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1 **Polymorphism identification in ovine *KISS1R/GPR54* gene**
2 **among pure and crossbreeds of Iranian sheep**

3
4 Sadreddin Anvari Majd ^a, Ahmad Ahmadi ^{a,*}, Reza Talebi ^a, Pezhman
5 Mahmoodi Koochi ^b, Stéphane Fabre ^c, Saber Qanbari ^{d,e}

6
7 ^a *Department of Animal Sciences, Faculty of Agriculture, Bu-Ali Sina*
8 *University, Hamedan, Iran*

9 ^b *Department of Pathobiology, Faculty of Para Veterinary Science, Bu-Ali*
10 *Sina University, Hamedan, Iran.*

11 ^c *GenPhySE, Université de Toulouse, INRA, ENVT, Castanet Tolosan, France.*

12 ^d *Animal Breeding and Genetics Group, Department of Animal Sciences,*
13 *University of Goettingen, Germany.*

14 ^e *Department of Animal Biotechnology, Agricultural Biotechnology Research*
15 *Institute of Iran (ABRII), Agricultural Research, Education and Extension*
16 *Organization (AREEO), Karaj, Iran.*

17
18 * Corresponding author's address: Department of Animal Sciences, Faculty of
19 Agriculture, Bu-Ali Sina University, Hamedan, Iran.

20 Email address: ahmadi@basu.ac.ir (Ahmad Ahmadi);

21 Postal address: Postal Code: 65178 33131; Tel: +98 81 3442 5401; Fax: +98
22 81 3442 5403.

23 **ABSTRACT**

24 Fertility traits have the greatest financial impact on sheep production. In this
25 study we aimed to characterize polymorphisms of the KISS1 receptor gene
26 (*KISS1R*), also known as the G-protein-coupled receptor 54 gene (*GPR54*) that
27 is reported to be involved in the control of puberty and reproductive function.
28 Genomic DNA were obtained from 156 ewes of pure Mehraban and Shal
29 Iranian native sheep and their crossbreeds with Romanov. The exploration of
30 polymorphisms of the *KISS1R/GPR54* gene (GenBank No: HM135393.1) was
31 performed by single-strand conformation polymorphism analysis (SSCP) and
32 Sanger sequencing. Seven single-nucleotide polymorphisms (SNPs) including
33 g.396T>G, g.456T>C, g.475C>A, g.571A>C, g.3431C>A, g.4108G>A and
34 g.4123C>A, were observed in the four breeds. Among these SNPs the
35 g.3431C>A in the exon 4 was the only amino acid altering variant (p.195
36 Phe>Leu). Subsequent statistical analysis revealed that the minor A allele at
37 this position had a significant ($P < 0.01$) negative effect on litter size (LS) and
38 birth weight (BW) and could be considered as a causal mutation impairing
39 these traits. No significant ($P > 0.05$) allelic association with the studied traits
40 was found at the position g.396T>G, g.456T>C, g.475C>A and g.571A>C. In
41 contrast, carrier ewes of the SSCP pattern F (homozygous reference; g.4108
42 G/G, g.4123 C/C) showed a significantly ($P < 0.01$) higher LS than ewes
43 carrying the patterns G (heterozygous; g.4108 G/A, 4123 C/A) or E
44 (homozygous variant; g.4108 A/A, g.4123 C/C). The results of the present
45 study provide additional evidences on the potential role of the *KISS1R/GPR54*
46 gene in controlling reproductive traits and particularly prolificacy in sheep.

47 **Keywords:** Causal mutation, Polymorphism, Prolificacy

48

49

50 **1. Introduction**

51 The traits with the greatest financial impact on sheep production are
52 ovulation rate (OR) and litter size (LS) (Notter, 2008). However, a classical
53 selection based on polygenic quantitative approach for improving reproductive
54 traits in sheep is difficult due to the sex-limited nature and low heritability of
55 these traits (Janssens et al., 2004). Therefore, breeding strategies based on
56 genetic information obtained from molecular techniques and marker-assisted
57 selection (MAS) have a high importance for improving reproductive
58 efficiency (El-Tarabany et al., 2017). Genetic studies in sheep have indicated
59 that OR and LS can be genetically regulated either by many loci, each having
60 a minor effect, or alternatively by the action of single genes with major effect
61 (Drouilhet et al., 2009). Several major genes controlling OR and LS, known as
62 *BMP15*, *BMPIB*, *GDF9* and *B4GALNT2* and so-called fecundity genes, have
63 been discovered in various ovine populations so far, with numerous causative
64 mutations identified (Vinet et al., 2012, Abdoli et al., 2016). When
65 heterozygous, all these mutations increase OR and LS, but depending on the
66 mutations and at the homozygous state, their effects may be opposite, from
67 hyper-prolificacy to sterility. Moreover, particular inheritance patterns are
68 observed for mutations carried by the X-linked *BMP15* gene compared to
69 mutations in the three other autosomal genes.

70 A further panel of loci, putatively implicated in reproduction physiology
71 has been suggested as candidate fecundity genes. Among them, the kisspeptin
72 G-protein coupled receptor (*KISS1R/GPR54*) is a major upstream regulator of
73 neurons secreting gonadotropin releasing hormone (GnRH) and thus affects
74 prolificacy (Navarro and Tena-Sempere, 2012). Kisspeptins are extremely
75 intense elicitors of luteinizing hormone (LH) and follicle-stimulating hormone
76 (FSH) secretion in various mammalian species (retrieved from El-Tarabany et
77 al., 2017). This pathway has been considered as a key gatekeeper of pubertal
78 development and reproductive function in mammals (Popa et al., 2005).
79 Moreover, polymorphisms of *KISS1R/GPR54* gene are reported in association
80 with precocious or late sexual maturity traits in Chinese (Feng et al., 2009;
81 Cao et al., 2011) and Indian goat breeds (Ahlawat et al., 2015). Two studies
82 have also reported preliminary association between different alleles of *GPR54*
83 gene and high LS in prolific Chinese Hu and Small Tail Han sheep breeds
84 (Chu et al., 2012; Tang et al., 2012).

