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ABSTRACT

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

Keywords: Causal mutation, Polymorphism, Prolificacy

1. Introduction

The traits with the greatest financial impact on sheep production are ovulation rate (OR) and litter size (LS) (Notter, 2008). However, a classical selection based on polygenic quantitative approach for improving reproductive traits in sheep is difficult due to the sex-limited nature and low heritability of these traits (Janssens et al., 2004). Therefore, breeding strategies based on genetic information obtained from molecular techniques and marker-assisted selection (MAS) have a high importance for improving reproductive efficiency (El-Tarabany et al., 2017). Genetic studies in sheep have indicated that OR and LS can be genetically regulated either by many loci, each having a minor effect, or alternatively by the action of single genes with major effect (Drouilhet et al., 2009). Several major genes controlling OR and LS, known as *BMP15, BMPR1B, GDF9* and *B4GALNT2* and so-called fecundity genes, have been discovered in various ovine populations so far, with numerous causative mutations identified (Vinet et al., 2012, Abdoli et al., 2016). When heterozygous, all these mutations increase OR and LS, but depending on the mutations and at the homozygous state, their effects may be opposite, from hyper-prolificacy to sterility. Moreover, particular inheritance patterns are observed for mutations carried by the X-linked *BMP15* gene compared to mutations in the three other autosomal genes.

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

As of 2008, Iran had the fifth largest sheep population in the world, with about 44 million animals (FAO Live Animals database, http://www.fao.org/faostat/en/#data/QA, Jowkar et al., 2016). More than twenty-seven distinctive breeds of sheep are distributed in a wide variety of habitats in Iran. They vary in their genetic potential for production traits including milk, meat, wool, disease resistance and fecundity. Although these breeds possess different performances regarding the fecundity traits, their overall performances are low (Tavakolian, 2000). Despite, many studies 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 *KISS1R*/*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 (Payapajoohesh 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

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

3. Results

3.1. PCR amplification and SSCP analysis

Agarose gel analyses of the PCR products have confirmed the size of the five expected fragments (923 bp, 850 bp, 925 bp, 321 bp and 257 bp) amplified from the ovine *KISS1R*/*GPR54* gene (Fig. 1).

The SSCP analysis of these fragments obtained from the four breeds revealed that only 3 of them were polymorphic. These corresponded to the 5′ UTR, exon 4 and exon 5 of *KISS1R*/*GPR54* (Fig. 2). We have observed two different SSCP patterns, called pattern A and B (Fig. 2A), for the 5′ UTR fragment for the Mehraban, Mehraban × Romanov and Shal × Romanov breeds, the Shal breed being not polymorphic at this location. Two SSCP patterns (C and D, Fig. 2B) were also observed for the exon 4. In contrast, three SSCP patterns (E, F and G, Fig. 2C) were observed for the exon 5.

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

In order to identify the polymorphisms that cause SSCP patterns, five random PCR samples from each SSCP pattern were sequenced. As shown in Fig. 3 and Table 2, the difference between SSCP patterns A and B was due to four SNPs located within the 5′ UTR fragment, named g.396T>G, g.456T>C, g.475C>A, and g.571A>C according to the 4263 bp sequence of the *KISS1R*/*GPR54* gene (GenBank No: HM135393.1). Based on the sequencing result, the SSCP pattern A corresponded to the homozygous reference form, while the SSCP pattern B was representative of the heterozygous form. From the five PCR products sequenced for pattern B, the all four SNPs were always heterozygous signing a possible haplotype. Within the exon 4, only one SNP was detected at position g.3431C>A. This SNP causes a non-conservative change in the protein sequence at the amino acid position 195 (p.195 Phe>Leu, Table 2). Interestingly, this change was predicted as probably deleterious for the protein function using the Polyphen2 predicting tool (Fig. 4). The SSCP pattern C corresponded to the heterozygous form C/A, while the SSCP pattern D signed the homozygous reference form C/C. Concerning the exon 5, we have discovered two SNPs within this fragment, g.4108G>A and g.4123C>A (Fig. 3). These two SNPs were silent polymorphisms at the level of the protein sequence as indicated in Table 2 (p.357Ala>Ala and p.362Thr>Thr). The SSCP pattern E was associated to the homozygous form of the variant alleles (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)

