Polymorphism Detection of GDF9 Gene and Its Association with Litter Size in Luzhong Mutton Sheep (Ovis aries)

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Abstract: Litter size is one of the most important economic traits in sheep. GDF9 and BMPR1B are major genes affecting the litter size of sheep. In this study, the whole coding region of GDF9 was sequenced, and all the SNPs (single nucleotide polymorphisms) were determined in Luzhong mutton sheep. The results suggested that two single nucleotide polymorphisms (SNPs), g.41768501A > G and g.41768485 G > A are associated with litter size. The g.41768485 G > A is a missense mutation which is predicted to affect the tertiary structure of the protein. Thus, these two mutations may be potential effective genetic markers to improve the litter size in sheep.

Keywords: sheep; GDF9 gene; FecB gene; litter size

1. Introduction

Litter size is one of the most important economic traits and is closely related to the economic benefits of modern sheep farming. Study on the key genes of litter size can provide useful information for marker-assisted breeding to increase litter size.

The growth differentiation factor 9 (GDF9) gene, encoding one member of the transforming growth factor β (TGF-β) superfamily, has important functions in the reproduction of ewes [1]. The gene was mapped on sheep chromosome 5 [2] and contains two exons separated by an intron. GDF9 is mainly expressed in oocytes and plays an important role in follicular development and ovulation in sheep [3]. In previous studies, many mutations in the gene were identified in Belclare sheep (FecG2) [4] of Europe, Iranian Afshari sheep (G2,
G3, and G4) [5] and Fat-Tailed sheep (G1, G2, G3, and G4) [6] of Middle Eastern origin, and Rahmani sheep (FecGH) [7] of Africa. It was reported that c.978A>G and c.994G>A, FecGH, c.1040T>C (Phe 347 Ser) were associated with litter size in Araucana creole, Rahmani, and Mongolia sheep [7–9], respectively.

BMPR1B was the first identified major gene for the fertility of sheep [10] and was mapped to chromosome 6 of sheep [11]. BMPR1B protein is also one of the members of TGF-β superfamily [12]. FecB is an A746G mutation in BMPR1B coding sequence which causes nonconservative substitution (Q249R), and this mutation is related to the prolific phenotype in Australian (Booroola sheep) and Chinese (Small Tailed Han sheep) breeds [13–16].

Luzhong mutton sheep is a new breed with high fertility and meat performance. It was developed by crossbreeding White-headed Dorper sheep from South Africa and Hu sheep from China. These two progenitor breeds have medium or high fecundity. Therefore, Luzhong mutton sheep is likely to carry FecB and mutations in the GDF9 gene simultaneously. Excavating mutations associated with litter size may help to discover new molecular markers to increase the efficiency of sheep reproduction. Thus, the objective in present study was to detect mutations in the GDF9 gene and determine the mutation loci associated with litter size in Luzhong mutton sheep.

2. Materials and Methods

2.1. Animals and DNA Extraction

Jugular blood samples of 154 Luzhong Mutton ewes with litter size records of two parities were collected from Ji’nan Laiwu Yingtai Agriculture and Animal Husbandry Technology Co. Ltd. (Ji’nan, Shandong, China). Genomic DNA of these ewes were extracted by the phenol–chloroform method and then dissolved in ddH2O. All experimental procedures involved in this study were approved by the Animal Welfare Division of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS) (Beijing, China). In addition, ethics approval was given by the animal ethics committee of IAS-CAAS (No. IAS2020-64) on 27 April 2020.

2.2. Full-Length Sequencing and Mutation Detection of GDF9 Coding Sequence

To amplify the GDF9 gene coding sequence in Luzhong mutton sheep, three pairs of primers were designed with Primer Premier software (version 5.0, PREMIER Biosoft international, San Francisco, CA, USA) software according to ovine GDF9 sequence (GenBank NC_040256.1). Detailed information of the primers is shown in Table 1. PCR products were sequenced using the Sanger sequencing method in Suzhou Genewiz Biotechnique Co. Ltd. (Suzhou, China) with the primer the same as the amplification primer. After sequencing the PCR products, the entire GDF9 coding sequence was assembled. Then, GDF9 gene sequences of 154 Luzhong Mutton sheep were aligned with the reference sequence of the ovine GDF9 gene (GenBank NC_040256.1) using MEGA7 software [17]. Finally, polymorphic loci of this gene were determined in Luzhong Mutton sheep and were subsequently searched in the Ensembl database (https://asia.ensembl.org/Ovis_aries/Gene/Variation_Gene/Table?db=core;g=ENSOARG00000013229;r=5:41841034-41843517;t=ENSOART0000014382, accessed on 27 May 2020) to check if these loci were novel mutations.