85 As of 2008, Iran had the fifth largest sheep population in the world, with
86 about 44 million animals (FAO Live Animals database,
87 <http://www.fao.org/faostat/en/#data/QA>, Jowkar et al., 2016). More than
88 twenty-seven distinctive breeds of sheep are distributed in a wide variety of
89 habitats in Iran. They vary in their genetic potential for production traits
90 including milk, meat, wool, disease resistance and fecundity. Although these
91 breeds possess different performances regarding the fecundity traits, their
92 overall performances are low (Tavakolian, 2000). Despite, many studies
93 conducted on known fecundity genes in different breeds of Iranian native

94 sheep (e.g., Abdoli et al., 2013; Zamani et al., 2015a,b; Ahmadi et al., 2016;
95 Nadri et al., 2016; Eghbalsaied et al., 2017; Talebi et al., 2018), no already
96 known prolific allele with a major effect has been reported. In a view of the
97 importance of the *KISSIR/GPR54* gene as a potential candidate gene for
98 controlling reproductive traits in sheep, the objectives of this study were 1) to
99 characterize the polymorphisms of *GPR54* gene in indigenous breeds of
100 Iranian sheep and 2) to evaluate the association of observed polymorphism
101 with the reproductive traits.

102

103 **2. Materials and methods**

104 *2.1. Animals, records and DNA isolation*

105 A total of 156 Iranian sheep blood samples (5 ml per ewe) were collected
106 from Mehraban (N=80), Shal (N=25) and their crosses with the Romanov
107 sheep, including Mehraban × Romanov (N=21), and Shal × Romanov (N=30).
108 Mehraban is a meat-type breed, predominant in western province Hamadan
109 with > 3 million heads of sheep. Mehraban samples were collected from the
110 research farm of Bu-Ali Sina University in Dastjerd. Shal is a dual-purpose
111 breed originated from central Qazvin province with an estimated population
112 size of ~200 thousand heads. Shal samples were collected from a personal
113 farm of Misagh in Famenin, capital of Famenin County in Hamadan province.
114 Romanov is a prolific breed of sheep from the Soviet Union that years ago has
115 been imported from Russia to Iran for commercial purposes. The Mehraban ×
116 Romanov and Shal × Romanov crossbreds were sampled from private flocks

117 respectively, located at Arak and Khomeyn Counties in Markazi province of
118 Iran.

119 Mehraban was the only group of the collected samples with LS (from 1 to 5
120 LS record/ewe) and lambs' birth weight (BW) records. LS was defined as the
121 number of lambs born alive per lambing and was considered as the prolificacy
122 trait of dams. These records were obtained between 2008 and 2016.
123 Accordingly, the information of maximum litter size (MaxLS) and average
124 litter size (ALS) have been recorded for each Mehraban ewe.

125 The blood sampling procedure was in accordance with the ethical
126 protocol of the Bu-Ali Sina University of Hamedan, Iran. Once collected,
127 blood samples were delivered back to the laboratory in an ice box at 4°C. The
128 genomic DNA was extracted from whole blood using the SinaClon kit (Sina
129 Clon Co, Iran) and stored at -20°C.

130

131 2.2. Designing PCR primers

132 The Primer 3 online program (Rozen and Skaletsky, 2000) was used to
133 design PCR primers based on the ovine *GPR54* gene sequence (GenBank No:
134 HM135393.1). Five pairs of primers were designed (Table 1) for the
135 amplification of a 923 bp fragment located in the proximal 5' UTR part of the
136 first exon, a 850 bp fragment encompassing the coding part of the first exon
137 and a part of the first intron, a 925 bp fragment with the exon 2, intron 2 and
138 exon 3, a 321 bp fragment encompassing the exon 4, and finally a 257 bp
139 fragment corresponding to the terminal part of the coding region of the exon 5.

140

141 *2.3. PCR amplification*

142 Polymerase Chain Reaction (PCR) was carried out in 20 µl reaction
143 volumes with 50 ng genomic DNA by mixing 10 µl of Taq DNA Polymerase
144 Master Mix (cat no. A140303) made in AMPLIQON manufacturer in
145 Denmark, 1 µl of genomic DNA, each of forward and reverse primers, 1 µl (10
146 pmoles/µl) and 7 µl distilled water. PCR was programmed as follows: initial
147 denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95
148 °C for 1 min, annealing for 45 s (specific temperature shown in Table 1) and
149 extension at 72 °C for 1 min. A final extension was performed at 72 °C for 10
150 min. The PCR products were controlled by electrophoresis on 0.7% agarose,
151 visualized by ethidium bromide and photographed under UV light using a
152 BTS-20.M gel documentation system (UVItec Ltd, UK).

153

154 *2.4. Polymorphism screening by PCR-SSCP*

155 Single-strand conformation polymorphism (SSCP) assay was performed
156 on all samples as previously described (Abdoli et al., 2013). Four µl of each
157 PCR product were mixed with 7 µL of SSCP gel loading dye (0.05%
158 bromophenol blue, 0.05% xylene cyanol, 95% formamide, 20 mM EDTA).
159 After heat denaturation at 98 °C for 10 min, the samples were immediately
160 chilled on ice to prevent hetero duplex formation and then run (22 h, 300 V, 5
161 °C) on 8% acrylamide:bis-acrylamide (29:1) gel, in 1x TBE buffer on a 21×22
162 cm gel casting vertical electrophoresis (Payapajooesh Pars, Iran). DNA
163 visualization was obtained by silver staining (Sanguinetti et al., 1994).

164

165 2.5. Validation of the PCR-SSCP assay

166 To evaluate the accuracy of the PCR-SSCP assay and to identify the
167 polymorphisms leading the each SSCP pattern, selected PCR amplicons (n = 5
168 for each SSCP pattern) were randomly examined by DNA Sanger sequencing.
169 The primers used for sequencing were the same as those for the PCR reaction
170 as illustrated in Table 1. The PCR products were sequenced by Bioneer Co.,
171 Korea. Sequenced reads were aligned against the sheep *KISS1R/GPR54* gene
172 (GenBank No: HM135393.1) using CLC Main Workbench Version 7.6.4
173 (www.clcbio.com) in order to assess for polymorphisms. Ultimately, potential
174 effect of the missense mutations, in terms of the structure and function of the
175 KISS1R protein, was predicted using PolyPhen-2 online software tool
176 (Adzhubei et al., 2010).

