (n = 46) | 0.128 (n = 40) | 0.725 (n = 226) |
| CNV3 | n = 313 | 0.153 (n = 48) | 0.300 (n = 94) | 0.547 (n = 171) |
| CNV4 | n = 229 | 0.223 (n = 51) | 0.332 (n = 76) | 0.445 (n = 102) |
| CNV5 | n = 302 | 0.013 (n = 4) | 0.017 (n = 5) | 0.970 (n = 283) |

**RNA Experiment: mRNA Expression**

A pair of primers for qPCR (FecB-F: 5’-GTGTCAGGAGGTATAGTGGAAGAA-R; FecB-R: 5’-ACACACGATCTCTTCATGCC-R) was designed according to the mRNA sequence (accession number: NM_001285575.1) of the goat FecB gene in the NCBI database (https://www.ncbi.nlm.nih.gov/gene) via primer premier 6.0 (Table 1). It has covered all exons of the goat FecB gene to ensure cDNA amplification. The qPCR reaction system (10 µl) comprised of 5 µl of cDNA, 0.5 µl of each primer, and 3 µl of ddH2O, using the same protocol as in our previous study (30). GAPDH was used as the reference gene (31). The results were then analyzed by 2^{-ΔΔCt} method (32, 33).

**DNA Experiment: Genotyping of CNV Within the FecB Gene**

To identify copy number variations (CNVs), we first searched for potential CNVs in the goat FecB gene in the Animal Omics database (http://animal.nwsuaf.edu.cn) and found five CNV loci. Additionally, the figure of the goat FecB gene was drawn using Adobe Illustrator 2021 (Adobe, USA) as shown in Figure 1. When we analyzed these CNVs in our detected population, five pairs of primers (Table 1) were designed by NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast), and qPCR was executed to further validate the CNVs. The qPCR reaction system (10 µl) contained 5 µl of 2× SYBR Premix Ex Taq (Takara Biotech, Dalian, China), 1 µl of cDNA, 0.5 µl of each primer, and 3 µl of ddH2O, using the same protocol as in our previous study (30). Then, the copy number of the goat FecB gene was calculated using 2^{ΔCt} method, where ΔCt = Ct target gene - Ct reference gene, and MC1R gene was the reference gene (28). Eventually, based on the value of 2^{ΔCt}, the CNVs were divided into three types: “loss” (2^{ΔCt} < 2), “median” (2^{ΔCt} = 2), and “gain” (2^{ΔCt} > 2).
Statistical Analyses

The genotype frequencies of the five CNVs in the tested population was calculated, and the haplotypes were constructed via SHEsis online program (http://analysis.bio-x.cn). After that, the genotype distributions of these CNVs in all single lamb and of fixed factors on goat litter size (Table 5) were analyzed using chi-square test (χ²).

Besides that, a linear model was used to estimate the effect of fixed factors on goat litter size (28): \( Y_{ijk} = \mu + a_i + b_j + e_{ijk} \), where \( Y_{ijk} \) is the litter size, \( \mu \) is the overall mean value for each trait, \( a_i \) is the fixed-factor age, \( b_j \) is the fixed-factor genotype, and \( e_{ijk} \) is the random error. For CNVs that displayed more than two genotypes, one-way ANOVA was used to assess their relationship with litter size; for \(<2\) group, \( t\)-test was performed.

RESULTS

mRNA Expression

mRNA expression was determined in the mammary gland, corpus luteum, large follicle, skeletal muscle, ovary, and uterus tissues. Our results demonstrated that \( Fec^B \) gene regulates sheep fecundity, while the specific mechanism of the goat \( Fec^B \) gene is still poorly understood and the first

![Figure 3](https://www.frontiersin.org)

**FIGURE 3** | Association analysis between litter size and copy number variations (CNVs) in Shaanbei white cashmere goats. (A) Association between CNV3 and litter size. (B) Association between CNV5 and litter size.