Table 1. Primers used for the amplification of the GDF9 gene.

| Primer Name | Primer Sequence (5’−3’) | Annealing Temperature/°C | Amplified Fragment/bp |
|-------------|--------------------------|--------------------------|-----------------------|
| GDF9-F1     | ACTGAATGAATAGGGTGTTG     | 58                       | 1553                  |
| GDF9-R1     | ATCTGTACCATATCTAAGTCC    | 57                       |                       |
| GDF9-F2     | GTTTGTTCGCACTTGGTTAGCC   | 63                       | 1507                  |
| GDF9-R2     | GGCGCCGCGATTTTACACTGG    | 58                       |                       |
| GDF9-F3     | TCAGCTGAAATGGGGACAA      | 60                       | 694                   |
| GDF9-R3     | ACACAGAATAATATGCCACACTAC| 62                       |                       |
2.3. Genotyping of FecB Mutation

The FecB mutation was genotyped in Luzhong mutton ewes using the Sequenom MassARRAY®SNP assay method previously described by Zhang et al. [18].

2.4. Statistical Analysis

Allele and genotype frequency, polymorphism information content (PIC), heterozygosity (He), and number of effective alleles (Ne) were calculated for GDF9 and BMPR1B genes using the following formula [19]:

\[
He = 1 - \sum_{i=1}^{n} p_i^2
\]

\[
Ne = 1 / \sum_{i=1}^{n} p_i^2
\]

\[
PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2
\]

where \( n \) is the number of alleles, \( p_i \) is the allele frequency of the \( i \)th allele, and \( p_j \) is the allele frequency of the \( j \)th allele.

The genotype distribution of each locus was tested for deviation from the Hardy–Weinberg equilibrium using the Chi-Square test [20]. The association of litter size with the genotypes of GDF9 and FecB was analyzed using the following fixed effects model in Luzhong mutton sheep, with least squares means used for multiple comparisons in litter size among the different genotypes:

\[
y = \mu + P + G1 + G2 + G1G2 + e
\]

where \( y \) is the phenotypic value of litter size, \( \mu \) is the population mean, \( P \) is the fixed parity effect (two levels), \( G1 \) is the fixed effect for GDF9 genotypes, \( G2 \) is the fixed effect for FecB genotype, \( G1G2 \) is the fixed interaction effect for GDF9 and FecB combined genotypes, and \( e \) is the random error effect of each observation. Analysis was performed using the general linear model procedure by R software (aov, Version 4.0.3) [21]. Mean separation procedures were performed using the Duncan test, with \( p \)-values < 0.05 considered to be significant.

2.5. Phylogenetic Analysis

A phylogenetic tree of GDF9 gene coding sequence in 10 sheep breeds and other five species (human, goat, Norway rat, chicken, and pig) was constructed by the maximum likelihood method (model: K2P + G4) with IQ-TREE software [22]. For Cele Black sheep, Hu sheep, Small Tail Han sheep, Prairie Tibetan sheep, Valley Tibetan sheep, Bayinbuluke sheep, Tan sheep, and Wuzhumuqin sheep, the GDF9 coding region sequences were extracted from the bam file of our previous whole-genome sequencing data with Samtools [23]. For Texel sheep, human, goat, Norway rat, chicken, and pig, sequences were obtained according to the NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 27 May 2020) Reference Sequence (NC_019462.2, NC_000005.10, NC_030814.1, NC_005109.4, NC_006100.5, NC_010444.4, respectively).

2.6. Protein Structure Prediction

The secondary structure of GDF9 with and without missense mutants was analyzed using SOPMA [24]. Prediction of the tertiary structure of GDF9 with and without missense mutants was performed using Swiss-Model [25] with homology modeling. The GDF9 mature protein region was predicted using uniprot [26].

