GENETIC ANALYSIS OF MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) CLASS I EXON 4-5 IN CATTLE AND BUFFALO USING MOLECULAR AND PHYLOGENETIC APPROACHES

Biswanath Patra[1], Manjit Panigrahi[1], Sheikh Firdous Ahmad[1], Pushpendra Kumar[1], Triveni Dutt[2] and Bharat Bhushan[1,*]

ABSTRACT

Characterization of major histocompatibility complex (MHC) Class I Exon 4-5 was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), single-strand confirmation polymorphism (SSCP) and sequencing techniques in crossbred cattle and buffaloes. Digestion of 559 bp fragment with *Hinf*I and *Hae*III restriction enzymes produced five and three distinct PCR-RFLP patterns in cattle and buffaloes, respectively. The genotype frequencies of *Hinf*I patterns ranged from 0.015 to 0.456 in crossbred cattle, 0.080 to 0.600 in Mehsana and 0.050 to 0.500 in Bhadawari breeds of buffaloes. Similarly, the genotype frequencies of *Hae*III patterns ranged from 0.045 to 0.636. The SSCP analysis showed that the BoLA-A and BuLA-A were highly polymorphic in cattle and buffaloes. A total of 10 alleles (AA, AB, BB, AC, AD, AE, AF, BC, DD and DB) were identified by PCR-SSCP. Four partial sequences, two each for crossbred cattle (AY790633 and AY790634) and buffaloes (AY785759 and AY785760), were submitted to the GenBank, NCBI. The sequencing results showed a number of amino acid changes in Exon 4-5 region. In conclusion, MHC Class I Exon 4-5 was found to be highly polymorphic in both cattle and buffaloes which may be exploited for association with traits of economic interest.

Keywords: *Bubalus bubalis*, buffaloes, BoLA-A, BuLA-A typing, Exon 4-5, MHC

INTRODUCTION

The major histocompatibility complex (MHC) is established to play a critical role in the immune system of all mammalian species. It is a large cluster of linked genes that are highly polymorphic and involved in tolerance to infectious diseases in livestock. Following the standard nomenclature, the MHC of cattle is known as bovine lymphocyte antigen (BoLA), while in buffalo it is referred as bubaline lymphocyte antigen (BuLA). These antigens are located on chromosome number 23 and 2 in cattle and buffaloes, respectively. Three genetic groups of histocompatibility antigens exist in mammals i.e., Class I, Class II, and Class III (Wieczorek *et al*., 2017). Among these, the first two classes of MHC antigens are mostly characterized on the molecular scale and have well-defined cell surface antigens. Class I antigens are found to be present on the cell surface of nearly all nucleated cells and in general, are involved in the cytotoxic T-cell response (García *et al*., 2012). The presence of hypervariable domains leads to the presence of

[1]Division of Animal Genetics, Indian Council of Agricultural Research, Indian Veterinary Research Institute, Uttar Pradesh, India, *E-mail: bhushan.drbharat@gmail.com

[2]Indian Council of Agricultural Research, Indian Veterinary Research Institute, Uttar Pradesh, India

a high-level polymorphism in MHC molecules (Ng *et al*., 2016). Two loci i.e., BoLA-A and BoLA-B, have been reported to be present in Class I region of cattle MHC, which are tightly linked within the range of 200 Kb genetic distance (Behl *et al*., 2012). The presence of 32 serologically defined alleles along with four more putative alleles for BoLA-A locus have also been reported (Sharma *et al*., 2004). On the other hand, Class II MHC presents its cell surface antigens to T-helper cells and subsequently activates CD4+ T-cells. Class III molecules are a group of complement proteins (Tizard, 2017). Besides having a profound role in intercellular recognition and with self-nonself discrimination, MHC has emerged as an important candidate gene cluster for tolerance/susceptibility studies in various diseases. The ability of an individual to respond to certain antigen is under genetic control through MHC. Previously, there were different immunologically defined sub-classes of polymorphic MHC Class I antigens because the complete information regarding the variation present among MHC alleles were not available. Understanding the structure, function, and diversity of MHC gene is of utmost importance in order to uncover the complexity of the immune system of vertebrate species (Behl *et al*., 2012). It provides an opportunity to understand disease dynamics in livestock (Castillo *et al*., 2010). Thus, understanding the molecular diversity and phylogenetic relationship(s) of MHC complex genes/alleles may prove pivotal in our understanding of varied response of a mammalian species towards various diseases. The technique of RAPD-PCR among others has been reported to be highly effective in the analysis of MHC polymorphism among different breeds (Sharma *et al*., 2004). Considering the importance of BoLA-A and BuLA-A genes in controlling immune response, the present investigation was aimed to study and molecularly characterize the Exon 4-5 in cattle and buffalo population.

