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Association of novel polymorphisms in follicle stimulating hormone beta (*FSHβ*) gene with litter size in Mehraban sheep

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Abstract Follicle-stimulating hormone (FSH) plays an important role in female reproduction by binding to a specific receptor (FSHR) through the β-subunit of FSH on the surface of the ovarian granulosa cells. This study aimed to characterize the FSH β-subunit gene (*FSHβ*) polymorphism and its association with litter size (LS) using a sample of 118 Mehraban sheep. Polymerase chain reaction (PCR) was performed to amplify fragments 300 bp and 431 bp of the ovine *FSHβ* gene (Oar_v4.0; Chr 15, NC_019472.2). Polymorphisms in the studied fragments were then explored using single strand conformational polymorphism (SSCP) and DNA sequencing methods. Seven single-nucleotide polymorphisms (SNPs), including g.59078564 C>G, g.59078624 T>C, g.59078655 T>C, g.59078691 T>C, g.59078754 C>A, g.59080186 G>C and g.59080365 C>T, were found among the six detected SSCP patterns A to F. Moreover, two novel indel polymorphisms called e.g., g.59078702del8-bp−ins64-bp and g.59078726ins54-bp were identified among the three different SSCP genotypes patterns G to I. We found significant differences in prolificacy categories between the SSCP genotypes patterns D, E and F ($P<0.01$) that simultaneously represented SNP polymorphisms of g.59078754 C>A, g.59080186 G>C and g.59080365 C>T. Similarly, novel indel polymorphisms revealed a significant difference in prolificacy categories between the SSCP genotypes patterns G, H and I ($P<0.05$). Our results suggested that *FSHβ is* a strong candidate gene to associate with the LS in sheep.

Keywords: *FSHβ* gene, litter size, Mehraban sheep, polymorphism, prolificacy

Introduction

Iran is home to more than 28 breeds of sheep, distributed in a wide range of climatic conditions. Mehraban sheep, a low-prolificacy breed, is one of the most important meat type breeds of economic importance, located in Hemedan province, in western Iran (Talebi et al., 2018). Mehraban sheep is well-adapted to the cold climate of Hamedan province (Ahmadi et al., 2021).

Litter size (LS) imposes the highest financial impact on the productive performance of sheep, and because of the increasing global demand for animal products, prolificacy is considered an important breeding objective (Gootwine, 2020). Due to sex-limited nature and low heritability of the reproductive traits in sheep, progress in genetic improvement, based on classical selection for quantitative polygenic traits, is slow (Janssens et al., 2004). Marker-assisted selection (MAS) for genetic improvement of the reproductive traits in sheep is a powerful scenario because MAS has the smallest founder allele representation among the favorable quantitative traits loci (QTL)

to avoid future inbreeding (Pedersen et al., 2009). Given the observed phenotypic variability, prolific sheep breeds have commonly been used to identify the prolificacy genes. Several major fecundity genes, such as *BMP15*, *BMPR1B*, *GDF9* and *B4GALNT2*, with numerous causative mutations and prolific alleles affecting the ovulation rate and litter size have been reported in various sheep populations (Abdoli et al., 2016; Majd et al., 2019; Nosrati et al., 2019; Vinet et al., 2012). Despite the efforts, none of the already known prolific alleles of the major fecundity genes such as *BMPRIB* (Abdoli et al., 2013; Talebi et al., 2018), *BMP15* (Zamani et al., 2015), *GDF9* (Abdoli et al., 2013; Ahmadi et al., 2016; Talebi et al., 2018), and *B4GALNT2* (Talebi et al., 2018) has been found in several Iranian native sheep. However, it must be emphasized that a constant effect of a gene or mutation on prolificacy is not expected for different breeds, because phenotypic expression of prolificacy, as a complex trait, is influenced by a large variety of biological mechanisms (Abdoli et al., 2018). Therefore, potential markers are still insufficient for genetic improvement of the litter size in Iranian sheep breeds.