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

4. Discussion

Several researches have been performed to find the known alleles at major genes affecting OR and LS (e.g., *BMP15/FecX*^{I,H,B,G,L,R,Gr,O,Bar}; *GDF9/FecG*^{V,E,NW,H,T}; *BMPR1B/FecB*^{B} and *B4GALNT2/FecL*^{L}) in Iranian indigenous sheep breeds. While some other polymorphisms in these major genes were more or less associated with variation of LS or other related reproductive traits (Abdoli et al., 2013; Eghbalsaied et al., 2014; Zamani et al., 2015a,b; Ahmadi et al., 2016; Nadri et al., 2016; Eghbalsaied et al., 2017; Talebi et al., 2018), no such a causal mutation has been confirmed so far. There is therefore a lack of potential markers for genetic management of prolificacy in Iranian breeds. In this study, we have investigated a new potential candidate gene that may control ovine prolificacy, i.e. the *KISS1R*/*GPR54* gene. This was the first exhaustive study conducted to investigate polymorphisms at the *KISS1R*/*GPR54* locus in pure and Romanov crossbreeds of Iranian sheep.

In the present work, we have detected seven SNPs in the *KISS1R*/*GPR54* gene located on ovine chromosomes 5 among the four studied Iranian breeds (Table 2). This gene displays numerous polymorphisms in livestock that have 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 *KISS1R*/*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 *KISS1R*/*GPR54* gene
306 within the coding region from exons 4 and 5 and in significant association

with reproductive traits in Mehraban breed. The first one (g.3431C>A) in the exon 4 is located at an already known polymorphic position of the *KISS1R* gene on the chromosome 5 (rs593807261, NC_019462.1, g.40846749C>T in OAR v3.1, or NC_019462.2, g.40775837C>T in OAR v4.0). Interestingly, while the C>T referenced variation is considered as a synonymous variant (p.195Phe=), the variant allele A detected in this study, leads to a non-conservative aminoacidic change p.195Phe>Leu. The variant effect of g.3431C>A [p.195Phe>Leu] predicted by PolyPhen2 is indicated as damaging with a score of 0.96 (Fig. 4). According to the sheep population genetics data available at ensembl.org, only the variant T allele is shared by many breeds including Iranian breeds. The A allele we evidenced presently seems specific to Mehraban, Shal and their crossbreeds. We established the genotypic and allelic frequencies of this locus but surprisingly it was not in Hardy-Weinberg equilibrium (Table 3), particularly in Mehraban breed where there is a deficit of homozygous A/A animals. The association analysis indicates that the minor allele g.3431A in the exon 4 has a significant negative effect on LS and BW, by reducing LS by -0.17 and BW by -0.08 (Table 4). Altogether, these results are a bundle of arguments in favor of the segregation of a causal mutation in the *KISS1R/GPR54* gene affecting negatively the ovarian function and/or the intrauterine development of the embryo approximated by LS and BW. Moreover, the lack of homozygous animals in the populations tested, could also indicate the presence of an embryonic lethal mutation and may reveal a negative impact of the homozygous g.3431A allele on the intrauterine

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

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

5. Conclusion

We detected seven variants in the *KISS1R*/*GPR54* gene, among which, 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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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

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

Figure legends

Figure 1. Amplified fragments of sheep *KISS1R*/*GPR54* gene (GenBank No: HM135393.1). **A.** 923 bp fragment located in the proximal 5′ UTR part of the exon 1. **B.** 850 bp fragment encompassing the coding part of the exon 1 and a part of the intron 1. **C.** 925 bp fragment with the exon 2, intron 2 and exon 3. **D.** 321 bp fragment encompassing the exon 4. **E.** 257 bp fragment corresponding to the terminal part of the coding region of the exon 5. The lanes of G1-G5 are five samples that have been amplified by PCR. MW: DNA molecular weight marker, informative size (bp) colored in white.