MATERIALS AND METHODS

Experimental animals

The present investigation was undertaken on a total of 113 animals from different breeds of cattle and buffalo. Among these, 68 animals from crossbred cattle (exotic inheritance from Holstein-Friesian, Brown Swiss, Jersey and indigenous inheritance from Hariana) were maintained at Cattle and Buffalo Farm, ICAR-Indian Veterinary Research Institute, Izatnagar; 25 Mehsana buffaloes at Gujarat Agriculture University, S.K. Nagar, Gujarat (India) and 20 Bhadawari buffaloes at Bhadawari Buffalo and Jamunapari Goat Breeding Farm, Etawah, Uttar Pradesh, India.

Sample collection and DNA isolation

Blood samples were collected from selected animals through jugular venipuncture. The collection was done under sterile conditions, involving EDTA as an anti-coagulant. Using the standard procedure as described by Sambrook and Russell (2001), genomic DNA was isolated from the blood samples. The DNA samples were checked *via* NanoDrop (spectrophotometry) method for their quality and concentration. DNA samples of good quality and optimal concentration were used for further analysis.

Amplification of Exon 4-5

A 559 bp fragment consisting of Exon 4-5 region of MHC Class I gene was amplified by employing the corresponding forward primer (5′-TGA CCC ATG GAA CAC AAA CTG A-3′) and

reverse primer (5′-GCC CTC CCT CCC TTC CCT ACC T-3′) designed from the nucleotide sequence available in GenBank using DNAStar software. The forward primer was located at intron 3 and the reverse primer at intron 5 in the region of corresponding exon-intron boundary.

PCR-RFLP analysis

For each sample, 10 µl of PCR product was digested, with overnight incubation at 37°C, using five units of restriction enzymes (*Hinf*I and *Hae*III) and 5 µl of the corresponding buffer in a final volume of 20 µl. The resolution of restriction fragments was achieved on 3% agarose gel electrophoresis at 30 V for 4 h in 1X TBE buffer and the same was documented through gel documentation system. The size of corresponding fragments was estimated using a 100 bp DNA ladder.

Single Strand Conformation Polymorphism (SSCP)

Samples having good PCR products without any smearing and artifacts were used for further analysis through SSCP. 3.5 µl of PCR product and 9.5 µl of SSCP stop solution with 96% formamide were mixed and denatured at 94°C for 5 minutes, followed by quick cooling on chilled water for 15 minutes. In presence of formamide, the denatured PCR product cannot get annealed and these were run on 8% polyacrylamide gel (PAGE). The ratio of acrylamide and bis-acrylamide used was 49:1. Electrophoresis was performed at a constant voltage of 120 V at room temperature for 12 h. Polyacrylamide gel electrophoresis (PAGE) results were passed through the procedure of silver staining for visualization and the same were documented through photography (Bassam and Gresshoff, 2007). Few selected alleles were sequenced from PCR products using forward and reverse primers. Sequencing was carried out using ABI PRISM 3100 and the results of Exon 4-5 sequences were analyzed with the help of DNAStar computer software.

Sequencing and phylogenetic analysis

Different alleles were sequenced from PCR products with M13 forward and reverse primers using an automated sequencer (ABI PRISM 3100) and the results of Exon 4-5 sequences were analyzed via DNAStar program. The phylogenetic tree analysis, based on UPGMA method, was done by aligning the corresponding sequences of BoLA-A/ BuLA-A for Exon 4-5 to identify the allelic lineage of characterized alleles with sequences available in the GenBank, NCBI.

RESULTS

PCR-RFLP of BoLA-A and BuLA-A

A fragment of 559 bp was amplified using two primers corresponding to the regions of Exon 4, Intron 4 and Exon 5 of BoLA-A and BuLA-A in crossbred cattle and buffaloes (Figure 1).

The digestion of 559 bp fragment with *Hinf*I restriction enzyme produced five BoLA-A alleles using RFLP analysis, which were designated as A, B, C, D and E (Figure 2).