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) control various stages of the ovarian cycle through their respective receptors on the granulosa cells of the ovarian follicles (Goodman and Inskeep, 2015). Among the four major genes associated with prolificacy in sheep, *BMPR1B* gene can lead to an increased density of the FSH and LH receptors with a concurrent reduction in apoptosis leading to increased ovulation rate in the ewe (Xu et al., 2018). FSH and LH are composed of α and β subunits and impose their biological effects via G protein–coupled receptors. Biological activities of FSH are manifested via binding with FSH beta subunit (FSHβ) (Trevisan et al., 2019). No mutation in α-subunit has been described yet, but polymorphisms in the *FSHβ* and *LHβ* subunits along with the mutations in the gonadotropin receptors are known as causes of delayed puberty and abnormal reproduction in males and females (de Kretser et al. 2016). Several studies have reported the relationship between polymorphisms in *FSHβ* gene and reproductive performance in women (Bianco et al., 2021; Rull et al., 2018; Trevisan et al., 2019), and with litter size in farm animals including the goat (An et al., 2010; Liang et al., 2006; Zhang et al., 2011), sheep (Gholiadeh and Najafi, 2017; Lameei et al., 2015; Nazifi et al., 2015), sow (Liu et al., 2009; Matoušek et al., 2005), and rabbit (Niu et al., 2019); however, there is less information about this gene in sheep compared with other species.

In view of the importance of the *FSHβ* gene, as a possible candidate gene associated with reproductive functions, the objective of the present study was to investigate the polymorphisms in *FSHβ* gene and their relation with reproduction in Mehraban sheep.

Materials and methods

Samples and DNA extraction

We compared three groups of Mehraban ewes with different reproductive histories, selected from three different flocks, in Hamedan County in Hamedan province, Iran. Ten ewes were barren in two consecutive lambing seasons (infertile group). Thirty-eight singleton lambing ewes were designated as the low prolificacy group, and 70 ewes, with litter sizes ≥ 2 as the high prolificacy group. Blood samples, collected from the jugular vein in tubes containing EDTA, were transferred to the Molecular Genetics Laboratory, Department of Animal Science, Bu-Ali Sina University, Hamedan, Iran. Genomic DNA was extracted from whole blood samples, based on the method described by Talebi et al. (Talebi et al., 2021).

PCR amplification of FSHβ gene for SSCP analysis

Two pairs of primers were designed using the Primer 3 (Ye et al., 2012), based on the published ovine FSHβ gene sequence (Oar_v4.0; Chr 15, NC_019472.2). The primers were designed for amplification of a 300 bp fragment in Exon2-partial Intron2 and a 431 bp fragment encompassing the Intron2-Exon3-partial Intron3 (see Table 1). PCR was programmed for initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 35-40 s (specific temperature shown in Table 1) and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 7 min.

SSCP analysis and polymorphism screening

PCR products (5 µL) were diluted with 10 µL of SSCP gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide, 20 mM EDTA), and after heating at 97 °C for 12 min, they were immediately placed on ice for 5 min. The mixture was electrophoresed for 24 h at 250 V and 5 °C. DNA visualization was obtained using the silver nitrate staining method (0.1%) for 30 minutes (Sanguinetti, 1994). To evaluate the accuracy of PCR-SSCP method and detection of the polymorphisms leading the each SSCP pattern, a total of five PCR products was randomly selected for each SSCP pattern and sent to Bioneer Co., Korea, for DNA sequencing. The primers used for sequencing were the same as those for the PCR reaction (Table 1). Sequenced reads were aligned against the ovine reference sequence (Oar_v4.0; NC_019472.2), using the CLC Main software Workbench 7.6.4 (www.clcbio.com) in order to identify polymorphisms.

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) was evaluated for the detected genotypes, using the POPGENE software version 1.31 (Yeh et al., 1999). Associations of the detected genotypes with litter size were evaluated using the Fisher's exact probability test. P values < 0.05

were considered as statistically significant levels. The GraphPad Prism version 9.2.0 (www.graphpad.com) was employed for the statistical analysis.

Table 1. Primer sequence, amplified region, product size and annealing temperature used in analyses of sheep FSHβ gene (Oar_v4.0; Chr 15, NC_019472.2)

[1] Primer sequence (5′ to 3′)	Amplified region	Product size (bp)	Annealing temperature (°C)
F: 5′-CTTGTTTGTTTCCCAGCCCA-3′ R: 5′-TGGCTAAAGGACTCATGGCT-3′	Exon2-partial Intron2	300	61
F: 5′-GTATTCAATCCCTGTCTCA-3′ R: 5′-GTAGGGTCTTCTGTGGTG-3′	Intron2-Exon3-partial Intron3	431	59

[1]Fwd and Rev : Forward and Reverse primers

Results and discussion

We selected the beta-subunit of the follicle stimulating hormone (*FSHβ*) gene as the candidate gene to scrutinize the effect of single-marker on litter size in Mehraban sheep. This gene is located on the ovine chromosome 15 and contains 3 exons and 2 introns (Hediger et al., 2009). The locus for the *FSHβ* gene including a 300 bp fragment located in the Exon2-partial Intron2 and a 431 bp fragment encompassing the Intron2-Exon3-partial Intron3, were successfully amplified by the designed primers from the ovine genomic DNA (see Figure 1 A and B).

