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Sequence Characterization of Ovine MHC Class II (DRB I Gene) in Indian Sheep Breeds

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A B S T R A C T

The major histocomtability complex in sheep is also called as Ovar- MHC and is a central molecule for antigen presentation coding for specialized antigen presenting glycoproteins. 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. The exon 2 of DRB 1 gene forms the part of antigen loading clamp of MHC and hence carries the greatest importance in relation to disease resistant research. We sequenced the target exon in four Indian sheep breeds, the sequences were submitted to NCBI. The sequences were then studied using bioinformatic tools to ascertain its structural features and phylogenetic relationship. Finally it was observed that the variation between the sequences is majorly present in intronic region which forms an excellent marker to study the inheritance of adjoining exon also. Variations within the exons were mostly neutral and didn't reflect in secondary structure as well as there was no change in the ligand binding site.

Introduction

The major histocompatibility complex in sheep is also called as Ovar- MHC and is a central molecule for antigen presentation coding for specialized antigen presenting glycoproteins (Dukkipati *et al.,* 2006). The MHC molecule is an important candidate gene for disease resistance studies in farm animals (Dukkipati *et al.,* 2006). The MHC is a group of closely linked genetic loci that tend to inherit together. In sheep the MHC is divided into three subgroups class I, class II and class III. The class I region however is poorly defined for the presence of number loci, that range from 2 to 4. The class II region however is much better characterized in sheep and consists of five loci viz., DR, DQ, DY, DM and DN/DO (Dukkipati *et al.,* 2006). The loci DR and DQ are most important and have been extensively used in disease resistant studies in sheep (Hulme *et al.,* 1991;

Litchfield *et al.,* 1993; Escayg *et al.,* 1997; Shrivastava *et al.,* 2015). The DR subgroup is further divided as DRA (coding for alpha chain of the peptide binding groove) and DRB (coding for beta chain of the peptide binding groove). The DRB loci is further divided into DRB1, DRB2, DRB3 and DRB4 regions among which DRB1 is coding and rest three are non coding genes (Dukkipati *et al.,* 2006; Shrivastava *et al.,* 2015). The DRB locus along with the DRA codes for the antigen binding groove of MHC therefore it displays a high degree of genetic variability to deal with variety of antigens (Tizzard, 2013). This diversity in its structure not only accounts for variety in antigens but also makes it a candidate gene for association studies especially involving disease / immune related traits (Li *et al.,* 2010). Beside the high degree of polymorphism exhibited by DRB locus in sheep one major characteristic feature of DRB region is presence of microsatellite repeat region (Dukkipati *et al.,* 2006). Because of its large size and presence of pseudogenes the full characterization of MHC gene is difficult. There are ample amount of MHC DRB sequences available of various sheep breeds from all over the world however data regarding sequence characterization in Indian sheep breeds is yet scanty. India possess a huge genetic diversity in sheep genetic resource consisting of 42 recognized sheep breeds (http://www.nbagr.res.in/). This high breed diversity is found scattered along 15 agro-climatic regions of the country (Kumar *et al.,* 2003). There are reports suggesting presence of unique morphological, physiological, productive, reproductive and health traits in these breeds. Some breeds like Garole (Nimbkar *et al.,* 2003), Magra, Bikaneri (Singh and Swarnkar, 2014) are known to possess inherent traits of being resistance to diseases especially to internal parasitic infestation that forms the major production and animal losses in sheep industry (Anonymous, 2012). 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 (Dukkipati *et al.,* 2006). Looking into these important facts we carried out sequencing and characterization of MHC DRB gene fragment in four sheep breeds of India viz., Muzaffarnagri, Chokla, Marwari and Malpura. The sequences were compared with existing sequences of major sheep breeds and were further analyzed on nucleotide and amino acid level using bioinformatic tools.