3. Results

3.1. Polymorphism Analysis of GDF9 Gene in Luzhong Sheep

To determine the polymorphism of GDF9 gene in Luzhong sheep with different litter sizes, the entire coding sequence of GDF9 gene in 154 ewes were sequenced. By
comparing their coding sequence with the known sequence of GDF9 gene in Rambouillet sheep (GenBank NC_040256.1), eight mutation loci were identified in the GDF9 gene, of which one mutation (g.41769606 T > G) was novel. The GDF9 gene coding sequence in Luzhong mutton sheep was shown in File S1. The mutation information is summarized in Table 1 and the sequencing profiles around eight mutation loci are shown in Figure 1. The combined results from sequence data and alignment analysis of GDF9 revealed five SNPs in intron 1 and three SNPs in exon 2 (Table 2). No mutation was detected in exon 1 of the GDF9 gene coding sequence. These mutations were different from the FecC^H mutation in African Rahmani sheep. By observing the genotypes of different mutation sites together, it was found that linkage exists between mutation loci g.41769246 A > G and g.41769002 A > G, g.41768501 A > G and g.41768485 G > A, and g.41769567 T > C and g.41769223 C > G, respectively. Of them, mutation g.41768485 G > A in exon 2 altered the amino acid (Val (V)–Ile (I)) at residue 332 and other two mutations in exon 2 were synonymous mutations (Table 2).

Figure 1. Sequencing profiles around eight mutation loci in the GDF9 gene of Luzhong sheep.
3.2. Population Genetic Analysis of SNPs in GDF9 and FecB

Population genetics analyses of eight SNPs in GDF9 and FecB were performed. Results showed that g.41769246 A > G, g.41769002 A > G, and FecB mutations had moderate polymorphisms (0.25 < \(PI\)C < 0.5), whereas six other loci (g.41769606 T > G, g.41769574 G > A, g.41769567 T > C, g.41769223 C > G, g.41768501 A > G, and g.41768485 G > A) had low polymorphisms (\(PI\)C < 0.25) in Luzhong mutton sheep (Table 3). In addition, the Chi-square test revealed that eight loci of GDF9 were under the Hardy–Weinberg equilibrium \((P > 0.05)\), except FecB \((P < 0.05)\) in Luzhong mutton sheep.

### Table 2. The information of eight mutation loci in the GDF9 gene of Luzhong sheep.

| Region | Genomic Location (Chr5: Oar_v4.0/Oar_v3.1) | Wild-Type | Mutant | Mutation in Ensembl Database | AA Coding Residue | Amino Acid Change |
|--------|------------------------------------------|-----------|--------|-------------------------------|-------------------|-------------------|
| Intron 1 | 41769606/41842523 | T | G | No | - | - |
|         | 41769574/41842491 | G | A | Yes | - | - |
|         | 41769567/41842484 | T | C | Yes | - | - |
|         | 41769246/41842163 | A | G | Yes | - | - |
|         | 41769223/41842140 | C | G | Yes | - | - |
| Exon 2  | 41769002/41841919 | A | G | Yes | 159 | Unchanged Leu (L) |
|         | 41768501/41841418 | A | G | Yes | 326 | Unchanged Glu (E) |
|         | 41768485/41841402 | G | A | Yes | 332 | Val (V)–Ile (I) |

### Table 3. Population genetic analysis of eight SNPs for GDF9 and FecB mutations in Luzhong mutton sheep.

| Gene | Locus | Genotype Frequency | Allele Frequency | \(PI\)C | He | Ne | Chi-Square Test (p-Value) |
|------|-------|-------------------|-----------------|------|----|----|-------------------------|
| \(GDF9\) | g.41769606 T > G | TT | 0.987 | 0.013 | 0.000 | 0.994 | 0.007 | 0.013 | 0.013 | 1.013 | 0.935 |
|       | g.41769574 G > A | GG | 0.987 | 0.013 | 0.000 | 0.994 | 0.007 | 0.013 | 0.013 | 1.013 | 0.935 |
|       | g.41769567 T > C | TT | 0.909 | 0.091 | 0.000 | 0.955 | 0.046 | 0.083 | 0.087 | 1.095 | 0.555 |
|       | g.41769246 A > G | AA | 0.078 | 0.416 | 0.506 | 0.286 | 0.714 | 0.325 | 0.408 | 1.690 | 0.821 |
|       | g.41769223 C > G | CC | 0.009 | 0.991 | 0.000 | 0.955 | 0.046 | 0.083 | 0.087 | 1.095 | 0.555 |
|       | g.41769002 A > G | AA | 0.078 | 0.416 | 0.506 | 0.286 | 0.714 | 0.325 | 0.408 | 1.690 | 0.821 |
|       | g.41768501 A > G | AA | 0.074 | 0.260 | 0.026 | 0.844 | 0.156 | 0.229 | 0.263 | 1.357 | 0.874 |
|       | g.41768485 G > A | GG | 0.074 | 0.260 | 0.026 | 0.844 | 0.156 | 0.229 | 0.263 | 1.357 | 0.874 |
| \(FecB\) | g.29315643 A > G | AA | 0.416 | 0.532 | 0.052 | 0.682 | 0.318 | 0.340 | 0.434 | 1.766 | 0.005 |

