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Genotyping of MHC class II DRB3 gene using PCR-RFLP and DNA sequencing in small ruminant breeds of Western Himalayan state of Himachal Pradesh, India

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ABSTRACT

Sheep and goat farming especially under migratory system is an important activity in state of Himachal Pradesh. DRB locus of major histocompatibility complex is widely studied for its association with disease resistance. Present study was planned to analyse genetic diversity of DRB3 locus in small ruminant breeds of Himachal Pradesh using PCR-RFLP and DNA sequencing. 63, 68, 80 and 73 true to the breed animals belonging to Gaddi sheep, Rampur-Bushair sheep, Gaddi goats and Chegu goats, respectively were utilized. Amplification of exon 2 of DRB3 gene yielded 285bp amplified product in different breeds. Three different digestion patterns corresponding to 2 alleles and seven digestion patterns corresponding to 4 alleles were observed with PstI and HaeIII restriction digestion. For Pst 1 digestion frequency of AA, AB and BB genotypes ranged from 0.09 to 0.22, 0.40 to 0.53 and 0.28 to 0.46 in different breeds studied. For HaeIII digestion frequency of five genotypes (AA, BB, CC, AB and AC) which were detected in all breeds varied from 0.18 to 0.32, 0.11 to 0.13, 0.07 to 0.17, 0.18 to 0.30 and 0.09 to 0.3 in different breeds, while frequency of CD and AD genotype unique to Rampur-Bushair sheep population was 0.09 and 0.06, respectively. The observed allele number (No) ranged from 5 to 6 in different breeds while effective allele number (Ne) ranged from 4.46 to 5.15 in different populations. H_{obs} and H_{exp} values ranged 0.42 to 0.49 and 0.55 to 0.59, respectively in different breeds. The nucleotide variability was detected using sequences from different breeds which was found at 36 places. In the present study the specific amplification of the exon 2 of DRB3 gene of native sheep and goat populations demonstrated marked polymorphism. Further association studies need to be carried out to investigate the association of these SNPs of DRB region with parasitic diseases.

Key words: Genetic polymorphism, Migratory system, Small ruminants.

INTRODUCTION

Small ruminants, sheep and goats, play a vital role not only in meeting increased human demand for animal origin protein foods but also in sustainability of overall farming system in general and livestock production system in particular. The major histocompatibility complex (MHC) is a large genomic region or gene family encoding different MHC molecules having pivotal role in immune system and autoimmunity. There are two general classes of MHC molecules: Class I and Class II. Class I MHC molecules are found on almost all cells and present proteins to cytotoxic T cells. Class II MHC molecules are found on certain immune cells themselves, chiefly macrophages and B cells, also known as antigen-presenting cells (APCs) (Traherne *et al.*, 2006). The MHC of the goat, also named the caprine lymphocyte antigen (CLA), has been shown to be similar to that of sheep and cattle. Class II MHC genes have been extensively characterized in sheep and cattle, whereas in goats only four groups have been identified till date. The DRB locus forms the part of antigen binding groove in MHC cluster and hence exhibits high degree of variability, which enables it to identify a wide range of antigens and elicit proper immune response. The high degree of variability at MHC loci is intended to be an outcome of balancing selection at this locus. In all species where the MHC loci have been analyzed, maps of highly polymorphic sites have been used to identify the genetic factors associated with disease resistance and susceptibility within this region, and perform comparative genomic studies (Jamshidi *et al.*, 2011).

The DRB fragment has been known to be associated with diseases conditions in sheep, and it has been used as a putative genetic marker in some sheep breeds for resistance/ susceptibility, especially against gastrointestinal nematodes. Sheep and Goat farming is important activity in state of Himachal Pradesh. The goat and sheep population in Himachal Pradesh is 1.12 and 0.81 million respectively (Livestock Census, 2012) constituting 39.8% of state's total livestock of 4.85 million. This highlights the importance of sheep and goat farming especially migratory farming in the economy of state. Animals reared under extensive and migratory system experiences parasitic infestation to a greater extent and significant losses do occur every year due to parasitic infestation. Therefore keeping in view the above factor present study was planned to analyse genetic diversity

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of exon 2 locus in small ruminant breeds of Himachal Pradesh using PCR-RFLP and DNA sequencing.