These alleles had fragment sizes as 200 bp; 193 bp; 218, 200, 193 and 153 bp; 218, 180 and 153 bp; 218, 200, 193 and 170 bp, respectively. It was observed that the AB allele shared fragments of both sizes, A as well as B. The genotype frequencies of *Hinf*I-A genotype were 0.118 and 0.120 in crossbred cattle and Mehsana buffaloes, respectively. The genotype *Hinf*I-B had the frequencies as 0.015, 0.200 and 0.250 in crossbred

cattle, Mehsana and Bhadawari breeds of buffaloes, respectively. The frequency of genotype *Hinf*I-C was found to be 0.456 in crossbred cattle, while in Bhadawari and Mehsana buffaloes it ranged from 0.200 to 0.600 (Table 1).

Highest genotype frequency was estimated for *Hinf*I-C in Mehsana buffalo followed by *Hinf*I-D in Bhadawari buffalo. The digestion of 559 bp fragment with *Hae*III restriction enzyme identified three alleles by RFLP analysis (Figure 3).

Allele 'A' had no specific fragment of size 191 bp, while it was present in allele 'B'. Allele 'C' had a specific fragment size of 200 bp. The frequencies of *Hae*III genotypes ranged from 0.045 (*Hae*III-B) to 0.636 (*Hae*III-A) in crossbred cattle (Table 1).

PCR-SSCP of BoLA-A and BuLA-A

The results revealed a high degree of polymorphic patterns in the region of Exon 4-5 of BoLA-A and BuLA-A genes as the PCR product size was 559 bp. In SSCP, a great variation was detected due to changes in a number of nucleotides and differential mobility of single-stranded secondary structures produced during denaturation of double-stranded PCR products. MHC Class I gene showed a very high level of nucleotide changes, hence most of the samples studied changed the configuration. Alleles identified by SSCP were AA, AB, BB, AC, AD, AE, AF, BC, DD and CC, which have been shown in Figure 4 and 5.

Sequencing of Exon 4-5

Nucleotide substitutions were observed at various positions in Exon 4 and 5, which made these regions highly polymorphic like other alleles of BoLA-A and BuLA-A genes. Sequences of this region were compared with the nucleotide sequences of Holstein-Friesian (HF) available in NCBI GenBank. Partial coding sequences of Exon 4 and 5 of crossbred cattle (AY790633 and AY790634) and Bhadawari buffaloes (AY785759 and AY785760) were submitted to GenBank.

Nucleotides substitution at positions 4 (CGT to CAT and CCT), 13 (ACC to CCC) and 55 (TCA to TTA) changed the amino acids at these positions from Arginine to Histidine and Proline; Threonine to Proline and Serine to Leucine, respectively in comparison to data already available for Holstein-Friesian. Similarly, changes in the genetic code were observed from CAG to CAA at position 67, CGC to CGT and TGC at position 70, GAG to GAT and AAT at position 73, GGG to GGA and GAG at 76, CAG to CTG at position 85 and CAG to TAG at position 91; and they also showed changes of a large number of amino acids in this region. A conserved region between the nucleotide from 94 to 126 bases was also seen in this exon region.

Another polymorphic region was observed from 127 to 232 bases due to changes in a large number of amino acids. Nucleotide substitutions were detected at positions 127 (GAC to GAT), 133 (AGC to ACC and AAC), 148 (GCA to GCG), 157 (GTG to GGG), 166 (TCT to TTT), 187 (ACG to AAG), 202 (CAC to CAT), 205 (AAA to GAG and AAG), 208 (GGG to GGT), 214 (CAG to AAG), 217 (GAG to GGG), 229 (CTG to CTC) and 232(ATA to AGA) in comparison to the sequence of Holstein-Friesian cattle available in the NCBI GenBank.

The intron region started with a conserved sequence GGTAA in Bhadawari buffaloes and crossbred cattle. After the conserved region, nucleotide substitution was seen at positions 243 (AGGA to GGAG) and 247 (G to C). A two base pair deletion was also detected at positions 251 to 252 in crossbred cattle and 253 to 254 in

Bhadawari buffaloes as well as crossbred cattle. Besides, a single nucleotide deletion was also seen in Bhadawari buffaloes at position 270.