3.3. Association Analysis between Eight Loci in GDF9 and FecB with Litter Size in Luzhong Sheep

The results indicated that the g.41768501A > G and g.41768485 G > A loci were significantly associated with litter size (Table 4). For the two loci, the litter size of ewes with heterozygous genotype was the highest and significantly higher than that of ewes with the homozygous genotype mutation \((p < 0.05)\). For the FecB mutation, the litter sizes of ewes with the mutant genotypes were significantly higher than that of ewes with wild genotype \((p < 0.05, \text{Table 4})\). Litter size of Luzhong sheep was significantly influenced by parity \((p = 0.022)\). Additionally, there was no significant interaction effect between FecB and the two loci associated with litter size \((p = 0.057)\).
Table 4. Least-square means and standard errors of litter size for different genotypes of nine loci in Luzhong ewes. Note: Different letters (a, b) for the groups indicate differences (p < 0.05).

| Gene | Locus | Genotype | Litter Size (Mean ± SD) |
|------|-------|----------|------------------------|
| GDF9 | g.41769606 T > G | TT       | 1.612 ± 0.680          |
|      |       | GT       | 2.000 ± 0.000          |
|      |       | GG       | 1.612 ± 0.680          |
|      |       | AG       | 2.000 ± 0.000          |
|      | g.41769574 G > A | TT       | 1.614 ± 0.684          |
|      |       | CT       | 1.642 ± 0.621          |
|      |       | CC       | 1.614 ± 0.684          |
|      |       | CG       | 1.642 ± 0.621          |
|      | g.41769567 T > C | TT       | 1.614 ± 0.684          |
|      |       | CT       | 1.642 ± 0.621          |
|      |       | CC       | 1.614 ± 0.684          |
|      |       | GG       | 1.603 ± 0.688          |
|      |       | AG       | 1.609 ± 0.679          |
|      | g.41769223 C > G | TT       | 1.614 ± 0.684          |
|      |       | CT       | 1.642 ± 0.621          |
|      |       | CC       | 1.614 ± 0.684          |
|      |       | GG       | 1.603 ± 0.688          |
|      |       | AG       | 1.609 ± 0.679          |
|      | g.41769246 A > G | GG       | 1.603 ± 0.688          |
|      |       | AG       | 1.609 ± 0.679          |
|      |       | AA       | 1.750 ± 0.608          |
|      | g.41769002 A > G | GG       | 1.603 ± 0.688          |
|      |       | AG       | 1.609 ± 0.679          |
|      |       | AA       | 1.750 ± 0.608          |
|      | g.41768501 A > G | GG       | 1.000 ± 0.000          |
|      |       | AG       | 1.825 ± 0.708          |
|      |       | AA       | 1.564 ± 0.656          |
|      | g.41768485 G > A | AG       | 1.825 ± 0.708          |
|      |       | GG       | 1.000 ± 0.000          |
|      |       | AA       | 1.000 ± 0.000          |
|      | FecB  | g.29315643 A > G | AA       | 1.094 ± 0.342          |
|      |       | AG       | 1.988 ± 0.596          |
|      |       | GG       | 2.000 ± 0.730          |

3.4. Construction of Phylogenetic Tree of GDF9 Gene for 10 Sheep Breeds and Other Five Animal Species

A phylogenetic tree of GDF9 gene coding sequence for ten sheep breeds and five other animal species was constructed using the neighbor-joining (NJ) method, showing that four prolific sheep breeds (Cele Black, Small Tail Han, Hu, Luzhong) were most closely related (Figure 2). The sequences of 10 sheep breeds and goat are clustered together, and the sequences of the other species showed relatively large differences between them.