MATERIALS AND METHODS

The experimental animals for the present study were taken from breeding tracts of different recognized sheep and goat breeds native to state of Himachal Pradesh. For screening of DRB 3 gene 63, 68, 80 and 73 true to the breed animals belonging to Gaddi sheep, Rampur-Bushair sheep, Gaddi goats and Chegu goats, respectively were utilized. The genomic DNA was isolated by phenol-chloroform extraction procedure (Sambrook and Russel, 2001). Concentration and purity of DNA were checked by using nanodrop spectrophotometer and DNA samples with OD 260/280 ratio of 1.7 to 1.9 were quality checked by using agarose gel electrophoresis. For amplification of CLA-DRB3*02 forward primer (5′ -TATCCCGTCTCTGCAGCAC ATTTC-3′) and reverse primer (5′ -TCGCCGCTGCACACT GAAACTCTC-3′) were used described by Amills *et al.*, (1995). A total of 50µl reaction mixture containing 100–200ng DNA template, 10 pm of each primer, 200µm of each dNTP, 2.0 mm MgCl, 1 U TaqDNA polymerase and 10x PCR assay buffer (Promega) was set up for amplification. The PCR amplification reaction consisted of an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 94°C for 45s, 63°C for 40 s and 72°C for 60s with a final extension step of 7 min at 72°C.

Amplified products obtained were subjected to agarose gel electrophoresis to see the amplification of desired region. The amplified PCR product was digested with two restriction enzymes: PstI and HaeIII (Fermentas, Hanover, MD). The reaction mixture was incubated at 37°C in water bath overnight, with the appropriate buffer. Digested products for PstI were subjected to agarose gel electrophoresis (2%), while HaeIII digested products were visualized using polyacrylamide gel electrophoresis (10%). Population genetic indices: gene heterozygosity (He), polymorphism information content (PIC) and effective allele numbers (Ne) were calculated utilizing PopGene software (Yeh *et al.*,1999). The test for genetic equilibrium was carried out by comparing observed genotypic frequencies with expected genotypic frequencies calculated from gene frequencies. The representative genotypes belonging to different breeds were subjected to Sanger sequencing after purification of amplified PCR products.

RESULTS AND DISCUSSION

The PCR amplification of exon 2 of DRB3 gene yielded 285 bp amplified product in different breeds (Fig 1) The PCR product of similar size has also been reported by Dongxiao and Yuan (2004) and Li and Zhao (2005) in Chinese local sheep and goat breeds; Ahmed and Othman (2006) in Egyptian goats; Baghizadeh *et al.*, (2009) in Raeini Cashmere goat; Hernandez (2011) in goats of the central highlands of Veracruz, Singh *et al.*, (2012) in Jamunapari breed of goat, Ashrafi *et al.* (2014) in Iranian Makuie sheep breed and Prakash *et al.*, 2014 in Marwari goats.

PCR-RFLP analysis of DRB3: The amplified product of DRB3 gene was digested with PstI and HaeIII restriction enzymes and digestion patterns obtained for respective enzymes are presented in Table 1. Two alleles (A and B) and three genotypic patterns (AA, AB and BB) were observed for Pst I digestion, while four alleles (A, B, C and D) and seven patterns (AA, BB, CC, AB, AC, CD, AD) were observed for Hae III digestion.

PCR-RFLP patterns using Pst I restriction enzyme: PCR-RFLP was detected with restriction enzymes PstI. On digestion three different digestion patterns (Fig 2) were observed with Pst1 (270 bp, 226bp/44bp and 270 bp/226 bp/ 44 bp) corresponding to two alleles A and B. The frequency of alleles and corresponding genotypes in different breed investigated are given in Table 2.

Allele frequencies of an allele was 0.65, 0.42, 0.31 and 0.46 and for B allele 0.35, 0.58, 0.69 and 0.54 in Gaddi sheep, Rampur-Bushair sheep, Gaddi goat and Chegu goat, respectively. The frequency of AA, AB and BB genotypes ranged from 0.09 to 0.22, 0.40 to 0.53 and 0.28 to 0.46 in different breed studied. Baghizadeh *et al.*, (2009) in Raeini cashmere goats detected 3 genotypes and 2 alleles similar to present study. In a study of 10 domestic goat breeds of China, Zhao *et al.*, (2011) also detected similar digestion pattern but one more pattern (158bp/79bp/48bp) was observed in their study which was not recorded in present investigation.