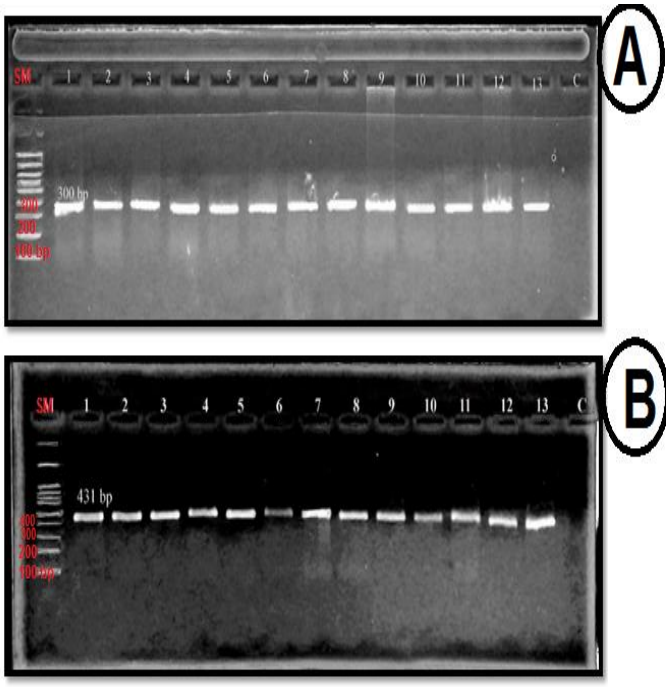

Figure 1. Amplified fragments of sheep FSHβ gene (Oar_v4.0; Chr 15, NC_019472.2). A. 300 bp fragment located in the Exon2-partial Intron2. B. 431 bp fragment encompassing the Intron2-Exon3-partial Intron3. The lanes of 1-13 are 13 samples that were amplified by PCR. Lane C is the negative control, a sample without gDNA. MW: DNA molecular weight marker, informative size (bp) colored in red.

In both amplicons, nine distinct SSCP patterns, called A to I were identified with three and six electrophoretic patterns related to the 300 and 431 bp segments, respectively (Figure 2). The largest proportion of prolificacy class (high prolificacy ewes, low prolificacy ewes and infertile ewes) belonged to pattern G (Figure 3). A study carried out by Nazifi et al. (2015) on exon 2 of *FSHβ* gene in three indigenous sheep breeds of Iran showed distinct SSCP patterns, AA and AC genotypes in Baluchi breed, AA and AB genotypes in Iran black breed, and AA homozygote genotype in Arman breed (Nazifi et al., 2015). In Baluchi sheep, a small breed of sheep in Iran, seven SSCP patterns at exon 1 of the *FSHβ* gene were identified, while no polymorphism were found for exon 3 (Gholiadeh and Najafi, 2017). It is unlike the results of the SSCP patterns in exon 3 of Mehraban sheep (Figure 2).

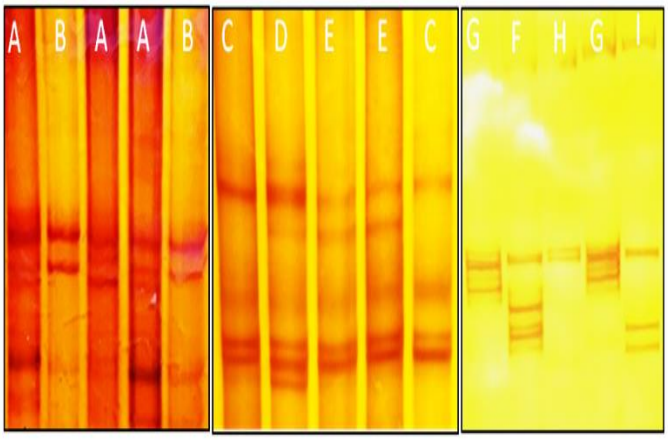

Figure 2. SSCP distinct patterns on the 10% acrylamide gel. Nine different SSCP patterns (A to I) for sheep FSHβ gene (Oar_v4.0; Chr 15, NC_019472.2) were identified in Mehraban breed.