Figure 2. The phylogenetic tree of the GDF9 gene in different species. The upper values refer to branch lengths values and the lower values refer to the bootstrap values measured in the number of substitutions per site. Note: The pink circle refers to the four prolific sheep breeds (Cele Black, Small Tail Han, Hu, Luzhong). The orange circle refers to mono-lamb sheep breeds (Prairie Tibetan, Tan, Wuzhumuqin, Valley Tibetan, Bayinbuluke, Texel). The light blue circle refers to different species.
3.5. Prediction of the Protein Structure

For one missense mutation found in GDF9 of sheep, the secondary and tertiary protein structures before and after the mutation at g.41768485 G > A were predicted, respectively. The results showed that secondary structure did not change significantly after the mutation (Figure 3A,B). Although the overall spatial structure of the protein did not change, there were obvious differences in the structure of the region containing V332I amino-acid substitution after the mutation (Figure 3C,D). Moreover, the locus is located in the mature protein region (Figure 3E), which may cause some changes in the function of this secreted factor in follicles.

Figure 3. Secondary protein structure and tertiary protein structure for the GDF9 product before and after the mutation at g.41768485 G > A based on its amino acid sequence. (A) Secondary protein structure before the mutation; (B) Secondary protein structure after the mutation; (C) Tertiary protein structure before the mutation; (D) Tertiary protein structure after the mutation. (E) The mutation is located in the GDF9 mature protein region. Note: The red arrow refers to the g.41768485 G > A locus.

4. Discussion

Several major genes affecting sheep prolificacy were identified, including BMPR1B (FecB) [13–16], BMP15 (FecX) [27–29], GDF9 (FecG) [2,4,30], B4GALNT2 (FecL) [31,32], and Woodlands [33]. BMPR1B has an important influence on follicular development and maturation, and its mutation A746G, namely FecB, can change primary follicle development and ovulation in sheep [10], mice [34], and goats [35]. BMP15 and GDF9 are the ligands of BMPR1B. FecB may lead to a weakened response of ewe granulosa cells to BMP15 and GDF9, which may lead to a lower level of granulosa cell proliferation in smaller follicles, and form an earlier LH (luteinizing hormone) response ability (the granulosa cells of the first mature follicle increase the production of steroids and thus inhibit the pituitary gland’s secretion of FSH (follicle-stimulating hormone), with only the follicles that express the LH receptor continuing to develop). Because more follicles have the ability to synthesize LH receptors, even if the FSH concentration drops, more follicles continue to develop during the preovulation period, eventually resulting in the ewe being able to release more eggs in an estrus period [36]. Furthermore, BMP4 and GDF5 can be used as natural ligands of BMPR1B, and the FecB mutation weakens the inhibitory effect of its ligand on steroid production of granulocytes, therefore, granulocytes can further differentiate and promote
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...follicle maturation [37]. The attenuation of the BMPR1B signal caused by FecB mutation leads to an increase in the density of FSHR and LHR in granulosa cells, which can reduce apoptosis of the granulosa cells and increase the ovulation rate [10]. Moreover, changes in amino acid metabolism affected by the FecB genotypes relating to different rates of protein biosynthesis might affect the growth of the developing oocyte in follicular fluid [38]. The FecB mutation was detected to be significantly related to litter size in Hu sheep and Small-Tail Han sheep [13,39,40]. The present study found that the litter sizes of ewes with the mutation genotypes were significantly higher than those of ewes with the wild genotype. Therefore, FecB mutation could also be used as a molecular marker to improve litter size in Luzhong sheep breeding.