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

Figure 3. Single nucleotide polymorphisms detected at *KISS1R*/*GPR54* gene (GenBank No: HM135393.1) among four breeds of sheep. **A.** SNPs whose were identified in pattern A (homozygous reference; g.396 T/T, g.456 T/T, g.475 C/C, g.571 A/A) and pattern B (heterozygous; g.396 T/G, g.456 T/C,

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

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

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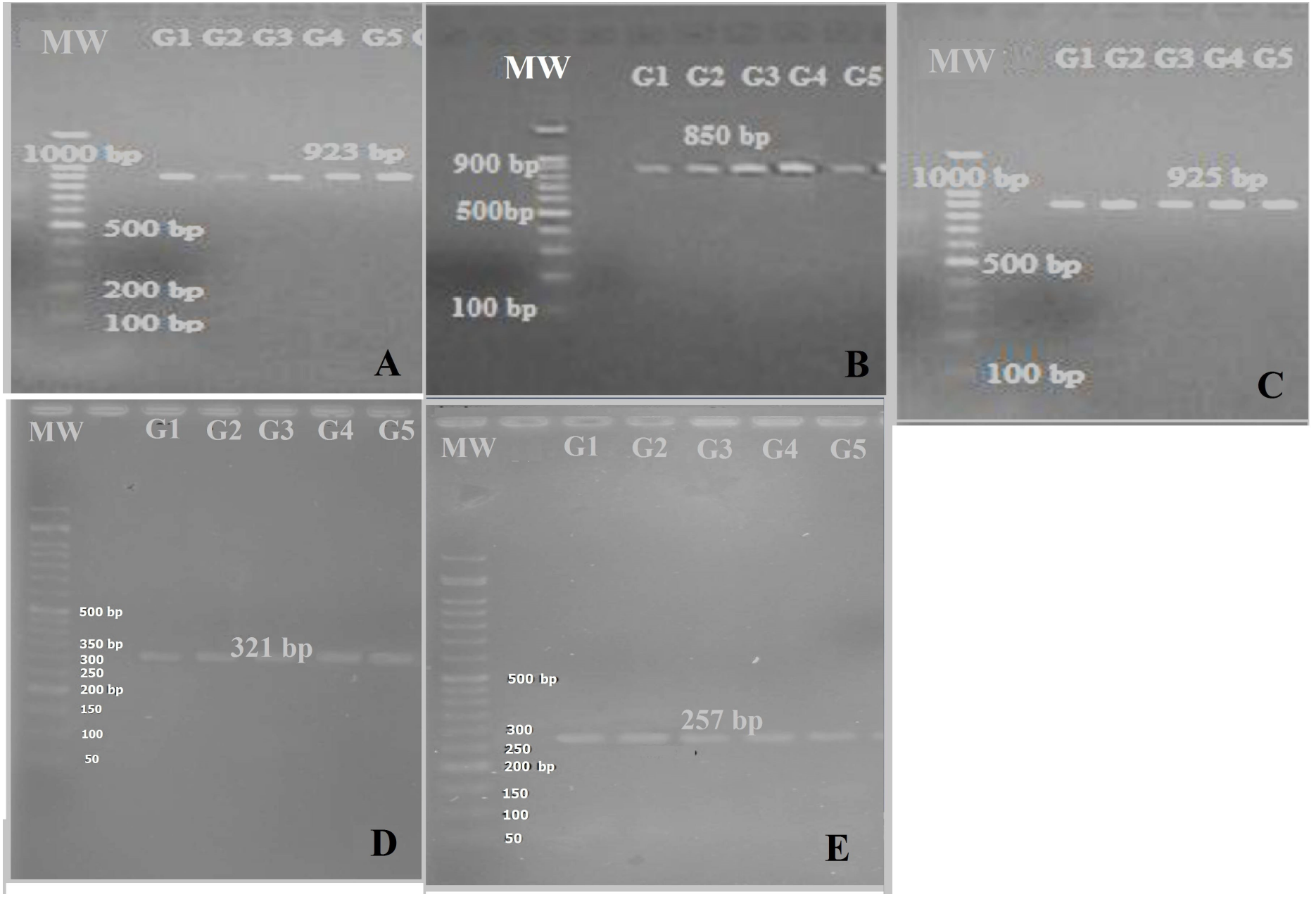
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200 bp
100 bp
A
MW
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900 bp
500bp
100 bp