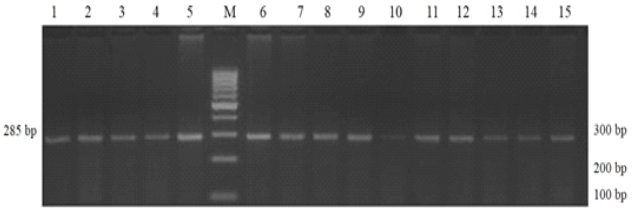


Fig 1: PCR products representing amplification of DRB3 gene in different breeds. Lane M: 100 bp ladder. Lanes 1 to 15, 280 bp PCR products. M: 100 bp DNA ladder.

Table 1 : Restriction pattern of exon2 of DRB 3 gene observed with different enzymes.

Restriction enzyme	Genotype of each restriction enzyme
PstI	AA: 270bp/15bp
	BB: 226bp/44bp/15bp
	AB: 270bp/226bp/44bp/15bp
HaeIII	AA: 154bp/131bp
	BB: 154bp/117bp/14bp
	CC: 154bp/65bp/52bp/14bp
	AB: 154bp/131bp/117bp
	AC: 154bp/131bp/65bp/52bp/14bp
	CD: 220bp/154/65/52/14
	AD : 220bp/154bp/131bp/65bp

Table 2: Allele and genotypic frequencies for Pst1 digestion of DRB3 gene in different breeds.

Breed	N	Allele frequencies		Genotypic frequencies		
		A	B	AA	AB	BB
Gaddi sheep	63	0.65	0.35	0.13(8)	0.44(28)	0.43(27)
Rampur-Bushair sheep	68	0.42	0.58	0.22(15)	0.40(27)	0.38(26)
Gaddi goat	80	0.31	0.69	0.09(7)	0.45(36)	0.46(37)
Chegu goat	73	0.46	0.54	0.19(14)	0.53(39)	0.28(20)

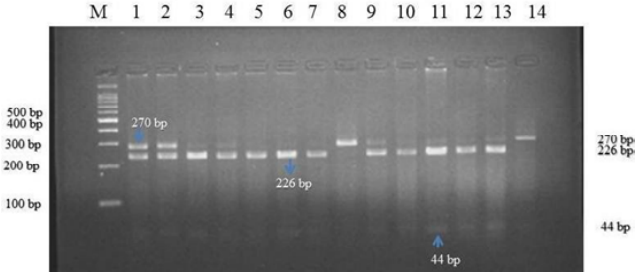


Fig 2: Electrophoretic pattern obtained after digestion of PCR product of DRB3 gene with PstI RE Lane 8-14: AA genotype with 270bp and 44bp fragments; Lane 1,2, 9 and 13 AB genotype with 270 bp, 226 bp, and 44 bp fragments; Lane 3-7, 10-12 BB genotype with 226bp and 44bp fragments. M:

Table 3: Allele and genotypic frequencies for HaeIII digestion of DRB3 gene in different breeds.

	Breed			
	Gaddi sheep	Rampur-Bushair sheep	Gaddi goat	Chegu goat
N	63	68	80	73
Genotype frequencies				
AA	0.32(20)	0.18(12)	0.23(18)	0.29(21)
BB	0.13 (8)	0.15(10)	0.20(16)	0.11 (8)
CC	0.17(11)	0.09 (6)	0.07 (6)	0.16(12)
AB	0.29(18)	0.18(12)	0.19(15)	0.30(22)
AC	0.09 (6)	0.26(18)	0.31(25)	0.14(10)
CD	—	0.09 (6)		
AD	—	0.06 (4)		
Gene frequencies				
A	0.51	0.43	0.48	0.51
B	0.27	0.24	0.29	0.26
C	0.23	0.26	0.23	0.23
D	—	0.07	—	—

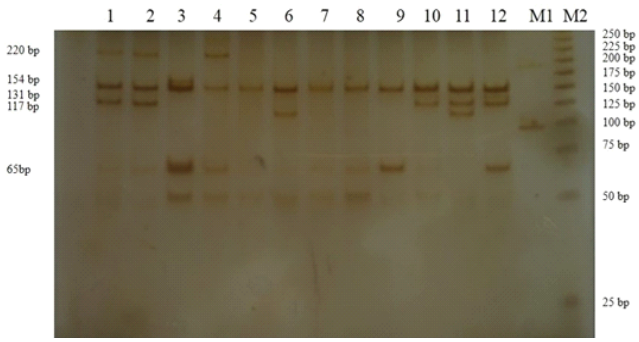


Fig 3: DRB gene Hae III digestion patterns repressing different genotypic patterns. M 25 bp DNA ladder.