The nucleotide substitutions were also observed at position 264 (CTT to TTT), 317 (ACT to GGT), 324 (T to C), 337 to 340 (AGCT to CCCC and CCTC) and 345 (T to C). The exon 5 is led by intron sequence TCCACA with initiation codon GAA at position 357. A large number of amino acid changes were seen due to nucleotide substitutions at positions 372 (TCC to TCT and TTT) Phenylalanine to Serine, 393 (GTT to ATT) Valine to Isoleucine, 396 (GTT to CTT) Valine to Leucine, 399 (GGC to GTC) Glycine to Valine, 402 (CTG to CAG) Leucine to Glutamine and 414 (CTG to GTG) Leucine to Valine in Bhadawari buffaloes as compared to the nucleotide sequence of Holstein-Friesian available in the NCBI GenBank, which led to polymorphism in this region.

Phylogenetic tree

A phylogenetic tree was produced by aligning our sequence with the sequence retrieved from GenBank. The results showed that the alleles of Exon 4-5 Bhadawari buffaloes were divergent to each other but had some closeness to those of crossbred cattle. The phylogenetic tree, based on amino acid sequences, revealed the differences in the closeness of the related alleles, as crossbred cattle showed closeness to Holstein-Friesian (Figure 6). The nucleotide dissimilarity varied to the extent of 2.4 and 8.9%.

DISCUSSION

Random amplification of polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) emerged as an effective tool and meticulously detected the presence of genetic variability in different populations. PCR-SSCP is a method of choice to study single nucleotide polymorphism (SNP) without knowing the restriction sites for any particular restriction enzyme. It was observed that the sensitivity was greatly influenced by the size of PCR product and very good results were obtained from the PCR products ranging between 100 and 560 bp. The present study of different cattle and buffalo alleles in the random samples showed that only a fraction of BoLA-A and BuLA-A diversity was present as compared to the global cattle and buffalo population. It is well documented in the literature that some form of balancing selection maintains the MHC polymorphism.

The most favored hypotheses include that of gene conversion, recombination, sexual selection or the host-pathogen co-evolution during the immune recognition of pathogens (Li *et al*., 2017). The presence of polymorphism in the Exon 4-5 region of MHC complex in these populations was revealed and this may add to the existing knowledge regarding the presence of polymorphism in other MHC regions. The polyphyletic lineage for different alleles did not support the trans-species persistence concept of allelic lineage between the alleles from two species. The trans-species persistence of allelic lineage in DRB alleles has already been reported (Van Den Bussche *et al*., 1999; Těšický and Vinkler, 2015). However, the results of the present study supported the findings of other researchers, who have reported characteristic patchwork pattern in DRB alleles (Brunsberg *et al*., 1996; De, 2000). The results showed that the alleles of Exon 4-5 in Bhadawari buffaloes were divergent to each other but have some closeness with those of crossbred cattle.

The present study revealed the presence

Table 1. Genotypic frequencies of BoLA-A/BuLA-A type specific restriction fragments from Exon 4-5 in cattle and buffalo.

Breed/ group	Genotype	Fragment size (bp)	Frequency
Crossbred cattle	*HinfI*-A	200	0.118
	HinfI-B	193	0.115
	HinfI-C	218, 200, 193 and 153	0.456
	HinfI-D	218, 180 and 153	0.147
	HinfI-E	218,200,193 and 170	0.015
	HinfI-AB	193, 200	0.250
Mehsana	*HinfI*-A	200	0.120
	HinfI-B	193	0.200
	HinfI-C	218, 200, 193 and 153	0.600
	HinfI-AB	193, 200	0.080
Bhadawari	*HinfI*-B	193	0.250
	HinfI-C	218, 200, 193 and 153	0.200
	HinfI-D	218, 180 and 153	0.500
	HinfI-AB	193, 200	0.050
Crossbred cattle	*Hae*III-A	90, 111, and 156	0.045
	*Hae*III-B	191	0.636
	*Hae*III-BC	200 and 191	0.318

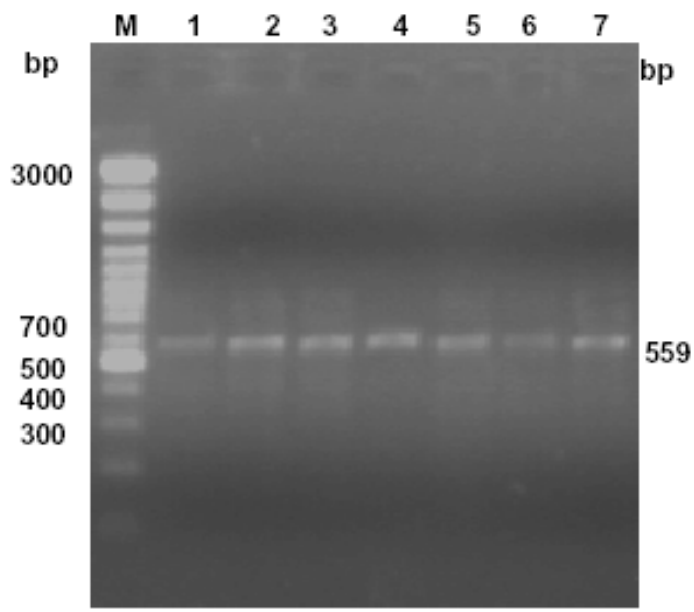

Figure 1. Amplification of Exon 4-5 (559 bp).