B
MW
G1 G2 G3 G4 G5
1000 bp
925 bp
500 bp
100 bp
C
MW
G1 G2 G3 G4 G5
500 bp
350 bp
300
250
200 bp
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D
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50
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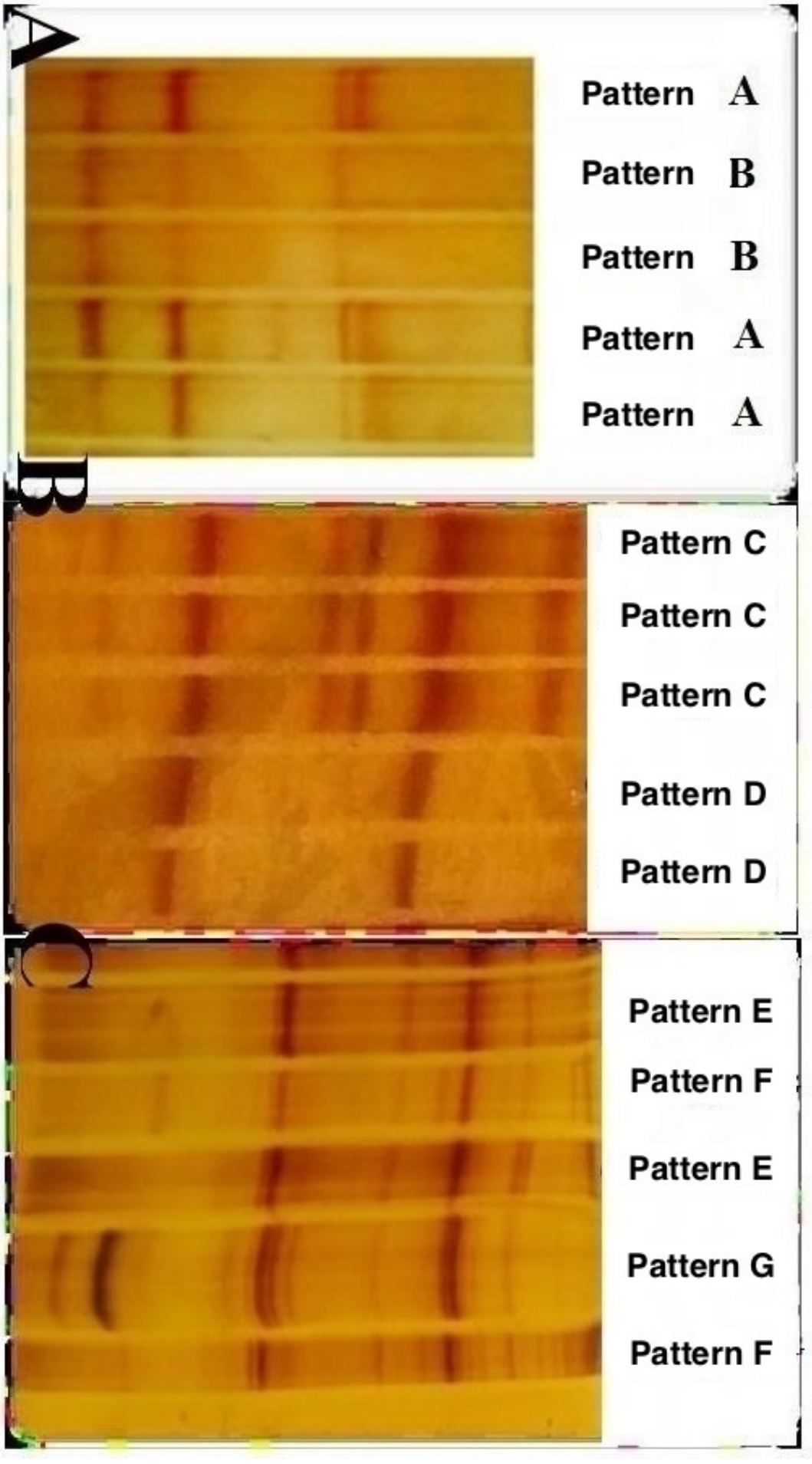
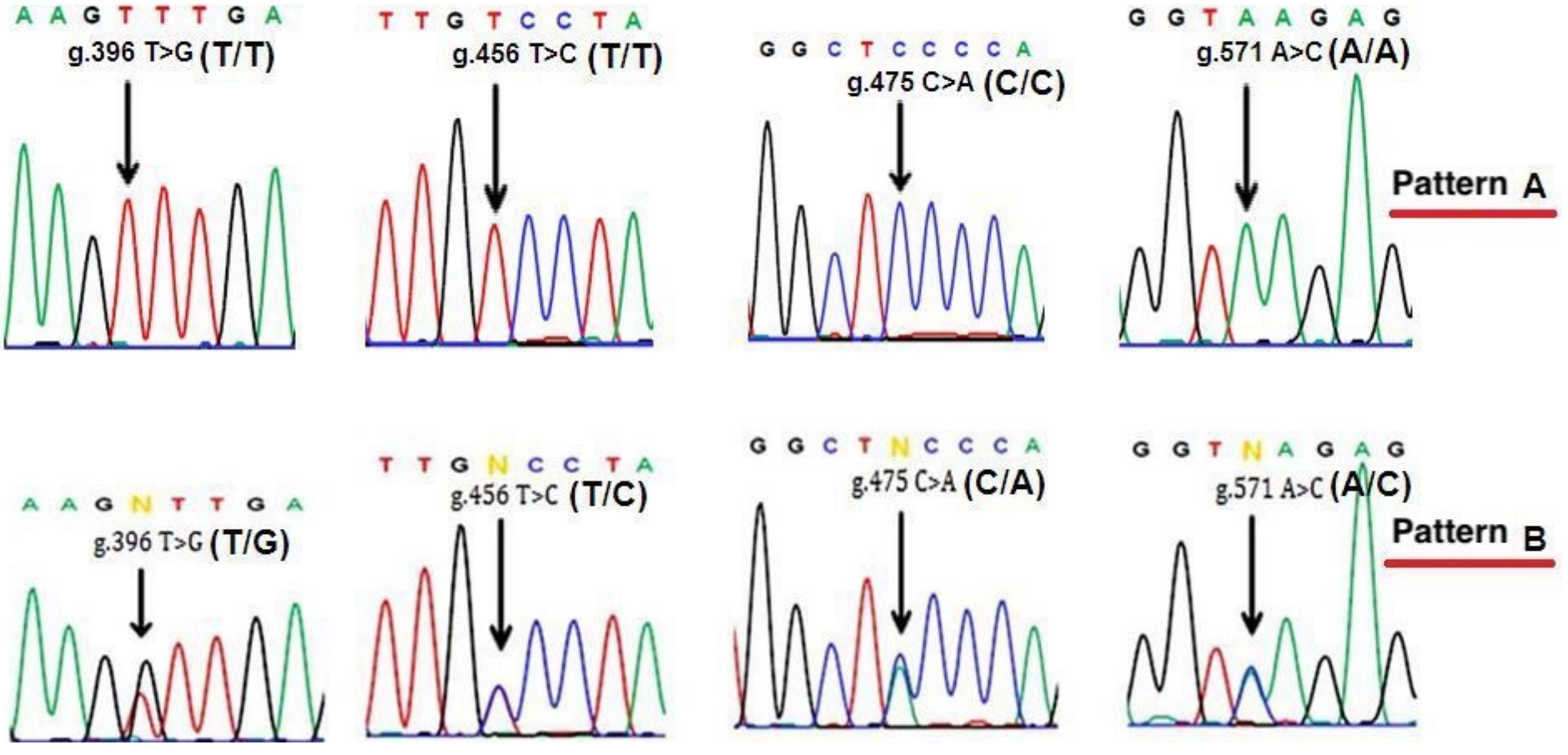
E