The results of present study are also in congruence with findings of Yadav *et al.* (2016) who reported similar amplification and digestion pattern in Sirohi and Barbari goat breeds of India with frequencies of genotypes AA, AB and BB as 0.53, 0.37 and 0.10, respectively in Sirohi breed and 0.73, 0.20 and 0.07, respectively in Barbari goat breed. Sheikh *et al.* (2006) in Changthangi goats reported genotypic frequencies of AA, AB and BB as 0.07, 0.72 and 0.20 with allelic frequencies of A and B alleles to 0.43 and 0.57, respectively. In Egyptian goats (Ahmed and Othman, 2006), Raeini cashmere goats (Baghizadeh *et al.*, 2009) and Jamunapari goats (Khobra *et al.*, 2013), the genotypic frequencies of AA, AB and BB were reported to be 0.00, 0.71 and 0.29; 0.21, 0.59 and 0.20; and 0.054, 0.22 and 0.72, respectively. Further, the allelic frequencies of A and B in the above three breeds was reported to be as 0.35 and 0.65; 0.51 and 0.49 and 0.17 and 0.83, respectively. Singh *et al.* (2012) in a study of Jamunapari breed of goat reported three genotypes with frequency 0.05, 0.23 and 0.73, respectively. In a study of on ten goat breeds of China (Zhao *et al.*, 2011) the genotypic frequency of AA varied from 0.0 to 0.59, AB varied from 0.10 to 0.80 and BB varied from 0.20 to 0.86. In one breed fourth genotype i.e. CC was also reported with a frequency of 0.57. These differences in allelic frequencies might be due to the fact that the different breeds/populations maintained under different environmental conditions are subjected to different evolutionary forces to varying degree**.**

PCR-RFLP patterns using HaeIII restriction enzyme: The PCR-RFLP was detected with HaeIII digestion. On digestion with Hae III restriction enzyme, the PCR-RFLP revealed 7 different digestion patterns (Fig 3) corresponding to alleles A, B, C and D. The frequency of different alleles and corresponding genotypes in different breeds are given in Table 3. The observed number of alleles for Gaddi sheep, Rampur -Bushair sheep, Gaddi goat and Chegu goat were 3, 4, 3 and 3 respectively and number of genotypes were 5, 7, 5 and 5 respectively. In Rampur-Bushair sheep population, allele D was observed, which was not detected in other three breeds. The allele frequency for A allele were 0.51, 0.43, 0.48 and 0.51, allele frequencies for B allele were 0.30, 0.24, 0.29 and 0.26 and allele frequency for C allele were 0.19, 0.26 0.23 and 0.23 in Gaddi sheep, Rampur-Bushair sheep, Gaddi goat and Chegu goat respectively. Allele D was detected only in Rampur-Bushair sheep with frequency of 0.07. The frequency of five genotypes (AA, BB, CC, AB

and AC) which were detected in all breeds varied from 0.18 to 0.32, 0.11 to 0.13, 0.07 to 0.17, 0.18 to 0.30 and 0.09 to 0.3 in different breeds. The frequency of CD and AD genotype unique to Rampur-Bushair sheep population was 0.09 and 0.06, respectively.

Similar digestion patterns were reported in a study of ten domestic goat breeds of China, Zhao *et al.* (2011), but 8 different alleles and 13 different patterns were observed. The more number of patterns observed were attributed to the fact that study involved ten goat populations thus expressing higher genetic diversity. The results of present investigation were also similar to Li *et al.* (2010) in Kazakh sheep revealing 6 different alleles. Konnai *et al.* (2003) in Suffolk sheep also reported 6 alleles of PCR-RFLP with HaeIII restriction enzyme.

Test for genetic equilibrium: The Chi-square value estimated for different breeds are presented in Table 4. The results revealed that for PstI patterns no significant deviation was observed from HWE. For HaeIII enzyme site, all breeds significantly deviated from HWE. Baghizadeh *et al.* (2009) in Raeini Cashmere goat observed no deviation from HWE for TaqI and PstI enzyme site. Similarly Zhao *et al.* (2011) in investigation of 10 domestic goat breeds of China observed that 4 breeds significantly differed from HWE for PstI enzyme site, while all breeds were out of HWE for HaeIII enzyme site. Similarly, Ashrafi *et al.* (2014) in Iranian Makuie sheep breed also observed no significant deviation from HWE. Yadav *et al.* (2016) also reported HWE at DRB locus in study of Sirohi and Barbari goats.