177

178 2.6. Statistical analysis

179 Genotypic frequencies of the four groups of animals were tested for
180 Hardy-Weinberg equilibrium (HWE) using the POPGENE software (Version
181 1.31; Yeh et al., 1999). Association analysis of the PCR-SSCP genotypes with
182 the reproductive traits was only evaluated in Mehraban sheep. The statistical
183 models used for analysis of four traits (LS, MaxLS, ALS and BW) were as
184 follows for *LS*, *MaxLS*, *ALS*: $Y_{ijmn} = \mu + Year_i + Npart_j + G_m + e_{ijmn}$; and

185 *BW*: $y_{ijklmn} = \mu + Year_i + LS_j + Nlamb_k + Sex_l + G_m + e_{ijklmn}$

186 In these models, y_{ijmn} or y_{ijklmn} are the phenotypic values of the studied traits; μ
187 is the overall mean; $Year_i$ is the fixed effect of the i th year (nine years from
188 2008 to 2016) of the ewe parturition; $Npart_j$ is the fixed effect of the j th

189 number of parturition (1, 2, 3 or 4); G_m is the fixed effect of the m th genotype
190 (patterns of A, B, C, D, E, F, G); LS_j is the fixed effect of the j th litter size (1,
191 2 or 3); $Nlamb_k$ is the fixed effect of the k th number of lamb born in all
192 parturition (1, 2 or 3); Sex_l is the fixed effect of the l th sex of lamb (male or
193 female); e_{ijmn} and e_{ijklmn} are random residual effects. While we admit that the
194 animals were sampled from different flocks, we were not able to include the
195 fixed effect of flock in the model due to limited records and the structure of
196 data. The GLM procedure of SAS software (SAS Institute, 2004) was used for
197 general linear model analysis and comparison of means.

198

199 **3. Results**

200 *3.1. PCR amplification and SSCP analysis*

201 Agarose gel analyses of the PCR products have confirmed the size of the
202 five expected fragments (923 bp, 850 bp, 925 bp, 321 bp and 257 bp)
203 amplified from the ovine *KISSIR/GPR54* gene (Fig. 1).

204 The SSCP analysis of these fragments obtained from the four breeds
205 revealed that only 3 of them were polymorphic. These corresponded to the 5'
206 UTR, exon 4 and exon 5 of *KISSIR/GPR54* (Fig. 2). We have observed two
207 different SSCP patterns, called pattern A and B (Fig. 2A), for the 5' UTR
208 fragment for the Mehraban, Mehraban \times Romanov and Shal \times Romanov
209 breeds, the Shal breed being not polymorphic at this location. Two SSCP
210 patterns (C and D, Fig. 2B) were also observed for the exon 4. In contrast,
211 three SSCP patterns (E, F and G, Fig. 2C) were observed for the exon 5.

212 Interestingly, all the SSCP patterns from C to G were present in the all four
213 breeds studied.

214 In order to identify the polymorphisms that cause SSCP patterns, five random
215 PCR samples from each SSCP pattern were sequenced. As shown in Fig. 3
216 and Table 2, the difference between SSCP patterns A and B was due to four
217 SNPs located within the 5' UTR fragment, named g.396T>G, g.456T>C,
218 g.475C>A, and g.571A>C according to the 4263 bp sequence of the
219 *KISS1R/GPR54* gene (GenBank No: HM135393.1). Based on the sequencing
220 result, the SSCP pattern A corresponded to the homozygous reference form,
221 while the SSCP pattern B was representative of the heterozygous form. From
222 the five PCR products sequenced for pattern B, the all four SNPs were always
223 heterozygous signing a possible haplotype. Within the exon 4, only one SNP
224 was detected at position g.3431C>A. This SNP causes a non-conservative
225 change in the protein sequence at the amino acid position 195 (p.195 Phe>Leu,
226 Table 2). Interestingly, this change was predicted as probably deleterious for
227 the protein function using the Polyphen2 predicting tool (Fig. 4). The SSCP
228 pattern C corresponded to the heterozygous form C/A, while the SSCP pattern
229 D signed the homozygous reference form C/C. Concerning the exon 5, we
230 have discovered two SNPs within this fragment, g.4108G>A and g.4123C>A
231 (Fig. 3). These two SNPs were silent polymorphisms at the level of the protein
232 sequence as indicated in Table 2 (p.357Ala>Ala and p.362Thr>Thr). The
233 SSCP pattern E was associated to the homozygous form of the variant alleles
234 (g.4108A and g.4123A), while the SSCP pattern F was the reference

235 homozygous form (g.4108G and g.4123C). SSCP pattern G corresponded to
236 the heterozygous form at the two SNPs here also considered as a haplotype.

237

238 3.2. Genotype frequency and allelic association with litter traits

239 Based on the SSCP patterns of the *KISS1R/GPR54* fragments and their
240 comprehensive genotypes we were able to establish the genotypic frequencies
241 of the different variants in the four Iranian sheep breeds studied (Table 3).
242 Based on these data we have observed several deviations from the Hardy-
243 Weinberg equilibrium of the genotype frequencies particularly at the 5' UTR
244 and exon 4 loci and for the four breeds. This may be explained by a lack of
245 observed homozygous animals and also the small number of animal
246 genotyped. At the exon 5 locus, only the pure breeds, Mehraban and Shal,
247 showed such significant deviation ($P < 0.01$, Table 3).

248 The association study between genotypes and litter traits was done only
249 using Mehraban breed data. We found no significant association for the SSCP
250 pattern A (homozygous reference; g.396T/T, g.456T/T, g.475C/C, g.571A/A)
251 and B (heterozygous; g.396T/G, g.456T/C, g.475C/A, g.571A/C) concerning
252 polymorphisms located in the 5' UTR of *KISS1R/GPR54* gene (Table 4). In
253 contrast, the C allele of the SNP g.3431C>A within the exon 4 (SSCP pattern
254 D) had a significant increasing effect on LS (LS: 1.3 vs. 1.13, C/C vs. C/A,
255 $P=0.005$, Table 4) and also on BW (BW: 3.92 vs. 3.84, C/C vs. C/A, $P = 0.02$,
256 Table 4). Similarly, homozygous ewes with reference alleles (g. 4108G/G and
257 g.4123C/C) at the exon 5 locus (SSCP pattern F) had significant ($P < 0.01$)
258 higher LS and higher MaxLS than heterozygous (g.4108 G/A, g.4123 C/A)

259 and homozygous variant ewes (g.4108 A/A, g.4123 C/C) (Table 4). Evaluation
260 of the associations between different genotypes and average litter size (ALS)
261 showed no significant association even if we have observed a trend ($P \leq 0.1$)
262 with SNP of the exons 4 and 5 similar to that observed for LS (Table 4).

263

264 **4. Discussion**

265 Several researches have been performed to find the known alleles at major
266 genes affecting OR and LS (e.g., *BMP15/FecX^{L,H,B,G,L,R,Gr,O,Bar}*;
267 *GDF9/FecG^{V,E,NW,H,T}*; *BMPR1B/FecB^B* and *B4GALNT2/FecL^L*) in Iranian
268 indigenous sheep breeds. While some other polymorphisms in these major
269 genes were more or less associated with variation of LS or other related
270 reproductive traits (Abdoli et al., 2013; Eghbalsaied et al., 2014; Zamani et al.,
271 2015a,b; Ahmadi et al., 2016; Nadri et al., 2016; Eghbalsaied et al., 2017;
272 Talebi et al., 2018), no such a causal mutation has been confirmed so far.
273 There is therefore a lack of potential markers for genetic management of
274 prolificacy in Iranian breeds. In this study, we have investigated a new
275 potential candidate gene that may control ovine prolificacy, i.e. the
276 *KISSIR/GPR54* gene. This was the first exhaustive study conducted to
277 investigate polymorphisms at the *KISSIR/GPR54* locus in pure and Romanov
278 crossbreeds of Iranian sheep.