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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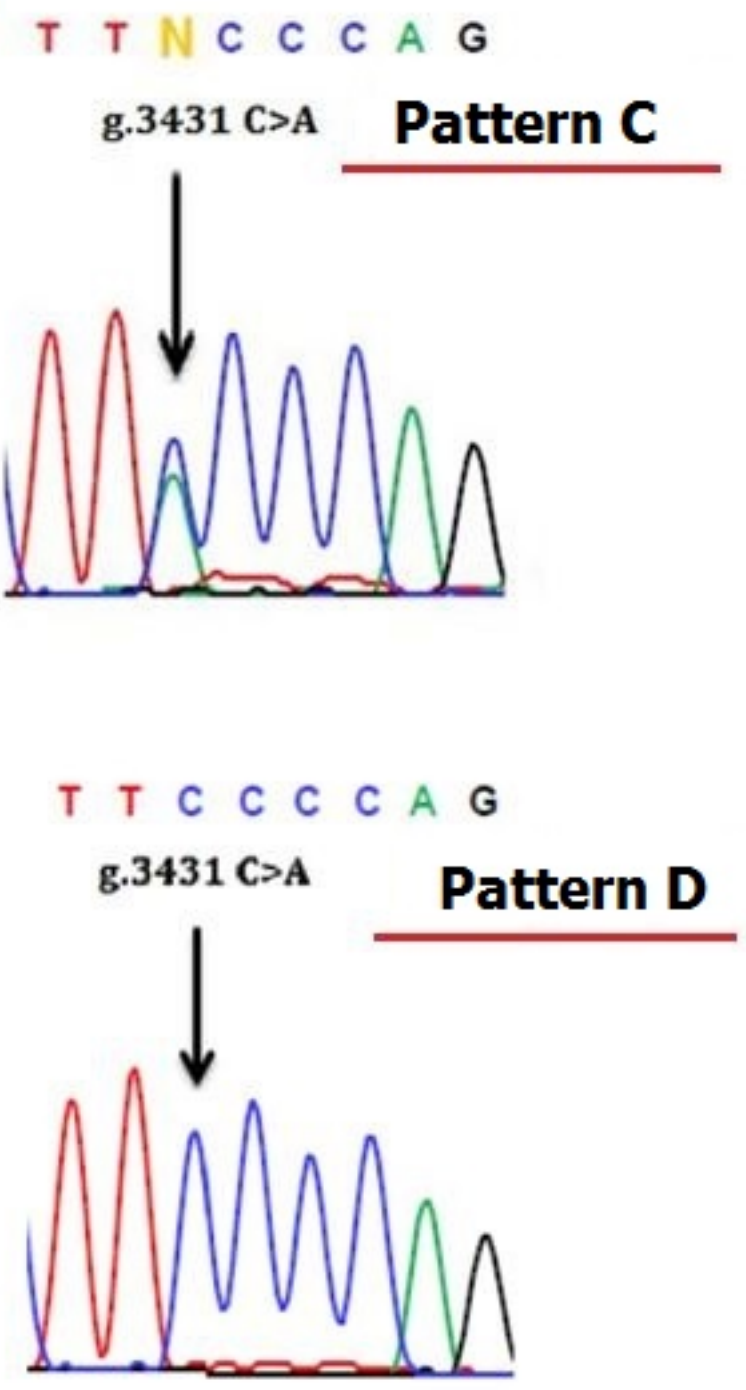
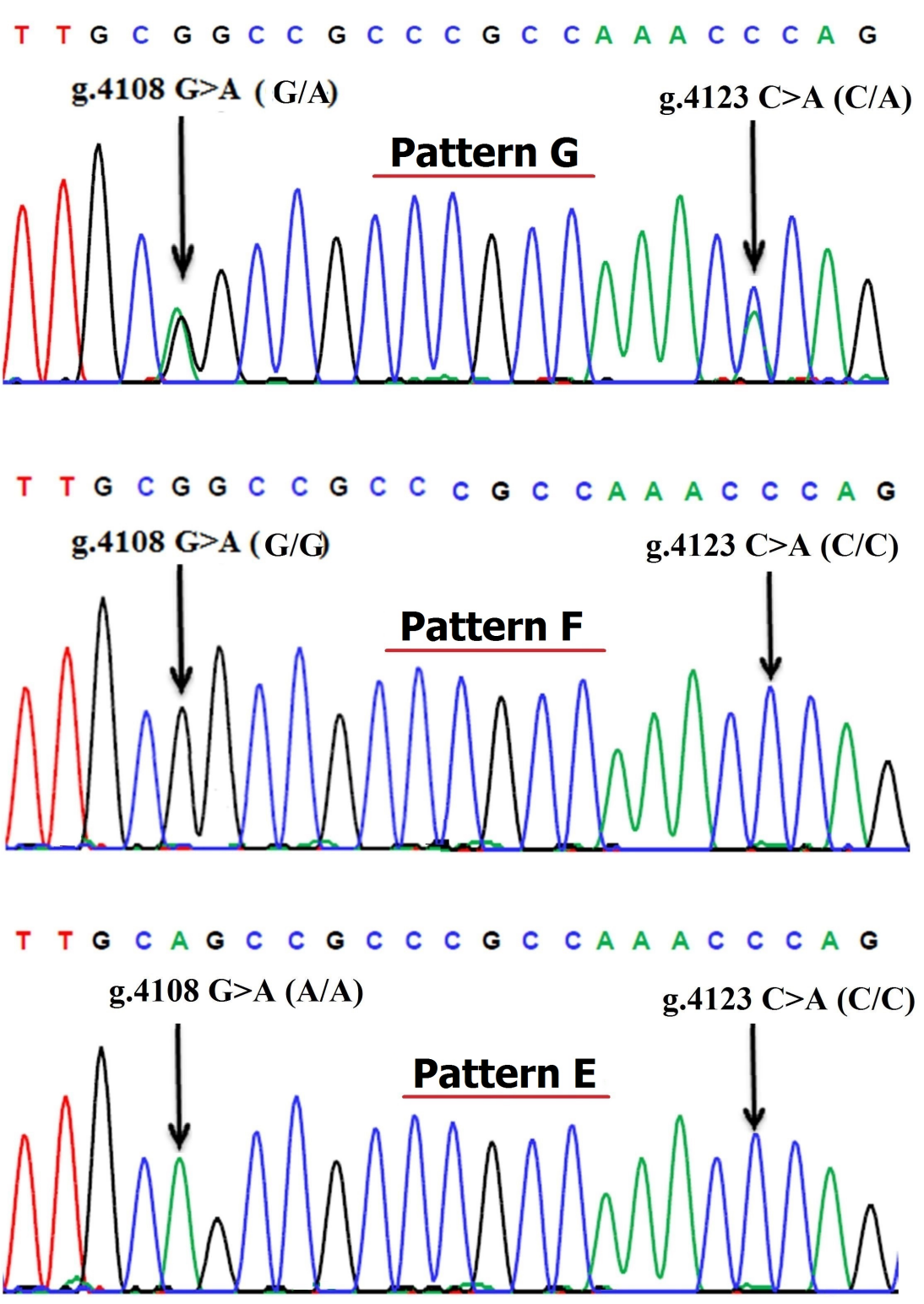
g.396 T>G (T/T)
T T G T C C T A
g.456 T>C (T/T)
G G C T C C C C A
g.475 C>A (C/C)
G G T A A G A G
g.571 A>C (A/A)
Pattern A
A A G N T T G A
g.396 T>G (T/G)
T T G N C C T A
g.456 T>C (T/C)
G G C T N C C C A
g.475 C>A (C/A)
G G T N A G A G
g.571 A>C (A/C)
Pattern B

B



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

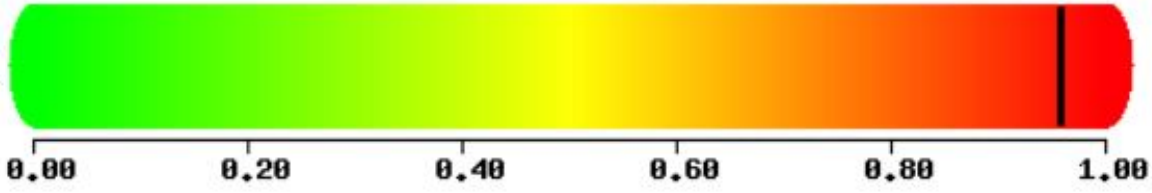


Table 1

Primer sequence, amplified region, product size and annealing temperature used in analyses of sheep *KISS1R*/*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′- CCAACTTCTCTGCCCACCTA -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

[1]Fwd and Rev : Forward and Reverse primers

Table 2

Identified SNPs in ovine *KISS1R*/*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 *KISS1R*/*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
	χ2		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
	χ2		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
	χ2		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; X^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
	P 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
	P 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
	P 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.