**TABLE 4** | Haplotype frequencies of the five copy number variations within the goat \( Fec^B \) gene.

| Haplotype | CNV1 | CNV2 | CNV3 | CNV4 | CNV5 | Frequencies |
|-----------|------|------|------|------|------|-------------|
| Hap 1     | M₁   | M₂   | L₃   | M₄   | G₅   | 0.062       |
| Hap 2     | M₁   | G₂   | L₃   | M₄   | G₅   | 0.062       |
| Hap 3     | M₁   | G₂   | M₃   | M₄   | G₅   | 0.062       |
| Hap 4     | M₁   | G₂   | G₃   | M₄   | G₅   | 0.062       |
| Hap 5     | G₁   | G₂   | G₃   | M₄   | G₅   | 0.125       |
| Hap 6     | G₁   | G₂   | M₃   | G₄   | G₅   | 0.188       |
| Hap 7     | G₁   | G₂   | G₃   | M₄   | G₅   | 0.188       |
| Hap 8     | G₁   | G₂   | G₃   | G₄   | G₅   | 0.251       |

**DISCUSSION**

The \( Fec^B \) gene, which serves as a major gene for high fecundity, has been shown to contribute to the multi-lambing phenomena in sheep. Currently, it is gradually becoming clear how the \( Fec^B \) gene regulates sheep fecundity, while the specific mechanism of the goat \( Fec^B \) gene is still poorly understood and the first
TABLE 5 | Association analyses between litter size and copy number variation types in Shaanbei white cashmere goats.

| Locus | Genotype (LSM ± SE) | P-values |
|-------|---------------------|----------|
|       | Loss                | Median   | Gain     |
| CNV3  | 1.25 ± 0.63B (n = 48)| 1.57 ± 0.51A (n = 94) | 1.33 ± 0.38B (n = 171) |
| CNV5  | 1.00 ± 0.11B (n = 4) | 1.00 ± 0.01B (n = 5)  | 1.43 ± 0.02B (n = 284) |

Values with different letters (A, B/a, b) within the same row differ significantly at P < 0.01/P < 0.05.

FIGURE 4 | Least squares mean and standard error for litter size of the different combination genotypes of CNV3 and CNV5 within the FecB gene in Shaanbei white cashmere goats. Combination genotypes with a sample size of less than five were not calculated.

major QTL still needs to be disclosed. Here we identified the expression of the FecB gene in six different goat tissues and strikingly found that it had the highest expression in the mammary gland, indicating that the FecB gene might also function as a lactation regulator of goats. According to a previous study, an increase in litter size stimulates the expression of RFamide-related peptide mRNA, which might contribute to lactational anestrus in rats (34). Additionally, as the litter size increases, milk yield increases (35–38), and so does the uptake of net daily amino acid by the mammary gland, which is the basis for milk yield and lactoprotein production (39). In short, the above results indicate that the lactation would be mediated by an increase in litter size. Thus, the FecB gene, which significantly affects litter size, also presumably has a function related to lactation in goats. This might be the reason why the FecB gene had the highest expression in the mammary gland in goats. To our best knowledge, this is the first time that the FecB gene has shown such a high expression in the mammary gland, which leads to an indication that it is worth investigating whether the FecB gene has a lactation-related function, especially in populations with multiple lambs. Then, following the mammary gland, high expression in the ovary indicated that the FecB gene might be involved in ovarian activity, thereby influencing goat fertility. However, whether the FecB gene could impact goat fecundity has not yet been sufficiently studied (17).

Until now, reported studies have found a series of polymorphisms associated with fecundity within the FecB gene in sheep. However, related research analyzing the association between mutations in the FecB gene and fertility in goat are relatively scanty—for instance, G773C was identified to be unique in Assam hill goat, while the association between goat fecundity still needed to be studied (19). T242C was uncovered

TABLE 6 | Least squares mean and standard error for litter size of different combination genotypes of CNV3 and CNV5 within the FecB gene in Shaanbei white cashmere goats.

| Locus | Genotype (LSM ± SE) | P-value |
|-------|---------------------|---------|
|       | Loss                | Median  | Gain     |
|       | 1.00 ± 0.01B (n = 22)| 1.36 ± 0.13B (n = 61) | 1.67 ± 0.06B (n = 115) |

Values with different letters (A, B) within the same row differ significantly at P < 0.01. Combination genotypes with a sample size of less than five were not calculated.