Sequencing of the amplified fragments showed seven SNPs, including g.59078564 C>G, g.5907864

T>C, g.59078655 T>C, g.59078691 T>C, g.59078754 C>A, g.59080186 G>C and g.59080365 C>T, in the six electrophoretic patterns (Table 2 and Figures 4 and 5). The SSCP pattern A corresponded to four SNPs compared to the homozygous reference sequence (g.59078564 CC, g.59078624 TT, g.59078655 TT, g.59078691 TT), but the SSCP patterns B and C had heterozygous (g.59078564 CG, g.59078624 TC, g.59078655 TC, g.59078691 TC) and homozygous variants (g.59078564 GG, g.59078624 CC, g.59078655 CC, g.59078691 CC), respectively (Figure 4).

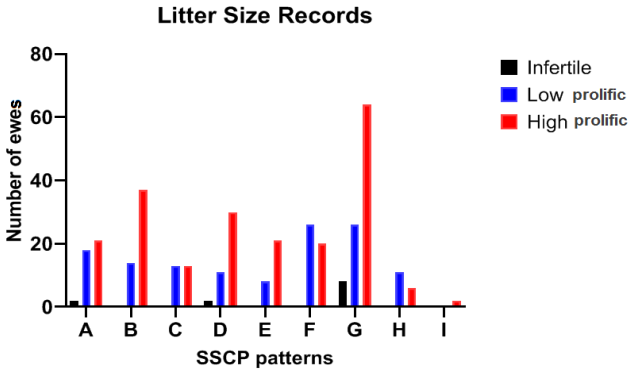

Figure 3. A schematic representation of SSCP patterns A to I among three prolificacy categories in Mehraban ewes. The proportion of ewes in three categories of prolificacy including high prolificacy, low prolificacy, and infertile is shown in red, blue and black colors, respectively.

Table 2. Identified SNPs in the ovine *FSHβ* gene (Oar_v4.0; Chr 15, NC_019472.2) in Mehraban sheep

Polymorphism ID	SNP gene location	SNP alleles	Protein position/substitution
rs423760863	Exon2	g.59078564 C>G	p.29 T>T
rs402353127	Exon2	g.59078624 T>C	p.49 Y>Y
rs417578162	Intron2	g.59078655 T>C	Non-coding
rs428750957	Intron2	g.59078691 T>C	Non-coding
Deletion/Insertion	Intron2	g.59078702del8-bp−ins64-bp	Non-coding
Insertion	Intron2	g.59078726ins54-bp	Non-coding
g.59078754 C>A	Intron2	g.59078754 C>A	Non-coding
rs1093551424	Intron2	g.59080186 G>C	Non-coding
g.59080365 C>T	Exon3	g.59080365 C>T	p.107R>R