Materials and Methods

DNA isolation and PCR amplification

Four Indian sheep breeds viz., Muzaffarnagri, Chokla, Marwari and Malpura were selected for the current study. DNA was isolated from blood using the standard Sambrook and Russell (2001) method, quality checked on 0.8 % agarose gel electrophoresis and nanodrop spectrophotometer. Only the good quality DNA showing intact band and $OD_{260/280}$ value ranging from 0.7-0.8 were further used for PCR amplification. For amplification of desired sequence (MHC DRB exon 2) oligonucleotide primers were used as reported by Amills *et al.,* (1998). Template DNA was amplified using standard PCR mix with 80 – 100 ng of genomic DNA, 12.5 µl of PCR master mix (ThermoFisher Scientific), 10 pmoles of each primer (forward and reverse) and nuclease free water to make volume 25 µl. The contents were thoroughly mixed and set up in a thermocycler (ABI thermocycler) with the following amplification programme; initial denaturation at 95°C for 5 min, followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 59.0°C for 45 s and extension at 72°C for 1 min, and finally the last extension at 72°C for 5 min. The PCR products were

checked by agarose gel electrophoresis using 1.0% agarose gel in 1X Tris-borate-EDTA buffer at 6 V/cm for 1 h.

Sequence analysis

Amplified PCR products were then cleaned using GeneJet PCR purification kit (Thermo Scientific) and rechecked using 1.0 % agarose gel electrohporesis. The samples were sent for sequencing by automated sequencer via outsourcing. The raw sequences were first assembled using bioedit and DNA Baser programme. The final assembled sequences were then aligned using Clustal W in MEGA 6.0 software. Related sequences were then downloaded in FASTA formats from NCBI using BLAST tool. Further analysis of sequences was carried out by MEGA 6.0 and DNAStar. For protein structure and binding site prediction two online servers viz., Phyre 2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley *et al.,* 2015) and 3D ligand site (http://www.sbg.bio.ic.ac.uk/3dligandsite/) (Wass *et al.,* 2010) were used.

Results and Discussion

The raw sequences obtained were assembled using Bioedit and DNA baser programme. The final assembled sequences were submitted to NCBI database and accession numbers were assigned to them as KT878759 (Marwari sheep), KT878758 (Malpura sheep), KT764074 (Chokla sheep; Haplotype 1), KT764075 (Chokla sheep; Haplotype 2), KT764076 (Chokla sheep; Haplotype 3), KT764077 (Chokla sheep; Haplotype 4), KT781587 (Muzaffarnagri sheep; Haplotype A), KT781588 (Muzaffarnagri sheep; Haplotype B). On the basis of dissimilarities after alignment the haplotypes were made, total four haplotypes were found in Chokla sheep designated from 1 to 4, and two were found in Muzaffarnagri sheep designated as A and B. There were total 98 dissimilarities between the sequences including the exons and introns, when the introns were trimmed and only exons were analysed the total sites of disagreement between the sequences was 48, which shows that a major portion of the variation was present in introns, specially in the repeat region. For comparison, sequences of the same region from Western sheep breeds, Merino (Acc No. U00227), Ramboullet (Acc. No. EU176819) and Texel (Acc. No. U00232) were downloaded from NCBI. The sequences were then aligned using Clustal W in MEGA 6.0 (Fig. 1). The exon two of MHC DRB spanned from 8th base pair to 277th base pair (bp) followed by the intron 2 region. The characteristic feature of the sequence was the tandem repeats present in intron 2 consisting of GT and AG dinucleotide repeats (Fig. 1). This repeat region is actually a microsatellite locus which is a prominent feature of introns in ovine MHC gene. A heat map was generated using heatmapper (Babicki *et al.,* 2016) to ascertain the pair-wise distance between the sequences. Within the Indian breeds, haplotypes of Chokla breed 3 and 2 were close in similarity and haplotypes 4 and 1 were distant apart. In Muzaffarnagri sheep, both the haplotypes were distant and the haplotype A was closer to Chokla haplotype 1 and 4 (Fig. 2).