279 In the present work, we have detected seven SNPs in the *KISSIR/GPR54*
280 gene located on ovine chromosomes 5 among the four studied Iranian breeds
281 (Table 2). This gene displays numerous polymorphisms in livestock that have
282 been associated with reproductive traits in cattle (Chen et al., 2011), goat

283 (Feng et al., 2009; Cao et al., 2011; Ahlawat et al., 2015; Othman et al., 2018),
284 sheep (Chu et al., 2012; Tang et al., 2012; Othman et al., 2018), and pig (Li et
285 al., 2008). In ovine, Tang et al. (2012) detected two mutations in the *GPR54*
286 gene in Small Tail Han and Corriedale sheep. The first one was a SNP
287 (g.125A>G, GenBank No: HM135393.1) whose alleles were not associated
288 with LS. The second one was a 5 bp deletion (g.163delTTCTT, GenBank No:
289 HM135393.1) for which the homozygous non-carrier animals showed a
290 significant increased LS by +0.66 ($P < 0.05$) compared to the deletion
291 homozygous animals. This deletion represents a potential effective DNA
292 marker to improve litter size in Small Tail Han sheep (Tang et al., 2012).
293 However, this polymorphism located in the 5'UTR of *KISSIR/GPR54* was not
294 evidenced in the 10 Iranian animals we sequenced for this region.
295 Nevertheless, in the same region we identified four SNPs, (g.396 T>G, g.456
296 T>C, g.475 C>A and g.571 A>C) already being referenced in the dbSNP
297 database (rs160071719, rs421814026, rs400364529 and rs161872366,
298 respectively) and known to segregate in various ovine populations and among
299 them Iranian individuals (see, Population genetics in ensembl.org). Altogether,
300 they created the two SSCP patterns A (homozygous reference; g.396T/T,
301 g.456T/T, g.475C/C, g.571A/A) and B (heterozygous; g.396T/G, g.456T/C,
302 g.475C/A, g.571A/C), but none had significant association ($P > 0.05$) with
303 fertility traits such as LS, MaxLS and ALS or BW obtained from Mehraban
304 ewes (Table 4).

305 In contrast, we identified three novel SNPs of the *KISSIR/GPR54* gene
306 within the coding region from exons 4 and 5 and in significant association

307 with reproductive traits in Mehraban breed. The first one (g.3431C>A) in the
308 exon 4 is located at an already known polymorphic position of the *KISSIR*
309 gene on the chromosome 5 (rs593807261, NC_019462.1, g.40846749C>T in
310 OAR v3.1, or NC_019462.2, g.40775837C>T in OAR v4.0). Interestingly,
311 while the C>T referenced variation is considered as a synonymous variant
312 (p.195Phe=), the variant allele A detected in this study, leads to a non-
313 conservative aminoacidic change p.195Phe>Leu. The variant effect of
314 g.3431C>A [p.195Phe>Leu] predicted by PolyPhen2 is indicated as damaging
315 with a score of 0.96 (Fig. 4). According to the sheep population genetics data
316 available at ensembl.org, only the variant T allele is shared by many breeds
317 including Iranian breeds. The A allele we evidenced presently seems specific
318 to Mehraban, Shal and their crossbreeds. We established the genotypic and
319 allelic frequencies of this locus but surprisingly it was not in Hardy-Weinberg
320 equilibrium (Table 3), particularly in Mehraban breed where there is a deficit
321 of homozygous A/A animals. The association analysis indicates that the minor
322 allele g.3431A in the exon 4 has a significant negative effect on LS and BW,
323 by reducing LS by -0.17 and BW by -0.08 (Table 4). Altogether, these results
324 are a bundle of arguments in favor of the segregation of a causal mutation in
325 the *KISSIR/GPR54* gene affecting negatively the ovarian function and/or the
326 intrauterine development of the embryo approximated by LS and BW.
327 Moreover, the lack of homozygous animals in the populations tested, could
328 also indicate the presence of an embryonic lethal mutation and may reveal a
329 negative impact of the homozygous g.3431A allele on the intrauterine

330 development of the embryo. However, the validation of such hypothesis will
331 need further work.

332 Two novel silent polymorphisms were further found at the end of exon 5
333 coding part. The g.4108G>A [p.357Ala>Ala] and g.4123C>A [p.362Thr>Thr]
334 (Table 2) were not referenced in dbSNP surely due to an apparent discrepancy
335 in the reference ovine genome (OAR v3.1 and v4.0) with our working
336 reference sequence of the *KISS1R/GPR54* gene (GenBank No: HM135393.1).
337 Nevertheless, among the three SSCP patterns formed by these two SNPs
338 (Table 4), the pattern F (homozygous reference; g.4108G/G, g.4123C/C) was
339 significantly associated ($P=0.007$) with increased LS by +0.18 and +0.25,
340 when compared to pattern G (heterozygous; g.4108 G/A, 4123 C/A) and
341 pattern E (homozygous variant; g.4108 A/A, g.4123 C/C), respectively.
342 Likewise, pattern F showed +0.39 elevated MaxLS than patterns G and E
343 (Table 4). These mutations are silent at the protein level, but could be in strong
344 linkage disequilibrium with a causal mutation nearby, like the g.3431C>A in
345 exon 4. However, a low correlation coefficient value ($r^2 = 0.25$) was estimated
346 between g.3431C>A in exon 4 and SSCP patterns of exon5. Furthermore, it
347 must be noted that, the sampled animals have been maintained under different
348 managements and we cannot rule out the possible effect of the flock in
349 evaluating observed associations.

350

351 **5. Conclusion**

352 We detected seven variants in the *KISS1R/GPR54* gene, among which,
353 g.3431C>A leads to an amino acid altering substitution p.195Phe>Leu with a

354 significant effect ($P < 0.01$) on LS and BW traits in sheep. Beside its
355 functional importance in controlling the reproductive function, our results
356 provide additional evidence that the *KISS1R/GPR54* gene is a promising
357 candidate to develop marker-assisted selection to genetically improve
358 prolificacy in sheep. In the precise case of the g.3431C>A marker, the strategy
359 would be the reduction of the frequency of the unfavorable allele A in the
360 Iranian sheep population in which it is segregating.