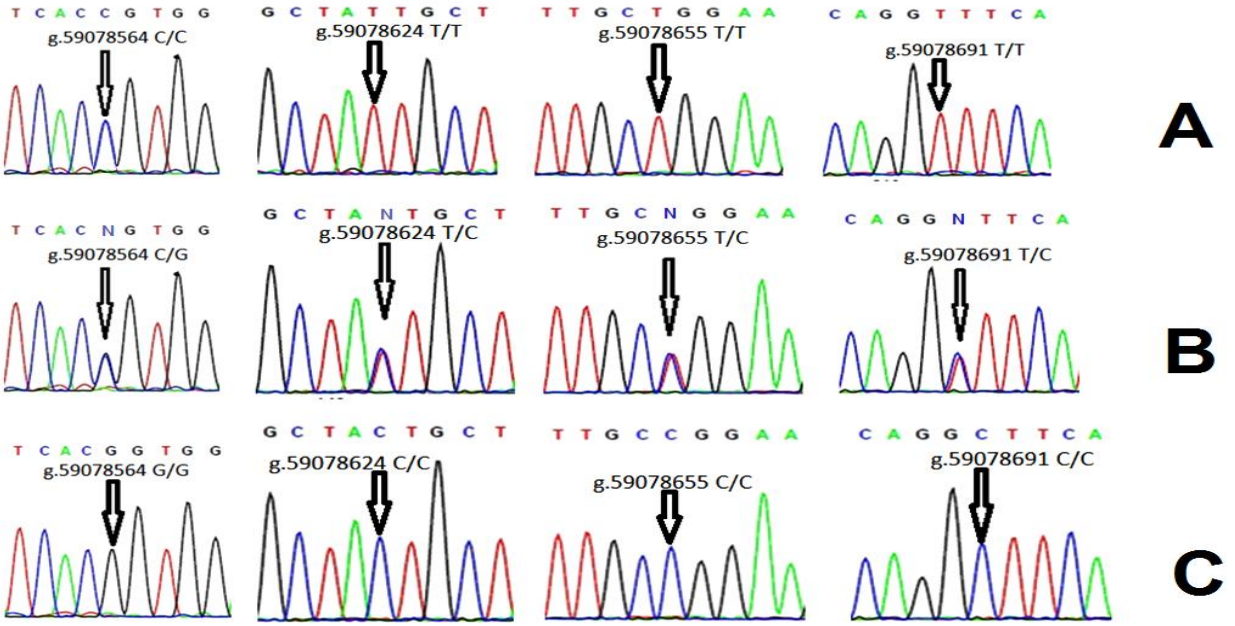

Figure 4. Single nucleotide polymorphisms detected at amplified fragment of Exon2-partial Intron2 in ovine *FSHβ* gene (Oar_v4.0; Chr 15, NC_019472.2). Identified SNPs in pattern A (homozygous reference; g.59078564 CC, g.59078624 TT, g.59078655 TT, g.59078691 TT), pattern B (heterozygous; g.59078564 CG, g.59078624 TC, g.59078655 TC, g.59078691 TC) and pattern C (homozygous variant; g.59078564 GG, g.59078624 CC, g.59078655 CC, g.59078691 CC).

For the three remaining SNPs, the SSCP pattern D had three SNPs compared to the homozygous reference form (g.59078754 CC, g.59080186 GG and g.59080365 CC), while the SSCP patterns E and F were representative of the heterozygous (g.59078754 CA, g.59080186 GC and g.59080365 CT) and homozygous variants (g.59078754 AA, g.59080186 CC and g.59080365 TT), respectively (Figure 5). The SNP polymorphisms of g.59078564 C>G, g.59078624 T>C, and g.59078655 T>C were previously identified on exon 1 of FSHβ gene among three SSCP patterns A to E in Baluchi Sheep (Gholiadeh and Najafi, 2017). In the present study, two novel SNPs g.59078754 C>A and g.59080365 C>T were respectively identified in intron 2 and exon 3 of the ovine FSHβ gene (Figure 4). However, these SNPs were silent polymorphisms at the protein sequence level as indicated in Table 2.