GDF9 plays an important role in controlling ovarian physiology and enhancing oocyte developmental competence [41]. The GDF9 mutation can affect the fertility and sterility by producing different effects on number of follicles and oocyte in ewes [1]. Previous studies showed that GDF9 mutants were highly associated with litter size in sheep, mainly including FecH, FecI, FecJ, and FecK [30,42–44]. Among them, FecH, FecI, and FecJ mutations increase the ovulation rate and FecK mutations have additive effects on ovulation and litter size. In some cases, ewes with the homozygous mutant of FecJ and FecK are infertile [30,43]. The FecK mutation can increase ovulation rate or litter size in heterozygote ewes and lead to infertility due to ovarian and uterine dysplasia in homozygote ewes. This infertility phenotype may be due to disruption of the PCSK (proprotein convertase subtilisin kexin) cleavage site of the GDF9 proprotein, preventing the conversion of GDF9 to dimeric, mature GDF9, which is the biologically active form of the protein [30]. For FecK, incomplete follicle development leads to infertility in homozygous ewes, despite apparently normal oocyte activation and expression of some oocyte-specific genes (including those involved in ZP (zona pellucida) formation) [43]. In addition, a new mutation FecA was detected in Araucana creole sheep, but this mutation was not significantly associated with litter size [8]. Another new mutation in exon 1 of the GDF9 gene was found in Egyptian sheep, which is related to litter size [45]. Previous studies indicated that mutations in the coding region of the GDF9 gene mainly exist in European sheep breeds [4,30,42], American sheep breeds [46], and a few Chinese indigenous sheep breeds. In the present study, eight mutations were detected in the GDF9 gene of Luzhong sheep, which were different from the FecI mutation found in African Rahmani sheep. Of them, one mutation (g.41769606 T > G) was novel and two tight linkage SNPs (g.41768501 A > G and g.41768485 G > A) were associated with litter size. The two loci g.41768501 A > G and g.41768485 G > A are similar to FecK and FecK, and the litter sizes of ewes with the heterozygous genotype are increased compared to homozygous individuals with mutated alleles. The mutations are located in the mature protein region of GDF9, which may affect the function and activity of the protein.

In order to further explore the changes in the structure of the protein after the mutation of g.41768485 G > A, the secondary structure and tertiary structure of the protein with and without the mutation were predicted, respectively. It was found that the region, including the mutation site, were always irregularly coiled before and after the mutation. However, there were some subtle differences in the three-dimensional structure. The slight changes in the three-dimensional structure may affect the binding affinity of GDF9 with its receptors, and further affect cumulus expansion and ovulation, which may have a final effect on ovulation rate or litter size in sheep; this needs further experimentation for verification.

BMP15, GDF9, and BMPR1B are located in the TGF-β pathway at the same time and can regulate cellular differentiation, follicular atresia, and oocyte maturation [47]. Specifically, BMP15 and GDF9 are secreted from oocytes and bind to the BMPR1B receptor on granulosa cells. So, it is possible that BMP15, GDF9, and BMPR1B have a synergistic effect on litter size in sheep. Demars et al. [27] found that BMP15 and GDF9 can form heterodimers, and the TGF-β pathway is inhibited after the two genes are mutated at the same time, eventually leading to an increase in ovulation as well. Moreover, GDF9 and BMP15 together can facilitate anti-Müllerian hormone expression, which is crucial for
ovarian function [48]. According to previous studies, the ewes carrying FecB and BMP15 mutants simultaneously showed higher litter sizes than individuals carrying the single mutant [13]. Hanrahan et al. [42] found that the ewes with mutants in both GDF9 and BMP15 had higher ovulation rates than those with any one mutation. However, little is known about the interactive effects of GDF9 and BMPR1B genes. It was speculated that GDF9 cannot bind to the BMPR1B receptor, while BMP15 likely binds to the BMPR1B receptor to transmit a signal through phosphorylation of SMAD2/3 [49], which may be consistent with the results regarding the absence of obvious interactive effect of GDF9 and BMPR1B genes on litter size in Luzhong mutton sheep.

Analysis of phylogenetic tree indicated that the genetic relationship of prolific sheep breeds is the closest and that they are clustered together. The reason for this is that their sequences are highly consistent, however, monotocous sheep breeds have one or more mutations in different loci compared with them. In this study, the evolutionary relationship of the GDF9 gene sequence among different species was similar to the previous results of Monestier et al. [50] and Ahmadi et al. [51].

5. Conclusions

The present study identified eight SNPs, including one novel mutation in the GDF9 gene of Luzhong sheep. Of them, g.41768501A > G and g.41768485 G > A were significantly associated with litter size in Luzhong sheep. The results preliminarily demonstrated that GDF9 is a major gene affecting the fecundity of Luzhong mutton sheep and the above two loci might be potential genetic markers for improving litter size.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2615/11/2/571/s1, File S1: The GDF9 gene coding sequence in Luzhong mutton sheep.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS) (Beijing, China) ((protocol code IAS2020-64 and data of approval 27 April 2020).

Data Availability Statement: The gene sequence data presented in this study are available in File S1.

Conflicts of Interest: All authors declare no conflicts of interest.

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