361

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367 **Conflict of interest statement**

368 The authors have declared that no competing interests exist.

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372 **Author contributions**

373 SAM, AA, RT and PMK conceived and designed the experiments. SAM
374 and RT collected the blood samples. SAM prepared the experimental records
375 and the laboratory results. SAM, RT and SF performed the polymorphisms
376 detection analyses. AA and RT performed the statistical analyses. SAM, AA
377 RT and PMK managed this experiment and its hypotheses. AA, RT, SF and

378 SQ wrote the manuscript. All authors approved the manuscript before
379 submission.

380

381 **Figure legends**

382 **Figure 1.** Amplified fragments of sheep *KISSIR/GPR54* gene (GenBank No:
383 HM135393.1). **A.** 923 bp fragment located in the proximal 5' UTR part of the
384 exon 1. **B.** 850 bp fragment encompassing the coding part of the exon 1 and a
385 part of the intron 1. **C.** 925 bp fragment with the exon 2, intron 2 and exon 3.
386 **D.** 321 bp fragment encompassing the exon 4. **E.** 257 bp fragment
387 corresponding to the terminal part of the coding region of the exon 5. The
388 lanes of G1-G5 are five samples that have been amplified by PCR. MW: DNA
389 molecular weight marker, informative size (bp) colored in white.

390

391 **Figure 2.** SSCP distinct patterns on the 8% acrylamide gel. **A.** Two possible
392 patterns A (homozygous reference) and B (heterozygous) for the proximal 5'
393 UTR part of the exon 1. **B.** Two possible patterns C (C/A) and D (C/C,
394 reference) for the exon 4. **C.** Three possible patterns G (heterozygous), F
395 (homozygous reference) and E (homozygous variant) for the terminal part of
396 the coding region of the exon 5.

397

398 **Figure 3.** Single nucleotide polymorphisms detected at *KISSIR/GPR54* gene
399 (GenBank No: HM135393.1) among four breeds of sheep. **A.** SNPs whose
400 were identified in pattern A (homozygous reference; g.396 T/T, g.456 T/T,
401 g.475 C/C, g.571 A/A) and pattern B (heterozygous; g.396 T/G, g.456 T/C,

402 g.475 C/A, g.571 A/C), in proximal 5' UTR part of the exon 1. **B.** SNP g.3431
403 C>A was identified in pattern C (C/A) and pattern D (C/C, reference), in exon
404 4. **C.** SNPs whose were identified in pattern G (heterozygous; g.4108 G/A,
405 4123 C/A), pattern F (homozygous reference; g.4108 G/G, g.4123 C/C) and
406 pattern E (homozygous variant; g.4108 A/A, g.4123 C/C), in terminal part of
407 the coding region of the exon 5.

408

409 **Figure 4.** Prediction of the amino acid substitutions impact (SNP g.3431 C>A:
410 p.195 Phe>Leu) on structure and function of the KISS1R codified
411 polypeptide.

412

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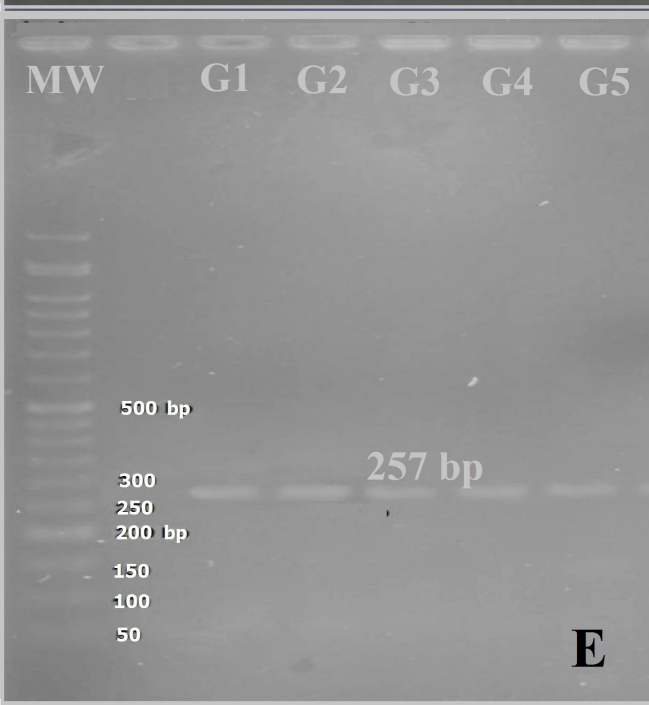
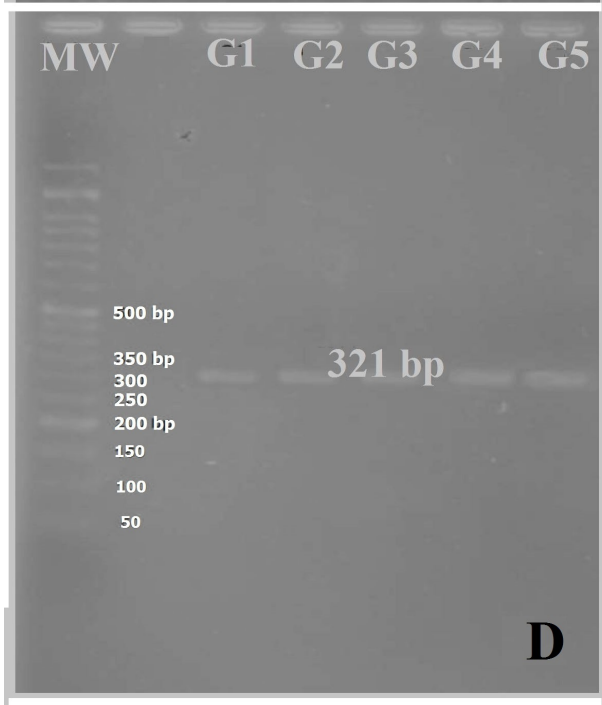
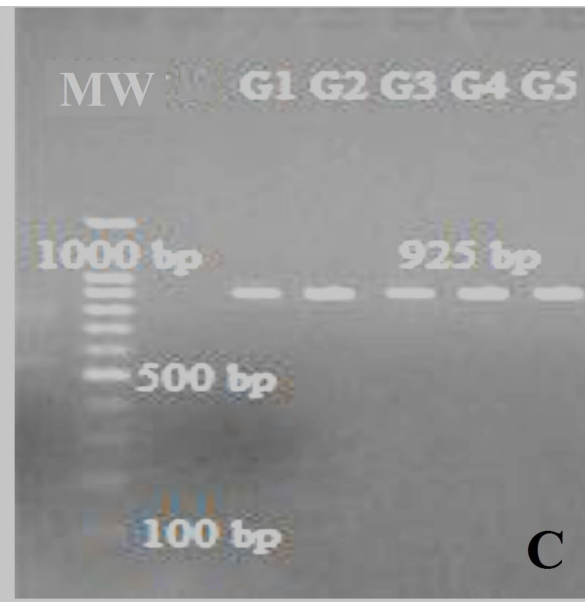
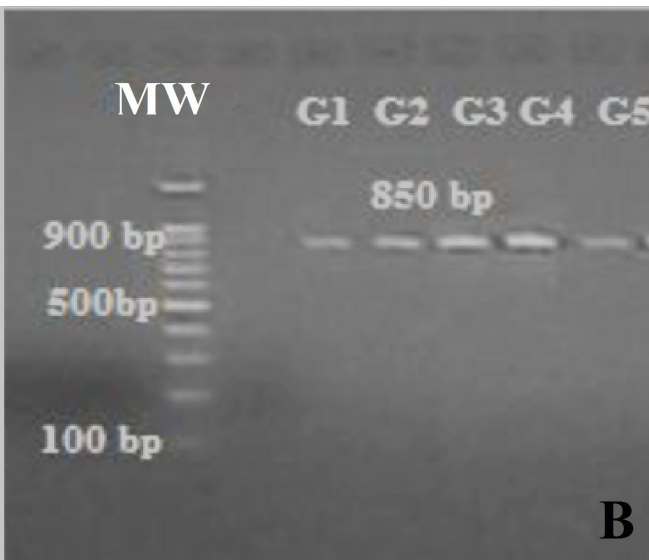
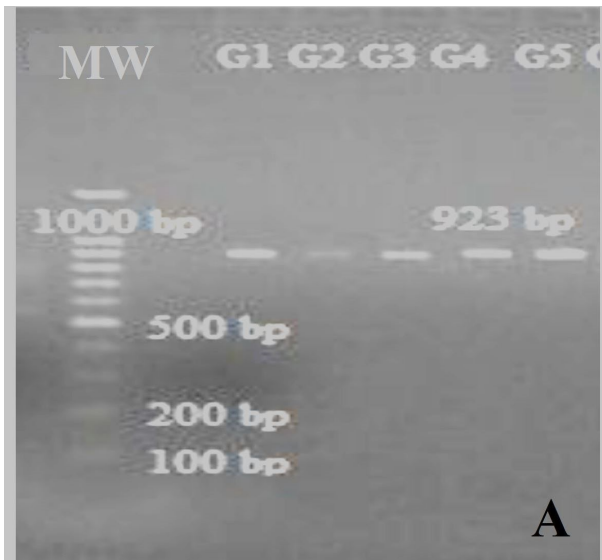
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511