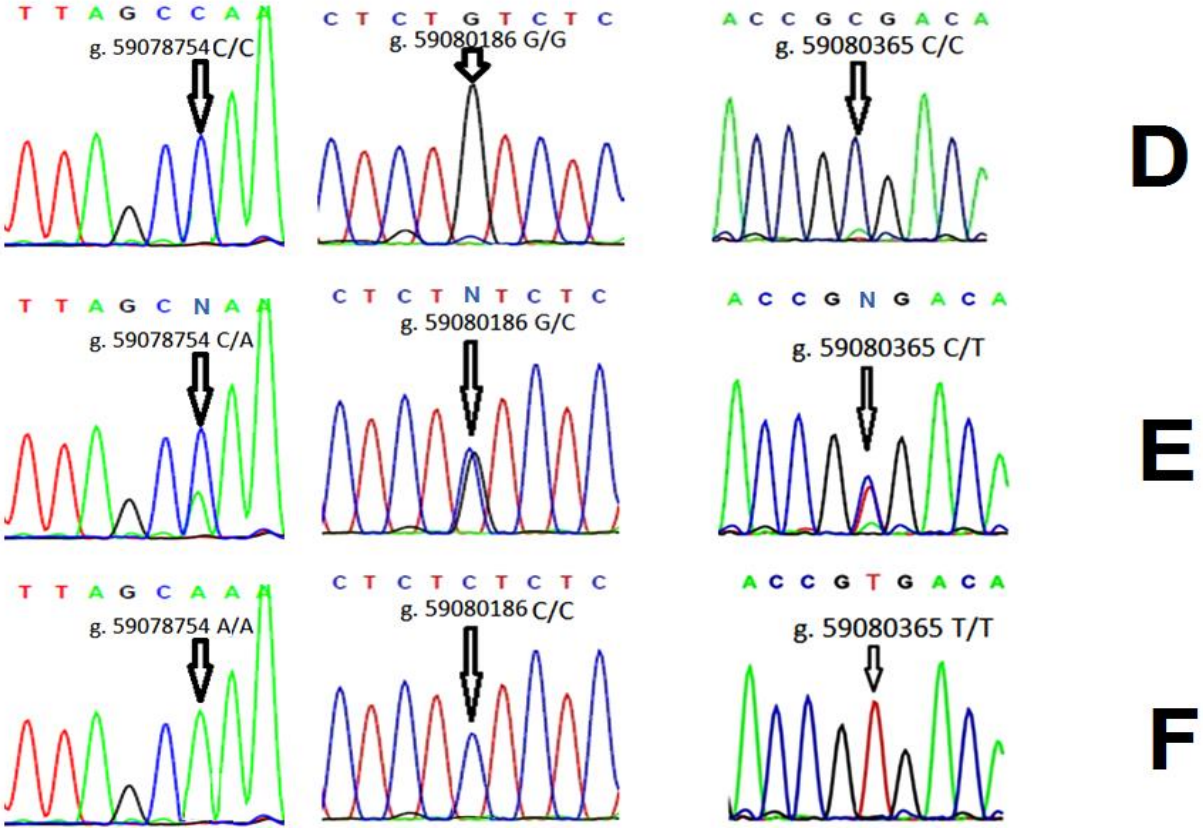

Figure 5. Single nucleotide polymorphisms detected at amplified fragment of Intron2-Exon3-partial Intron3 in ovine *FSHβ* gene (Oar_v4.0; Chr 15, NC_019472.2). Identified SNPs in pattern D (homozygous reference; g.59078754 CC, g.59080186 GG and g.59080365 CC), pattern E (heterozygous; g.59078754 CA, g.59080186 GC and g.59080365 CT) and pattern F (homozygous variant; g.59078754 AA, g.59080186 CC and g.59080365 TT).

A novel insertion/deletion (indel) polymorphism as the simultaneous state for the 8-bp deletion and 64-bp insertion in position 59078702bp, called g.59078702del8-bp−ins64-bp and a novel 54-bp insertion polymorphism called g.59078726ins54-bp were identified among the three different SSCP genotype patterns G to I (Table 2 and Figure 6). As illustrated in Figure 6, patterns G to I corresponded to the insertion/deletion (indel) polymorphisms in homozygous reference form (pattern G, not shown), heterozygous (pattern H, simultaneous state for the 8-bp deletion and 64-bp insertion in position 59078702bp, called g.59078702del8-bp−ins64-bp) and homozygous variant (54-bp insertion in position 59078726bp, called g.59078726ins54-bp).

The electrophoretic patterns D, E and F that corresponded to novel SNPs (g.59078754 C>A and g.59080365 C>T) revealed significant deviation from the Hardy-Weinberg equilibrium ($P<0.01$, Table 3). This observation is probably due to the previous selection programs on reproductive traits, and some possible genetic stratifications (Niu et al., 2019). Accordingly, the highest and lowest genotype frequencies belonged to the pattern G (~ 83%) and pattern I (~ 2%), respectively (Table 3).