Genetic diversity analysis: Various measures of genetic diversity analysis are given in Table 5. The observed allele number (No) ranged from 5 to 6 in different breeds while effective allele number (Ne) ranged from 4.46 to 5.15 in different populations. H_{obs} and H_{exp} values ranged 0.42 to 0.49 and 0.55 to 0.59, respectively in different breeds. Results of present finding are in close agreement with study of Zhao *et al.* (2011) in 10 different Chinese goat populations who reported No and Ne ranging from 5-9 and 3.15-6.30 respectively. The observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}) and PIC ranged from 0.34 to 0.45, 0.50 to 0.54, and 0.30 to 0.41, respectively in different populations. In present study H_{obs} was highest in Gaddi goat followed by Rampur-Bushair sheep, Chegu goat and Gaddi sheep which indicates that at the studied locus polymorphism and genetic variation was highest in Gaddi goat and lowest in Chegu goat. The high H_{obs} and PIC value indicated the usefulness of locus in genetic diversity studies and indicate that sufficient genetic diversity exist at the studied locus. Baghizadeh *et al.* (2009) documented H_{obs} and H_{exp} as 0.51 and 0.53, in Raieni cashmere goats which were almost similar to those observed in present study. Zhao *et al.*, (2011) in 10 different Chinese goat populations reported H_e and PIC ranging from 0.36 to 0.63 and 0.32 to 0.55, respectively. Ashrafi *et al.* (2014) also reported No and He as 3.72 and 0.73, respectively in Iranian Makuie sheep breed. Shrivastva *et al*. (2015) reported He and PIC value as 0.28 and 0.33 in Rohilkhandi goats, which was lower than those observed in present study. The present study also revealed highly polymorphic nature of DRB3 gene exon 2 in different populations studied. Earlier reports by several researchers in both sheep and goat also documented extensive polymorphism at DRB locus (Jugo and Vicario 2000 in Latxa and Karrantzar sheep; Gruszczynska *et al.,* 2004 in Polish sheep; Li and Zhao 2005 in Chinese goat; Brujeni *et al.,* 2009 in Iranian Shaul sheep; Riggio *et al.*, 2013 in Scottish Black sheep).

Table 4: Test for genetic equilibrium of studied breed for different restriction enzymes.

Breed	Restriction enzyme	
	PstI	HaeIII
Gaddi sheep (GS)	0.05(0.81)	58.28(<0.01)*
Rampur-Bushair sheep (RB)	2.50(0.11)	33.91(<0.01)*
Gaddi goat (GG)	0.14 (0.71)	29.67(<0.01)*
Chegu goat (CG)	0.34 (0.55)	34.74(<0.01)*

*Significantly deviating from HWE.

Sanger sequencing of PCR products: Purified PCR products representing genotypes in different breeds were got sequenced. The sequences were subjected to BLAST analysis to ascertain that they belong to DRB 3 gene and to check accuracy of PCR-RFLP detection. Chromatograms representing different alleles detected by PCR-RFLP are presented in Fig 4 and 5. The representative sequenced samples were analyzed with DNASTAR and consensus sequences for different breeds were generated from sequenced sample. Sequence of each breed is aligned with reference sequence from NCBI database by using MegAlign and aligned sequences are presented in figure. The nucleotide variability wad detected using sequences from different breeds which was found at 36 places (Fig 6).

Table 5: Genetic variation for DRB3 gene (Pst1 and HaeIII digestion) in different breeds.

Breed	No	Ne	H_{obs}	H_{exp}	PIC	F_{IS}
Gaddi sheep (GS)	5	4.46	0.42	0.55	0.51	0.18
Rampur-Bushair sheep (RB)	6	5.15	0.49	0.59	0.54	0.06
Gaddi goat (GG)	5	4.49	0.48	0.53	0.49	0.16
Chegu goat (CG)	5	4.63	0.49	0.56	0.52	0.08

No: observed no of allele; Ne: effective no of alleles; He: Heterozygosity; PIC; Polymorphic information content: F_{IS} : Fixation index.

The protein profile of PstI allele studied in present investigation has clearly established amino acid change as reported earlier by Amills *et al.,* 1996. The presence of PstI site has been associated with TAC codon (Tyrosine) whereas its absence was linked to TGT codon (cysteine). The replacements of this region are thought to be functionally important for antigen recognition by antigen binding site. In fact, these proteins are primarily responsible for synthesis of antigen presenting domain of cell surface and the changes of protein structure probably influence the stringency of epitope binding and ultimately affect the host immune response against the causative organism (Amills *et al.,* 1996).