Amino acid comparison was only done between the studied Indian breeds. The nucleotide codons were translated and aligned using MEGA 6.0. The total length of amino acid chain was 89 amino acid long with 26 sites of disagreement between the sequences (Fig. 3), and amino acid composition analysis showed maximum percentage of arginine (12.08%) followed by glutamic acid (11.66%) and methionine was found least (nil) (Table 1). The overall average evolutionary divergence was low *i.e*., 0.13 among the sequences. The secondary structure prediction using phyre2 server showed that all the sequences contained a majority of beta sheet

structure, ranging from 38% – 44 %. The secondary structures were then used to predict possible ligand binding site prediction and it was found that in all the sequences, amino acid serine (at position 83) and glutamine (at position 87) were having the highest confidence (Fig. 4).

Fig.1 Nucleotide alignment of all the studied sequences as done by Mega 6.0

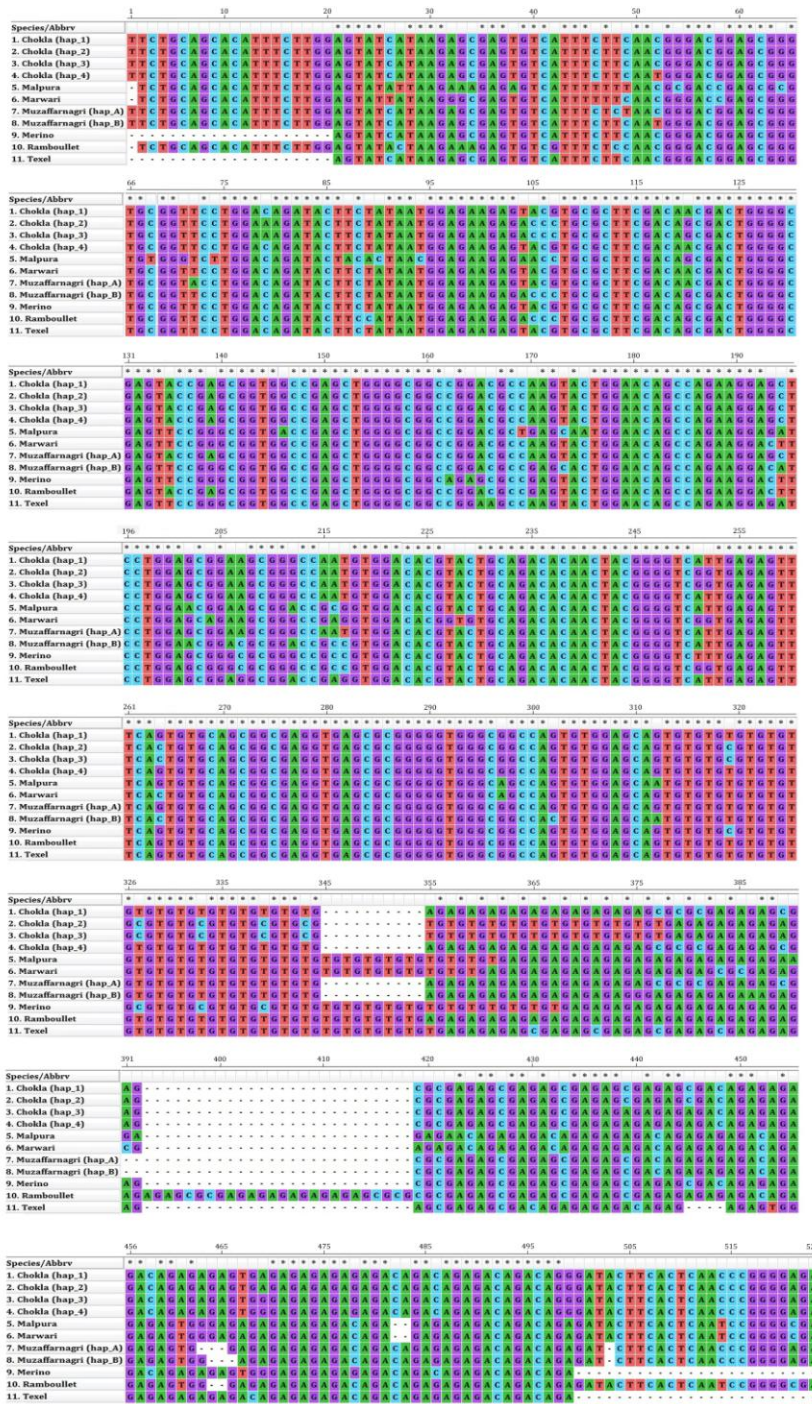
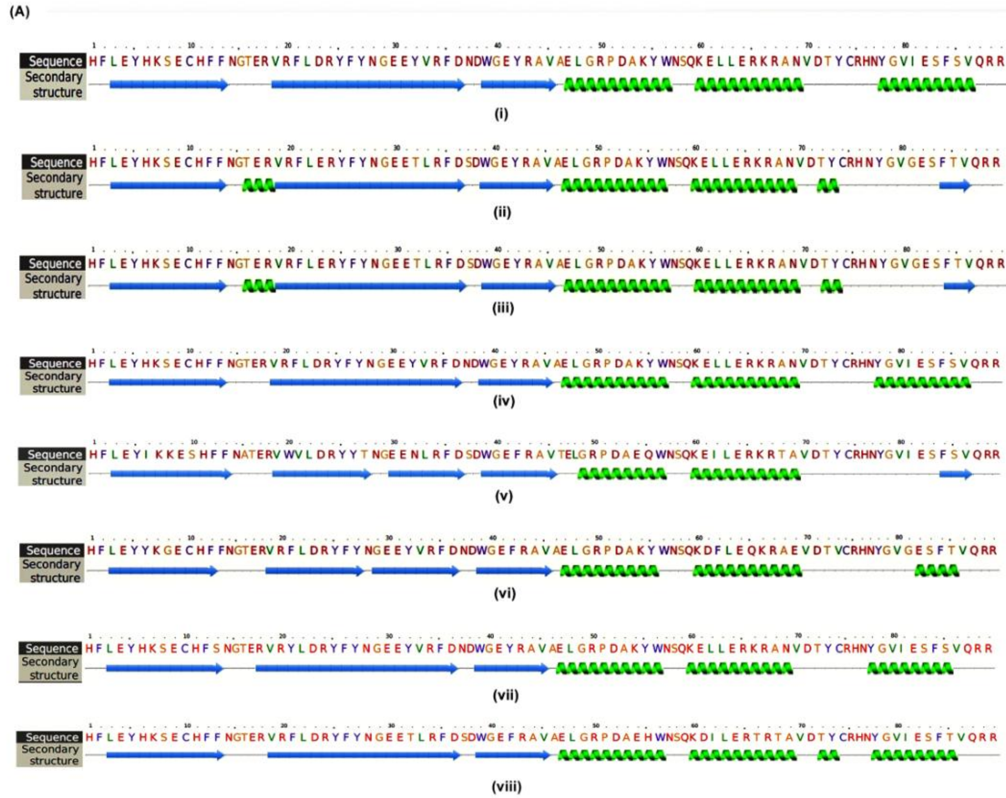