A



Pattern A

Pattern B

Pattern B

Pattern A

Pattern A

B



Pattern C

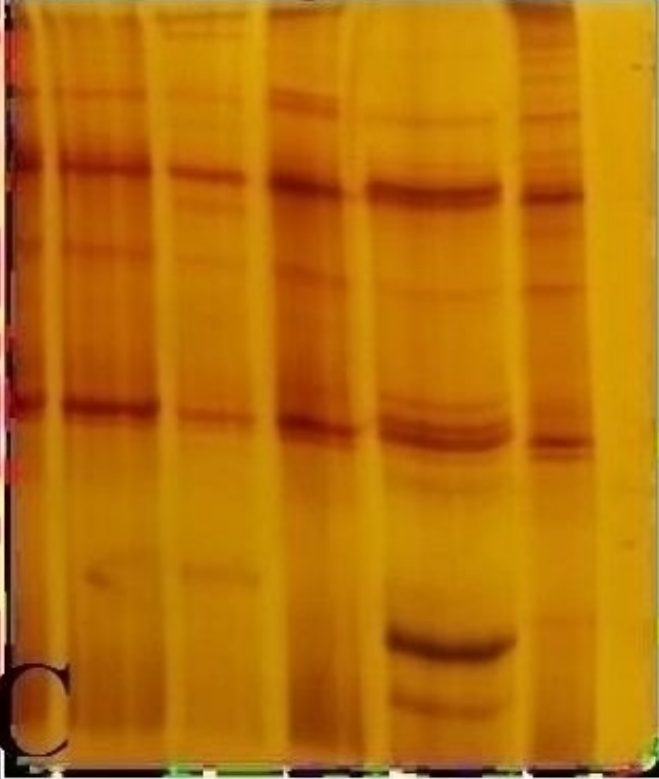
Pattern C

Pattern C

Pattern D

Pattern D

C



Pattern E

Pattern F

Pattern E

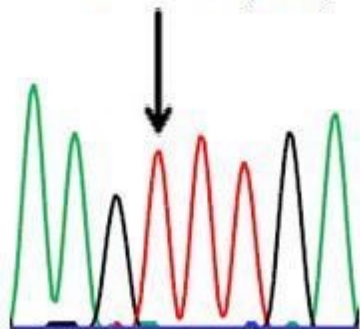
Pattern G

Pattern F

A

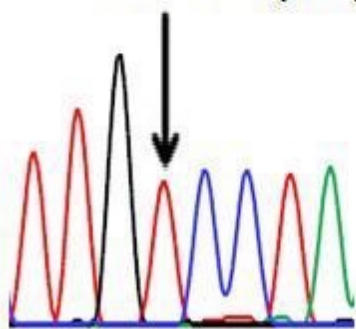
A A G T T T G A

g.396 T>G (T/T)



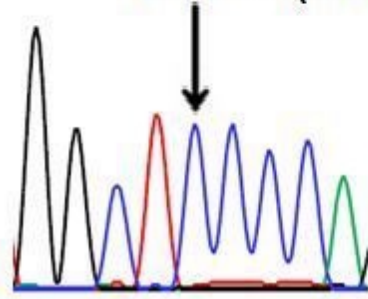
T T G T C C T A

g.456 T>C (T/T)



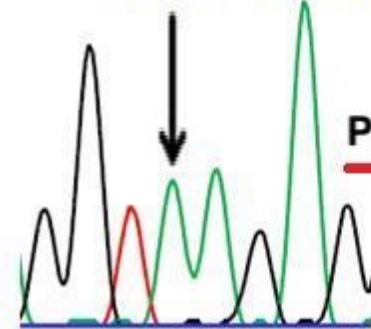
G G C T C C C C A

g.475 C>A (C/C)