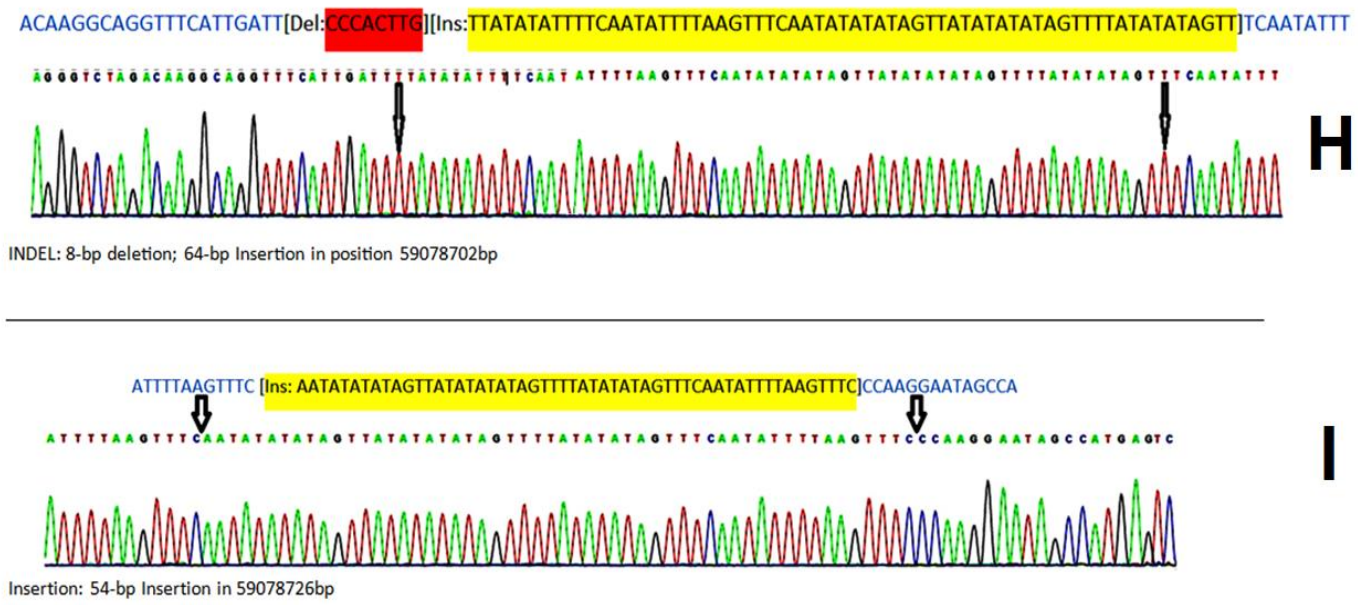

Figure 6. Insertion/deletion (indel) polymorphisms detected at intron 2 of the amplified fragment of Intron2-Exon3-partial Intron3 in ovine *FSHβ* gene (Oar_v4.0; Chr 15, NC_019472.2). Identified indel polymorphisms in pattern G (homozygous reference; not shown), pattern H, (heterozygous; g.59078702del8-bp−ins64-bp) and pattern I (homozygous variant; g.59078726ins54-bp).

Table 3. Percentage of genotypic frequencies of identified SSCP variants among amplified fragments of sheep *FSHβ* gene (Oar_v4.0; Chr 15, NC_019472.2)

Locus of *FSHβ*	SSCP pattern	Numbers	Frequencies	Chi-Squared values	P-value
Exon2-partial Intron2	A	41	34.75	1.74	0.19
	B	51	43.22		
	C	26	22.03		
Intron2-Exon3-partial Intron3	D	43	36.44	30.47	0.00
	E	29	24.58		
	F	46	38.98		
	G	98	83.05		
	H	17	14.41	1.43	0.23
	I	2	1.69		

The association study between the genotypes and litter size was done based on differential analysis using the Fisher's exact probability test on prolificacy categories (Table 4). We found significant differences in prolificacy categories between SSCP genotypes patterns D, E and F (P<0.01) that simultaneously represented the SNP polymorphisms of g.59078754 C>A, g.59080186 G>C and g.59080365 C>T (Table 4). Prolificacy categories indicated a significantly higher frequency of SSCP genotypes patterns D (g.59078754 CC, g.59080186 GG and g.59080365 CC) and E (g.59078754 CA, g.59080186 GC and g.59080365 CT) in high prolificacy than in low prolificacy ewes (P<0.01, Table 4). These mutations are silent at the protein level, but could be in strong linkage disequilibrium with a causal mutation nearby (Talebi et al., 2018). The SNP g.59078624 T>C in exon 2 of FSHβ gene already being referenced in the dbSNP database (rs402353127) did not exhibit significant association with the litter size in Mehraban sheep in accordance with the results obtained in Iran-black sheep (Nazifi et al., 2015). This composite breed has been synthesized through the first cross of Chios ra-

ms with Iranian Baluchi ewes (Mokhtari et al., 2014). However, Gholiadeh and Najafi (2017) showed significant association (P<0.05) of silent polymorphisms in exon 1 with litter size for parity 2 of Baluchi ewes in a way that pattern E had the most phenotype compared with pattern A as reference while the less phenotype was detected for pattern B (Gholiadeh and Najafi, 2017).

Table 4. Differential analysis of the identified genotypes among amplified fragments of the *FSHβ* gene (Oar_v4.0; Chr 15, NC_019472.2) based on prolificacy categories in Mehraban sheep

Locus of *FSHβ*	Genotype patterns	Prolificacy categories: Infertile (LS = 0)	Low prolificacy (LS = 1)	High prolificacy (LS ≥ 2)	
Exon2-partial Intron2	A	2	18	21	Chi-Squared = 8.89 P-value = 0.064 ns
	B	0	14	37	
	C	0	13	13	
Intron2-Exon3-partial Intron3	D	2	11	30	Chi-Squared = 13.70 P-value = 0.008 **
	E	0	8	21	
	F	0	26	20	
	G	8	26	64	
	H	0	11	6	Chi-Squared =11.48 P-value = 0.022 *
	I	0	0	2	