Fig.2 Heat map of pair wise comparison of the studied sequences done by Heat mapper. Colour from blue to yellow indicates similarity scale, blue being the indicator for similarity

Fig.3 Amino acid alignment of the translated exon portion of Indian sheep breeds as done by Mega 6.0

Species/Abbrv	Sequence
1. Chokla_Hap_1	HFLEYHKSECHFFNGTERVRFLDRYFYNGEEYVRFDNDWGEYRAVAELGRPDAKYWNSQKELLERKRANVDTYCRHNYGVIESFSVQRR
2. Chokla_Hap_2	HFLEYHKSECHFFNGTERVRFLERYFYNGEETLRFDSDWGEYRAVAELGRPDAKYWNSQKELLERKRANVDTYCRHNYGVGESFTVQRR
3. Chokla_Hap_3	HFLEYHKSECHFFNGTERVRFLERYFYNGEETLRFDSDWGEYRAVAELGRPDAKYWNSQKELLERKRANVDTYCRHNYGVGESFTVQRR
4. Chokla_Hap_4	HFLEYHKSECHFFNGTERVRFLDRYFYNGEEYVRFDNDWGEYRAVAELGRPDAKYWNSQKELLERKRANVDTYCRHNYGVIESFSVQRR
5. Muzaffarnagri_Hap_A	HFLEYHKSECHFSNGTERVRYLDRYFYNGEEYVRFDNDWGEYRAVAELGRPDAKYWNSQKELLERKRANVDTYCRHNYGVIESFSVQRR
6. Muzaffarnagri_Hap_B	HFLEYHKSECHFFNGTERVRFLDRYFYNGEETLRFDSDWGEFRAVAELGRPDAEHWNSQKDILERTRTAVDTYCRHNYGVIESFTVQRR
7. Malpura	HFLEYIKKESHFFNATERVWVLDRYYTNGEENLRFDSDWGEFRAVTELGRPDAEQWNSQKEILERKRTAVDTYCRHNYGVIESFSVQRR
8. Marwari	HFLEYYKGECHFFNGTERVRFLDRYFYNGEEYVRFDNDWGEFRAVAELGRPDAKYWNSQKDFLEQKRAEVDTVCRHNYGVGESFTVQRR

Fig.4 (A) Secondary protein structure prediction of the translated amino acids; (i) Chokla hap 1, (ii) Chokla Hap 2, (iii) Chokla Hap 3, (iv) Chokla Hap 4, (v) Malpura, (vi) Marwari, (vii) Muzaffarnagri Hap A and (viii) Muzaffarnagri Hap B; (B) Ligand binding site prediction of translated amino acid sequence taking Malpura breed as representative



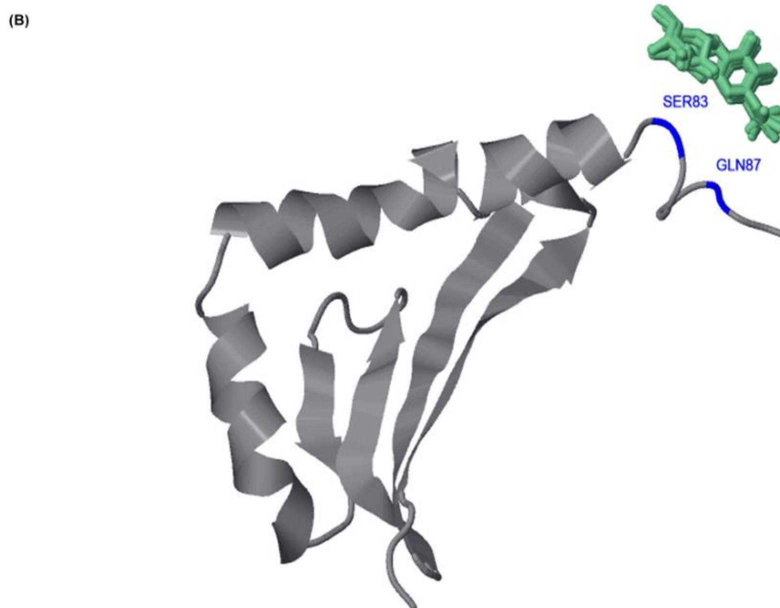


Table.1 Amino acid composition of all the studied sequences (values are expresses as percentage)