G G T A A G A G

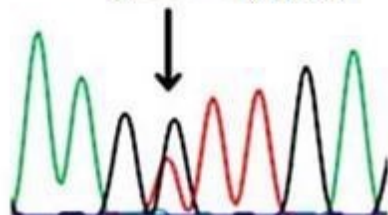
g.571 A>C (A/A)



Pattern A

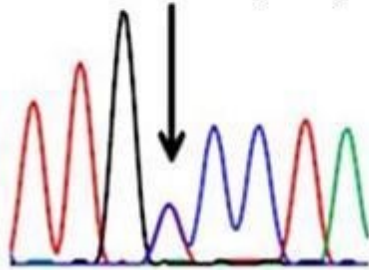
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g.396 T>G (T/G)



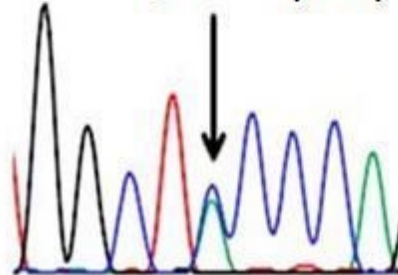
T T G N C C T A

g.456 T>C (T/C)



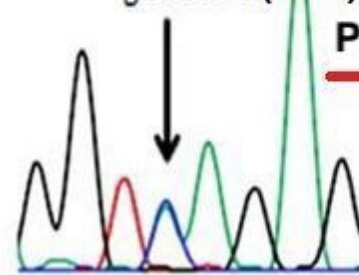
G G C T N C C C A

g.475 C>A (C/A)



G G T N A G A G

g.571 A>C (A/C)

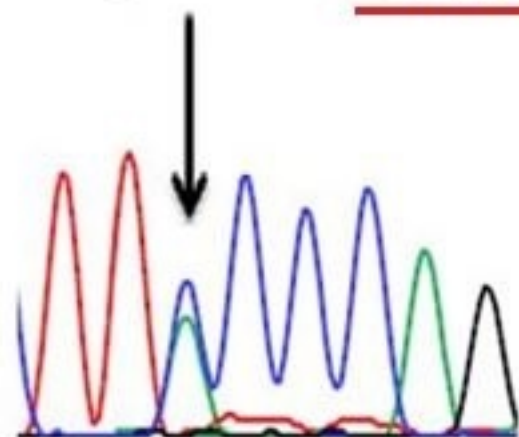


Pattern B

B T T N C C C A G

g.3431 C>A

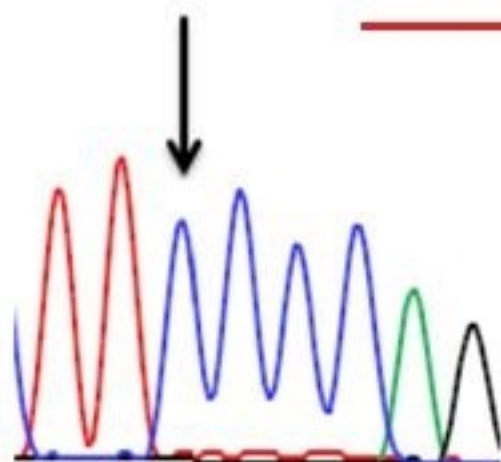
Pattern C



T T C C C C A G

g.3431 C>A

Pattern D



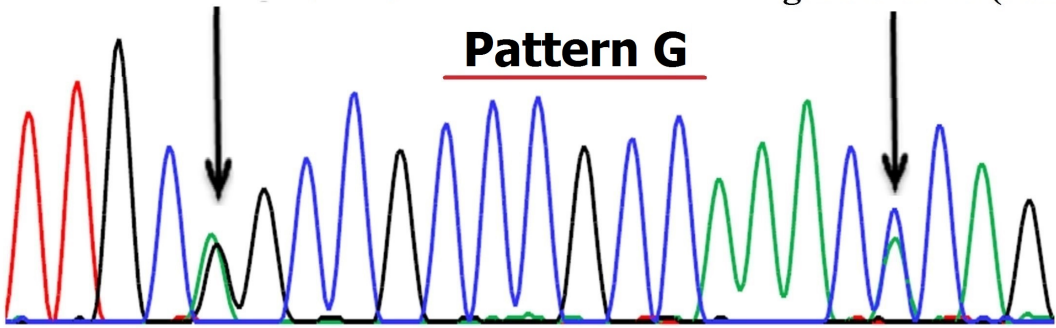
C

T T G C G G C C G C C C G C C A A A C C C A G

g.4108 G>A (G/A)

g.4123 C>A (C/A)

Pattern G

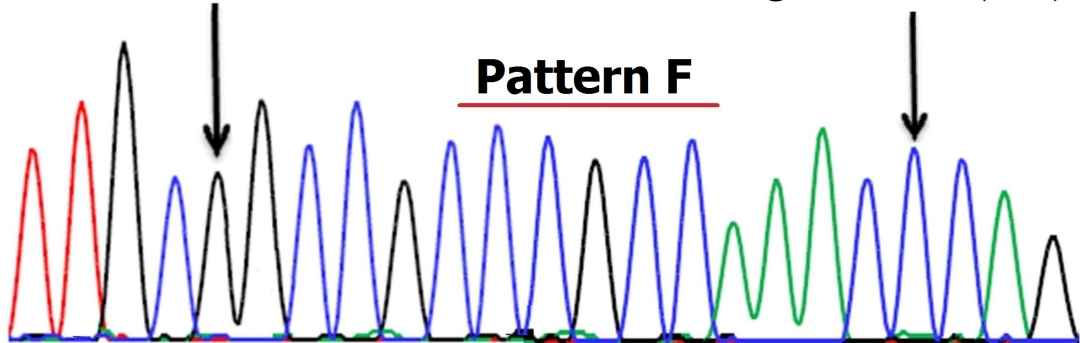


T T G C G G C C G C C C G C C A A A C C C A G

g.4108 G>A (G/G)

g.4123 C>A (C/C)

Pattern F

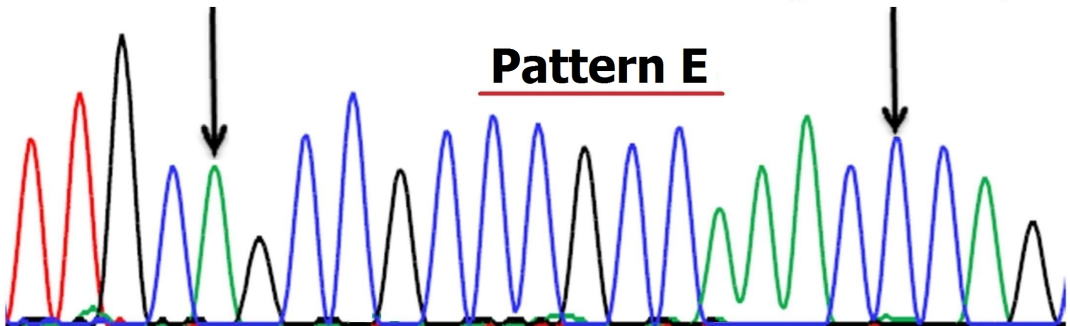


T T G C A G C C G C C C G C C A A A C C C A G

g.4108 G>A (A/A)

g.4123 C>A (C/C)