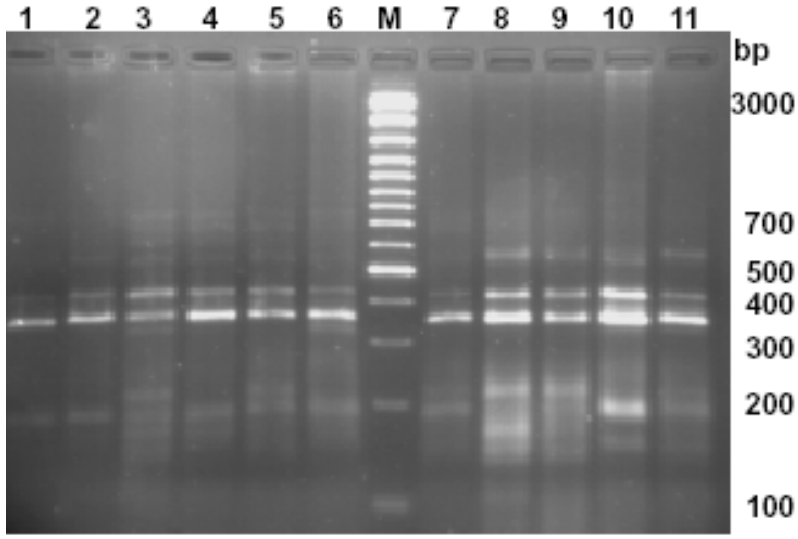

Figure 2. *HinfI* digestion patterns of Exon 4-5.
Lane M: 100 bp DNA ladder,
Lane 1 and 2: Allele AA,
Lane 3 and 5: Allele CC,
Lane 4, 6, 7, 10 and 11: Allele AB,
Lane 8 and 9: Allele DD.

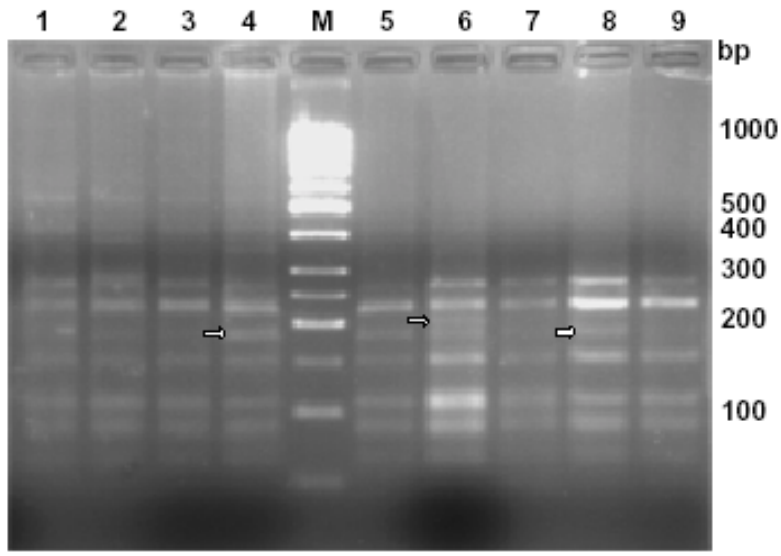

Figure 3. *Hae*III digestion patterns of Exon 4-5.
Lane M: 50 bp DNA ladder,
Lane 1, 4, 5 and 8: Allele BB,
Lane 2, 3, 7 and 9: Allele AA,
Lane 6: Allele BC.