Name	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr
Marwari	4.49	2.25	6.74	11.24	10.11	7.87	3.37	0.00	4.49	4.49	0.00	5.62	1.12	3.37	11.24	2.25	3.37	7.87	2.25	7.87
Malpura	4.49	1.12	5.62	12.36	6.74	4.49	3.37	3.37	4.49	5.62	0.00	5.62	1.12	3.37	11.24	5.62	5.62	6.74	3.37	5.62
Chokla Hap 4	4.49	2.25	5.62	11.24	7.87	5.62	4.49	1.12	4.49	5.62	0.00	6.74	1.12	2.25	12.36	4.49	2.25	6.74	2.25	8.99
Chokla Hap 3	4.49	2.25	4.49	12.36	7.87	6.74	4.49	0.00	4.49	6.74	0.00	5.62	1.12	2.25	12.36	4.49	4.49	5.62	2.25	7.87
Chokla Hap 2	4.49	2.25	4.49	12.36	7.87	6.74	4.49	0.00	4.49	6.74	0.00	5.62	1.12	2.25	12.36	4.49	4.49	5.62	2.25	7.87
Chokla Hap 1	4.49	2.25	5.62	11.24	7.87	5.62	4.49	1.12	4.49	5.62	0.00	6.74	1.12	2.25	12.36	4.49	2.25	6.74	2.25	8.99
Muzaffarnagri Hap A	4.49	2.25	5.62	11.24	5.62	5.62	4.49	1.12	4.49	5.62	0.00	6.74	1.12	2.25	12.36	5.62	2.25	6.74	2.25	10.11
Muzaffarnagri Hap B	4.49	2.25	6.74	11.24	8.99	5.62	5.62	2.25	2.25	5.62	0.00	4.49	1.12	2.25	12.36	4.49	6.74	5.62	2.25	5.62
Average	**4.49**	**2.11**	**5.62**	**11.66**	**7.87**	**6.04**	**4.35**	**1.12**	**4.21**	**5.76**	**0.00**	**5.90**	**1.12**	**2.53**	**12.08**	**4.49**	**3.93**	**6.46**	**2.39**	**7.87**

The MHC class II DRB 1 gene carries immense importance as it forms the antigen binding cleft and presents these exogenous peptides to the T cell receptors of CD4+ helper T cells (Germain and Margulies, 1993). The DRB gene codes for the beta (β) chain of the MHC heterodimer and at least four DRB loci has been identified i.e., DRB1 which is the coding gene and DRB2, DRB3, DRB4 (non coding) (Dukkipati *et al.,* 2006). The region has characteristic GT and AG repeat sequence, which we were able to identify, this microsatellite (or simple sequence repeat) region forms an excellent marker for studying the inheritance of this gene and its association with disease resistance traits in sheep (Buitkamp *et al.,* 1994; Schwaiger *et al.,* 1995; Charon *et al.,* 2002; Sayers *et al.,* 2005; Marshall *et al.,* 2009; Castillo *et al.,* 2011). The exon region spanning from 8th base pair to 277th also forms a potential region for SNP (single nucleotide polymorphism) studies. As we have observed there are variations at amino acid level, however, there was no change in ligand binding site affinity and a less amount of variation was seen at secondary structure level. This confers that variations at exons may not be necessarily translated into proteins and even the variations within the same species in not affecting the predicted structure. Therefore, this variation may have their effect during binding or switching the structure of antigen loading clamp as per the exogenous peptide. However, at nucleotide level the exon regions showed 48 points of dissimilarities between the sequences, this illustrates that the exon region forms a potential candidate for PCR – RFLP studies (Brujeni, *et al.,* 2009; Jamshidi *et al.,* 2011; Shrivastava *et al.,* 2015; Ilhan *et al.,* 2016). The region has been extensively studied for association of polymorphism at restriction cutting sites with resistance/ susceptibility to diseases viz., hydatidosis in Kazakh sheep (Li *et al.,* 2010), echinococcosis in Chinese merino sheep (Shen *et al.,* 2014), faecal egg count in Iranian Ghezel sheep (Valilou *et al.,* 2015).

In conclusion, the importance of ovine MHC class II, DRB region is unequivocal. The presence of repeat region in introns was a constant feature among different breeds. There was ample amount of variation within the sequence (in both intron and exon) that can be tapped in association studies. The variation however, was not carried in protein secondary structure and in ligand binding site indicating that it will be more beneficial to study the variability of intronic region, which can be better utilized to study the inheritance of whole region (*i.e*., intron and the adjacent exon).

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