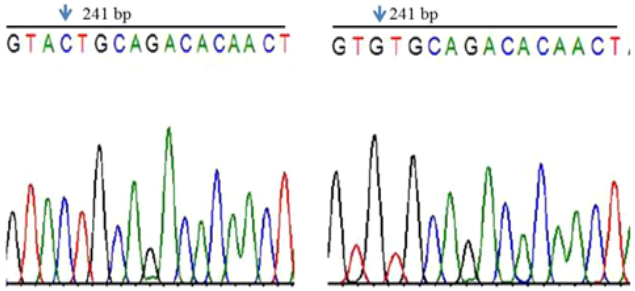


Fig 4: Chromatogram representing C/G substitution in DRB gene (Pst1 restriction digestion).

These SNPs have demonstrated enormous potential to be used as marker for identifying the animals as susceptible or resistant to disease (Hohenhus and Outteridge 1995; Outteridge 1996). Sayer *et al.,* (2005) reported association of DRB genotype with faecal egg count in Suffolk sheep. Li *et al.,* (2010) reported significant association of DRB genotypes with hyadatidosis in Kazakh sheep. In the present study the specific amplification of the exon 2 of DRB3 gene native sheep and goat populations demonstrated marked polymorphism.

Studies related to internal parasitic infestations and their association with genetic diversity in MHC region has suggested that some alleles of MHC DRB gene might augment the immune response against parasitic antigens and also may hamper parasitic fecundity inside the host (Shrivastava *et al.,* 2018a). The SNP observed in the present study are similar to those reported by Shrivastava *et al.,* (2018b) in Indian goat breeds, where significant association was reported with faecal egg count (FEC) to mixed natural infection of *Haemonchus contortus*. Thus, further association studies need to be carried out to investigate the association of these SNPs of DRB region with parasitic diseases in native