TABLE 7 | Type distribution between mothers of single lamb and multi-lamb in Shaanbei white cashmere goats.

| CNV loci | Genotypes | Single lamb | Multi-lamb | Independent χ² | P-values |
|----------|-----------|-------------|------------|----------------|---------|
| CNV1     | Loss      | 1           | 0          | $\chi^2 = 3.372$ | df = 2, $P = 0.155$ |
|          | Median    | 13          | 9          |                |         |
|          | Gain      | 36          | 53         |                |         |
| CNV2     | Loss      | 24          | 22         | $\chi^2 = 2.237$ | df = 2, $P = 0.327$ |
|          | Median    | 27          | 13         |                |         |
|          | Gain      | 139         | 87         |                |         |
| CNV3     | Loss      | 36          | 12         | $\chi^2 = 20.213$ | df = 2, $P = 4.1E-5$ |
|          | Median    | 40          | 54         |                |         |
|          | Gain      | 115         | 56         |                |         |
| CNV4     | Loss      | 28          | 29         | $\chi^2 = 0.436$ | df = 2, $P = 0.804$ |
|          | Median    | 35          | 29         |                |         |
|          | Gain      | 58          | 50         |                |         |
| CNV5     | Loss      | 4           | 0          | $\chi^2 = 6.759$ | df = 2, $P = 0.034$ |
|          | Median    | 5           | 0          |                |         |
|          | Gain      | 160         | 123        |                |         |
in Barbari, Beetal, Black Bengal, Ganjam, Jhakana, Osmanabadi as well as Sangamneri goat breeds, but the effect of genotypes was non-significant on litter size (40). Moreover, C94T within the goat FecB gene was revealed to be significantly associated with litter size in Liaoning cashmere goats (18). Interestingly, a related work investigated the FecB mutation in goat since its significant influence on multiple lambing in sheep. However, the results pointed out that the FecB mutation did not exert the same effect in goat, which led to a hint that novel significant mutations still need discovering (20). Many reports altogether revealed lots of polymorphisms within the FecB gene associated with fecundity in sheep, while potential mutations still need discovery in goats. No related research also paid attention on the CNV within this gene. In this study, we first identified five potential CNVs in the FecB gene and measured their effects on litter size in goats. Our results pointed out that the “gain” genotype, rather than another two genotypes, displayed the highest frequency in all five CNVs. Identically, the G1G2G3G4G5 haplotype was also the dominant haplotype with the highest frequency. In addition to the distribution of the five CNVs, we further analyzed their effect on goat litter size, which was the main purpose of this work. The results based on a large experimental population showed that CNV3 and CNV5 exerted significant effects on litter size in goats (P < 0.05), with “median” and “Gain” displaying a superior phenotype, respectively. In addition, the combination genotypes of CNV3 and CNV5 could also have the same effects on goat litter size. Notably, the combined genotype of “median” (CNV3) and “gain” (CNV5) performed superior litter size, which is consistent with the abovementioned results. Thus, CNV3 and CNV5 could serve as effective DNA markers applied to marker-assisted selection breeding, and individuals, especially with combined genotype “M3G5” should be selected to improve the litter size of goats.

Given that previous investigations revealing intronic variations could influence the interaction between transcription factors and host genes, we hypothesized that CNV3 and CNV5 might influence the combination ability of DNA sequence with transcription factors to indirectly influence the expression of the FecB gene (41, 42). To add, with BMPs acting as the key intraovarian factors regulating ovarian function (43–45), their biological effects will be mediated after binding to membrane-bound receptors. Mutations might influence the process BMPs combing with DNA sequence, thereby changing the related functions. However, how the two CNVs within the FecB gene influence the goat litter size still needs to be studied.

In conclusion, we have identified five CNVs within the FecB gene in SBWC goat population and found that CNV3 and CNV5 significantly influenced the litter size of goats. Moreover, this is the first report on the effect of CNVs within the FecB gene on litter size. Despite this, the specific mechanisms require further investigation.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the International Animal Care and Use Committee of the Northwest A&F University (IACUC-NWAFU; protocol number NWAFAC1008).

AUTHOR CONTRIBUTIONS

YB, XL, and CP came up with idea and revised the manuscript. YB wrote the manuscript and performed the experiments. WF, YK, KW, and YY collected the goat samples and isolated the genomic DNA. YB, YK, and LQ analyzed the data. All authors approved the final version of the manuscript for submission and contributed to the article and approved the submitted version.

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