An et al. (2010) reported one polymorphic locus occurring in exon 2 of *FSHβ* gene with three genotypes (EE, EF and FF) in Xinong Saanen and Boer goats where the EE genotype had significantly higher litter size than EF and FF genotype. They observed the heredity of litter size was mainly influenced by genetic additive effect, and the EE genotype was a favorable marker genotype for litter size in goats. Furthermore, a mutation (A2645G) in exon3 of *FSHβ* in goats which changed glutamine (Glu) to arginine (Arg) was significantly associated with the litter size in Boer ewes (Zhang et al., 2011) and with libido and semen quality traits in Boer male goats (Nikbin et al., 2018). For Rex rabbits, Niu et al. (2019) reported g.284 G > T mutation at exon 1 *FSHβ* gene and g.2963 G > A mutation at exon 3 *FSHβ* gene were both associated with reproductive traits e.g., total number of born kits and number of alive born kits (Niu et al., 2019).

We identified two novel indel polymorphisms in *FSHβ* gene intron 2 locus in Mehraban sheep (Table 2), called g.59078702del8-bp−ins64-bp (shown in SSCP pattern H, Figure 6) and g.59078726ins54-bp (shown in SSCP pattern I, Figure 6). In our study, homozygous ewes with reference alleles (SSCP pattern G) and homozygous variant ewes (SSCP pattern I) had significantly (P=0.02) higher LS in high prolificacy ewes compared to the low prolificacy ewes (Table 4). Similarly, a single cytosine insertion in *FSHβ* gene intron 2 locus (g.110insACT, GenBank No: S. 64745.1) suggested an association with the higher litter size in Naeinian and Baluchi sheep breeds (Gholiadeh and Najafi, 2017; Lameei et al., 2015). Thus, the novel indel polymorphisms of *FSHβ* gene e.g., g.59078702del8-bp−ins64-bp and g.59078726ins54-bp that revealed a significant difference on prolificacy categories (Table 4), could be possible DNA markers in controlling prolificacy in Iranian indigenous sheep breeds. In our previous studies, an extra ovulation increased per estrus for a novel allele at exon 7 *BMPR1B* gene (Talebi et al., 2018) and also a novel allele at exon 4 *KISS1R*/*GPR54* gene was considered to be a potential causal mutation affecting the litter size and body weight traits in Mehraban sheep (Majd et al., 2019). However, there were no evidence for the presence of relationship between *FSHβ* gene and body weight traits in Iran-black, Arman and Baluchi sheep (Nazifi et al., 2015).

As a final remark, we would like to highlight an abundance of GWAS peaks for litter size in sheep that have been mapped on chromosome 15 in the vicinity of *FSHβ* gene. This locus had the strongest effect with a difference of ~1 lamb between the alternative homozygotes in Baluchi sheep (Gholizadeh et al., 2014). In addition to the associations with litter size in sheep that were discussed above, the *FSHβ* region also harbors GWAS hits for the reproductive functions in women e.g., menstrual cycle length, and with gynecological diseases such as polycystic ovary syndrome, and endometriosis (Laisk et al., 2018). Studies revealed that the T-allele of the *FSHβ* gene −211 G>T in women was associated with significantly higher serum levels of FSH and LH (Bianco et al., 2021; Rull et al., 2018). However, the *FSHβ* gene −211G>T polymorphism in men resulted in significant reductions in FSH level, FSH:LH ratio, testicular volume and sperm count (Mitchell, 2012).

In conclusion, we identified seven SNPs and two indel polymorphisms of ovine *FSHβ* gene based on the observed SSCP patterns in Mehraban sheep. Two novel SNPs e.g., g.59078754 C>A and g.59080365 C>T, revealed interesting deviations from the Hardy-Weinberg equilibrium, and were significantly related to LS in Mehraban sheep. Moreover, we discovered two large in-

del polymorphisms related to LS, including g.59078702del8-bp−ins64-bp and g.59078726ins54-bp, which do not affect the Hardy-Weinberg equilibrium. Our results indicated that ovine *FSHβ* gene may be a promising candidate for developing marker-assisted selection to genetically improve the prolificacy in sheep. However, further association analyses using a larger number of individuals and more extensive records and in the genome-wide scale are required to verify the results and contribute to detecting potential genetic markers influencing LS in Mehraban sheep.

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Conflict of interest statement

The authors declare that they do not have any conflict of interest.

Author's contributions

AA and PZ conceived and designed the study. ZM collected the blood samples, prepared the experimental records and the laboratory results. ZM and RT performed the polymorphisms detection analyses. AA and RT performed data analysis, interpretation and manuscript writing. RT and MRG edited the manuscript. All authors approved the manuscript before submission.

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