Pattern E



This mutation is predicted to be **PROBABLY DAMAGING** with a score of **0.957** (sensitivity: **0.78**; specificity: **0.95**)

p.195 Phe>Leu

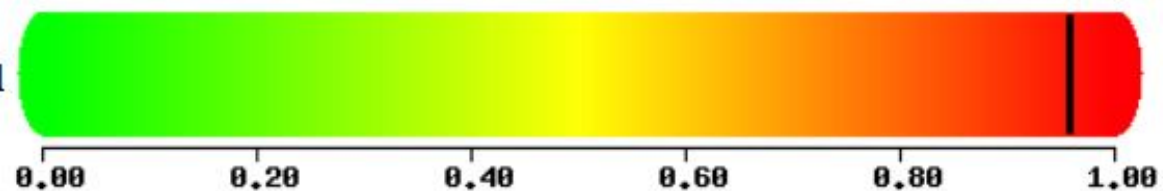


Table 1

Primer sequence, amplified region, product size and annealing temperature used in analyses of sheep *KISSIR/GPR54* gene (GenBank No: HM135393.1).

¹ Primer sequence (5' to 3')	Amplified region	Product size (bp)	Annealing temperature (°C)
Fwd: 5'- GCAGACTCCTAACCACGAGA-3' Rev: 5'- ACTGTCGTGACTTTCCCGAT-3'	Proximal 5' UTR region	923	64
Fwd: 5'- ATTTTGTAGCGCATCCCTGG -3' Rev: 5'- CACACTCTCCAACCTCCCTT -3'	Exon 1- partial Intron 1	850	62
Fwd: 5'- CCAACTTCTCTGCCCCACCTA -3' Rev: 5'- CTGAACTCACCCACCCAGAT -3'	Exon 2-Intron 2-Exon 3	925	60
Fwd: 5'-GGGTCTTCAACAGGGCTCT-3' Rev: 5'-GTAGATGCGCCTCACTCCC-3'	Exon 4	321	62
Fwd: 5'-CAAGATCTGGGCACACTTCA-3' Rev: 5'- CCTCAGAACGCACAGCATG -3'	End of Exon 5 ORF	257	61

¹Fwd and Rev : Forward and Reverse primers

Table 2

Identified SNPs in ovine *KISSIR/GPR54* gene (GenBank No: HM135393.1).

SNP gene location	SNP alleles	Protein position/substitution
5' UTR	g.396 T>G	Non-coding
	g.456 T>C	Non-coding
	g.475 C>A	Non-coding
	g.571 A>C	Non-coding
Exon 4	g.3431 C>A	p.195 Phe>Leu
Exon 5	g. 4108 G>A	p.357 Ala>Ala
	g. 4123 C>A	p.362 Thr>Thr

Table 3

Percentage of genotypic frequencies of identified SSCP variants among amplified fragments of sheep *KISSIR/GPR54* gene (GenBank No: HM135393.1).

Locus of GPR54	SSCP Pattern	numbers /frequencies	Sheep Breeds			
			Mehraban	Shal	Mehraban×Romanov	Shal×Romanov
5' UTR	A	N	51	25	9	14
		Frequency	0.64	1	0.43	0.47
	B	N	29	0	12	16
		Frequency	0.36	0	0.57	0.53
χ^2		3.92	-	3.36	3.97	
P value		0.048	ND	0.07	0.046	
Exon 4	D	N	41	11	6	15
		Frequency	0.51	0.44	0.29	0.5
	C	N	39	14	15	15
		frequency	0.49	0.56	0.71	0.5
χ^2		8.31	3.78	6.48	3.33	
P value		0.003	0.05	0.01	0.07	
Exon 5	G	N	25	6	7	11
		Frequency	0.31	0.24	0.33	0.37
	F	N	33	10	8	7
		Frequency	0.41	0.4	0.38	0.23
E	N	22	9	6	12	
	Frequency	0.27	0.36	0.29	0.4	
χ^2		10.54	6.74	2.24	1.81	
P value		0.001	0.009	0.13	0.18	

N, number of genotypes/variants in SSCP patterns; χ^2 : The chi-square test; ND, no significant difference.

Table 4

Association of the identified genotypes and variants of sheep *KISS1R/GPR54* gene (GenBank No: HM135393.1) with reproductive traits in Mehraban sheep breed

Locus name	Genotype patterns	N=	LS	MaxLS	ALS	BW (kg)
5' UTR	Pattern A (Homozygous reference)	51	1.28	1.37	1.14	3.9
	Pattern B (Heterozygous)	29	1.16	1.2	1.07	3.89
	SEM		0.24	0.12	0.2	0.23
	<i>P</i> value		0.07	0.25	0.31	0.67
Exon 4	Pattern D (C/C, reference)	41	1.3 ^a	1.4	1.15	3.92 ^a
	Pattern C (C/A)	39	1.13 ^b	1.21	1.06	3.84 ^b
	SEM		0.13	0.11	0.2	0.23
	<i>P</i> value		0.005	0.17	0.1	0.02
Exon 5	Pattern G (heterozygous)	25	1.22 ^b	1.22 ^b	1.09	3.92
	Pattern F (homozygous reference)	33	1.4 ^a	1.61 ^a	1.21	3.96
	Pattern E (homozygous variant)	22	1.15 ^b	1.22 ^b	1.08	3.76
	SEM		0.24	0.12	0.19	0.23
	<i>P</i> value		0.007	0.007	0.09	0.53

N=: number of ewes at each genotype; LS: litter size; MaxLS: maximum litter size; ALS: average litter size; BW: birth weight; ^{a,b}: The means with same letter in each part of each column were not significantly different in Duncan's test at error level of 0.05.

Highlights

- Seven SNPs were evidenced in ovine *KISS1R/GPR54* gene among Iranian sheep breeds
- Three novel SNPs lying in the coding region of ovine *KISS1R/GPR54* gene were associated with prolificacy of Mehraban sheep
- The SNP g.3431C>A leading to the amino acid altering variant p.195Phe>Leu could be considered as a causal mutation impairing sheep prolificacy.