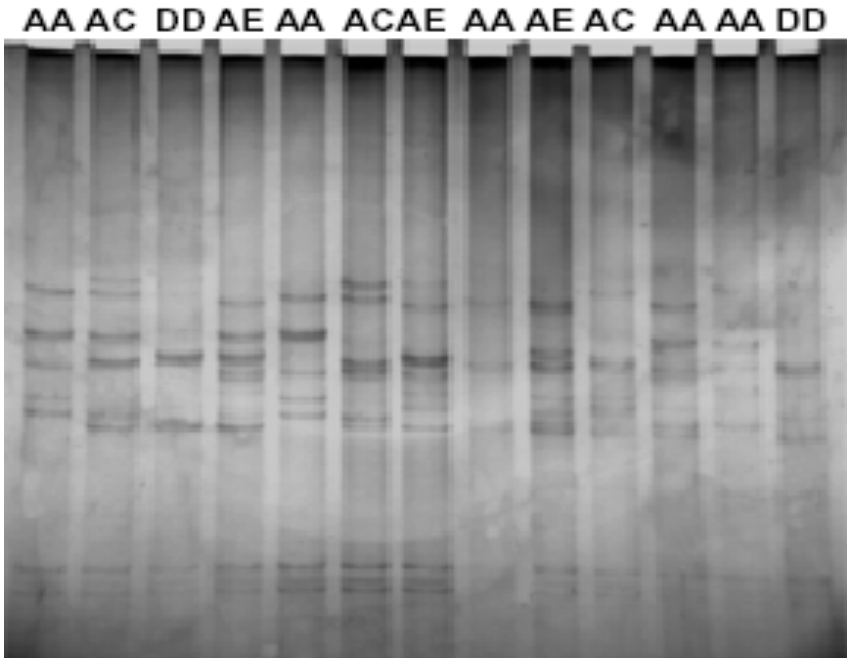

Figure 4. PCR-SSCP patterns of Exon 4-5 in cattle.

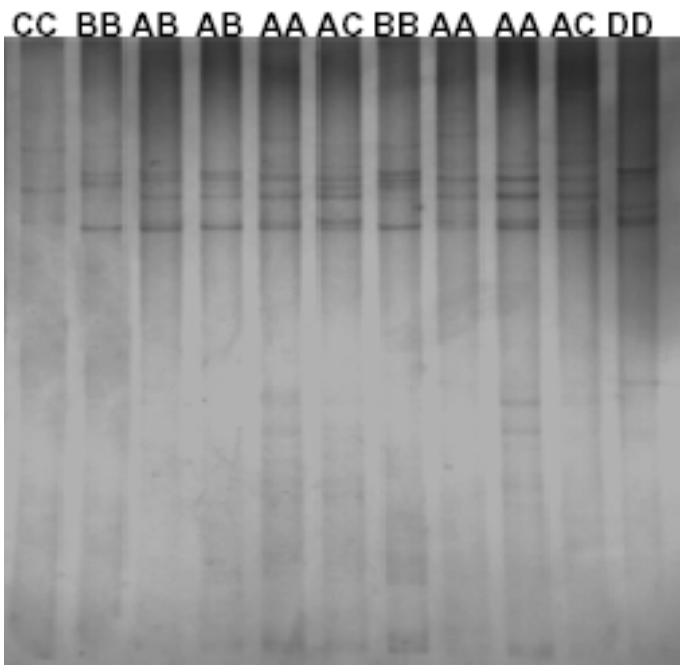

Figure 5. PCR-SSCP patterns of Exon 4-5 in buffalo.

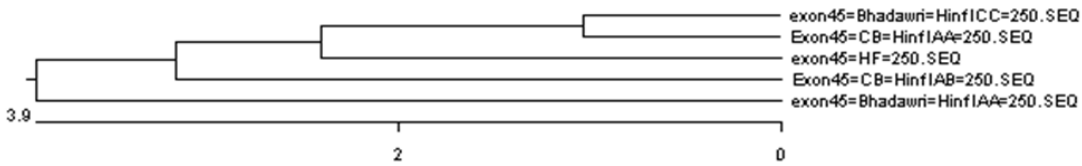

Figure 6. Phylogenetic tree (UPGMA method) based on nucleotide sequences of BoLA-A/BuLA-A alleles, Exon 4-5.

of high scale polymorphism in Exon 4-5 region of MHC of cattle and buffaloes. SSCP results revealed that most of the cattle and buffalo population characterized were heterozygous. Sequencing of this region confirmed the changes in the nucleotides in crossbred cattle and buffaloes as compared to Holstein-Friesian cattle. The MHC polymorphism and its association with the tolerance/susceptibility of animal population to various diseases may prove to be an asset for genetic selection for disease traits.

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