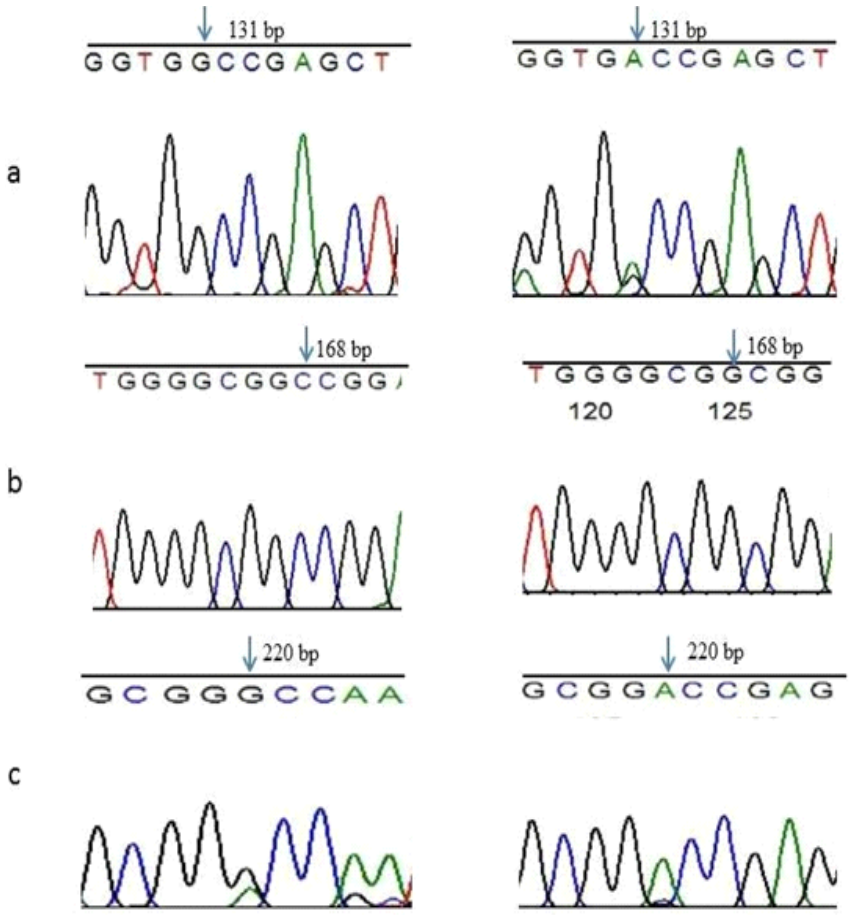
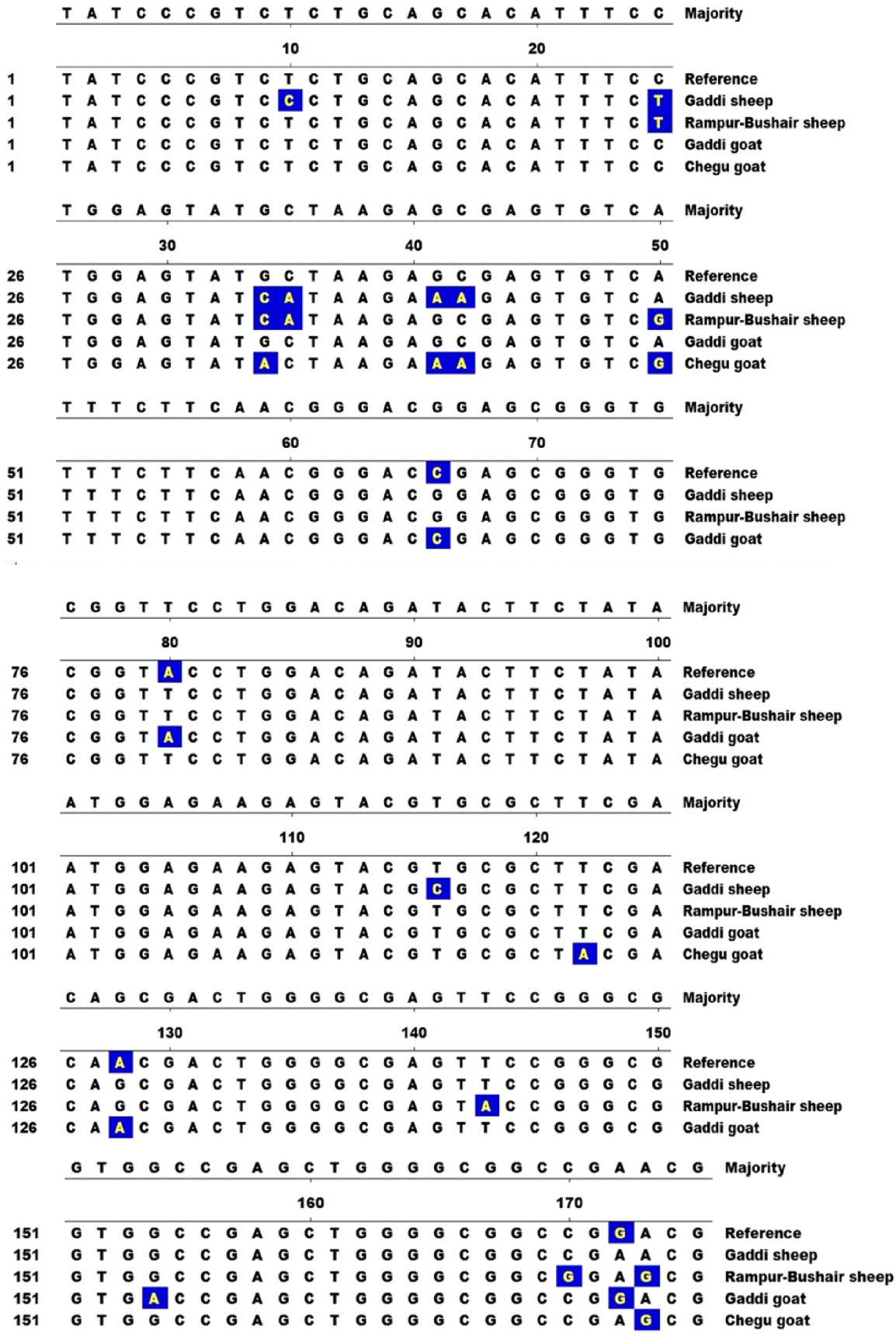


Fig 5 (a-c): Chromatogram representing G/A (a; 154bp), C/G (b; 168bp) and G/A(c; 220 bp) substitutions in DRB gene (Hae III restriction digestion.


Majority
10
20
1 Reference
1 Gaddi sheep
1 Rampur-Bushair sheep
1 Gaddi goat
1 Chegu goat
Majority
30
40
50
26 Reference
26 Gaddi sheep
26 Rampur-Bushair sheep
26 Gaddi goat
26 Chegu goat
Majority
60
70
51 Reference
51 Gaddi sheep
51 Rampur-Bushair sheep
51 Gaddi goat
Majority
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90
100
76 Reference
76 Gaddi sheep
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76 Chegu goat
Majority
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101 Reference
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101 Rampur-Bushair sheep
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151 Rampur-Bushair sheep
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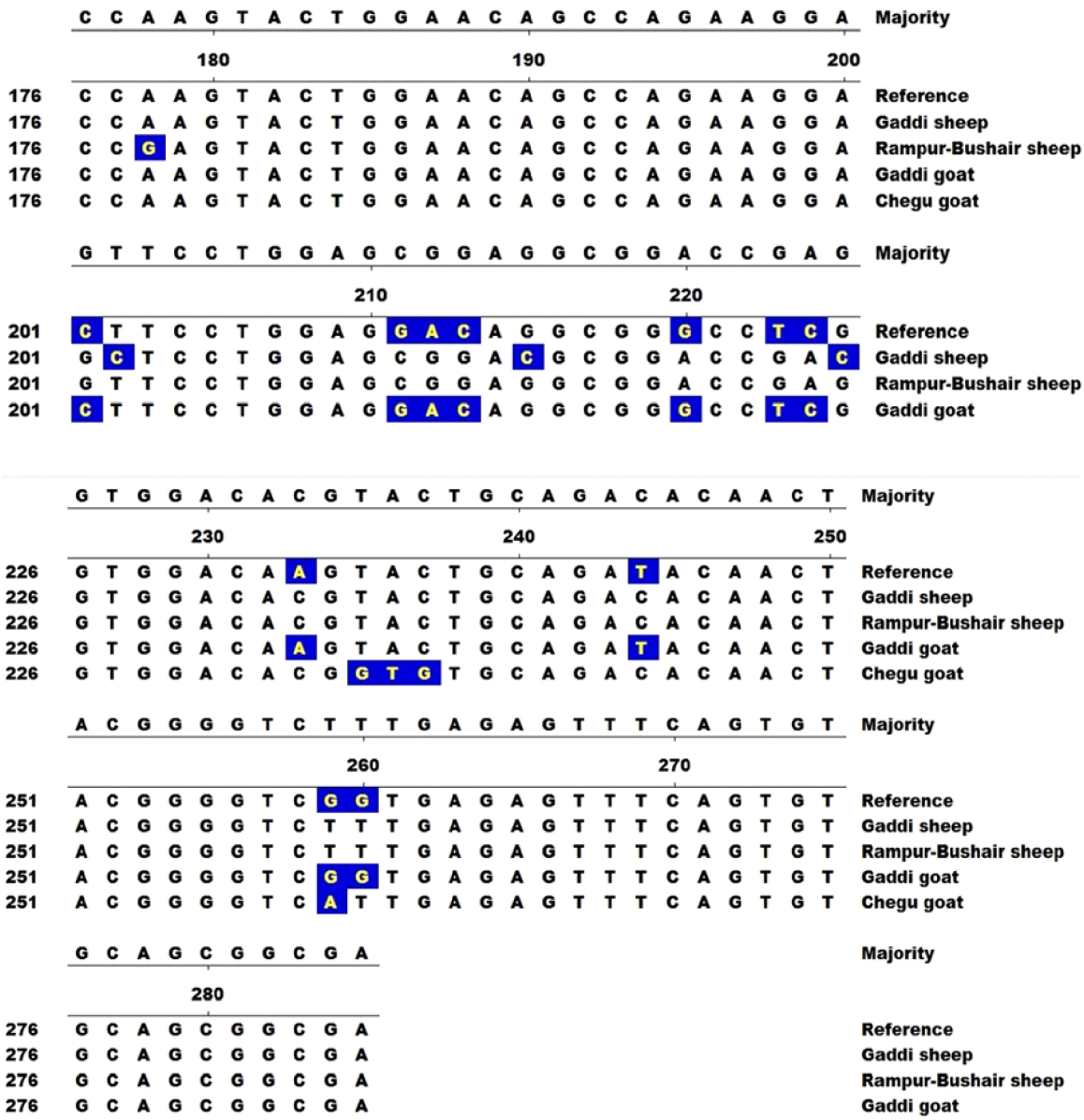

Decoration 'Decoration #1′: Shade (with solid bright cobalt) residues that differ from the Consensus.

Fig 6: Sequence alignment using MegAlign for DQB gene in studied breeds highlighting residue substitutions.

sheep and goat breeds of Himachal Pradesh. Since the populations studied are reared under extensive and migratory system which experiences parasitic infestation to a greater extent and significant losses do occur every year due to parasitic infestation, the future association studies will be of great help in designing suitable breeding strategies to improve parasitic resistance.

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