MOLECULAR CHARACTERIZATION OF EXON 2-3 OF MAJORHISTOCOMPATIBILITY COMPLEX (MHC) CLASS I IN BUFFALO (*BUBALUS BUBALIS*)

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ABSTRACT

Present investigation was undertaken to characterize the exon 2 to 3 of major histocompatibility complex (MHC) class I genes in buffaloes (BuLA-A) of Murrah, Mehsana and Bhadawari breeds using Polymearse chain reaction-restriction length fragment polymorphism (PCR-RFLP). Two primers corresponding to the region of exon 2 intron 2 and exon 3 of BoLA-A were used to amplify the fragment of 714 bp. The PCR-RFLP patterns with respect to DdeI, TaqI, and HinfI restriction enzymes produced distinct polymorphic patterns in all the breeds. Two new patterns with the fragment sizes as 614, 60, 39 bp and 417, 187, 78, 21, 12 bp with DdeI digestion and 608, 97, 9 bp and 609, 106 bp with TaqI digestion were observed. The selected genotypes were cloned and sequenced to find out any change in nucleotides or amino acids with respect to the Holstein Friesian sequence available in the NCBI GenBank. The sequencing results showed that the exon 2- exon 3 products varied from 713 to 715 bp in buffaloes. Polymorphism in exon 2 occurred mostly due to changes of nucleotides at the positions from 62 to 72, 121 to170 and 184 to 237. The exon 3 initiated at 468 position of nucleotide after intron 2, which had purine rich region conserve sequence CGGGTCA. The phylogenetic tree predicted changes of higher degree in nucleotides as well as amino acids of exon 2- exon 3 and the nucleotide dissimilarity ranged from 3.0 to 14.7%. The results of the present study revealed that the exon 2 to 3 was found polymorhic in the buffaloes.

Keywords: buffalo, *Bubalus bubalis*, cloning, major histocompatibility complex (MHC), PCR-RFLP, sequencing

INTRODUCTION

In recent years research efforts are directed towards the development of herds, which are genetically disease resistant so as to reduce the losses from devastating and harmful diseases. It is a known fact that indigenous buffalo breeds are more tolerant to the endemic diseases than exotic breeds. This tolerance may be due to their continuous exposure to pathogens for a long period and natural selection for thousands of years. Therefore, Indian native germplasm offers a great opportunity to evaluate basis of genetic resistance to diseases. The major histocompatibility complex (MHC) is a chromosomal region consisting of a series of closely linked loci or so called gene families. The MHC of buffalo is known as buffalo lymphocyte antigen (BuLA) and located on chromosomes number 2. Among the gene product of MHC region, there are two molecularly well-

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defined classes of cell surface antigens. Till date, high degree of genetic polymorphism in MHC class I and II genes are documented in a number of different species (Klei, 1975; Behl *et al*., 2012; Bhushan *et al*., 2007; Naskar *et al*., 2012; Gowane *et al*., 2013). Expression of MHC genes is an essential component of studies on immune response and resistance/susceptibility to diseases (Shirley *et al*., 1999).

Class I antigens are found on the surface of nearly all cell type and these antigens are in general involved in the cytotoxic T cell response e.g. as restricting elements in the elimination of virus-infected cells. These molecules are highly polymorphic and their amino acid sequences are very variable. These variations are concentrated in three to four discrete hypervariable regions within the α1 and α2 domains. Usually only two or three different amino acids are found at each position, and one is usually predominant.

The BoLA-A locus has 32 serologically defined alleles and at least 4 more putative alleles, although there is a high frequency of null alleles (Tizard, 1998). Thus BoLA-A typing defines a class I haplotypes rather than an allele (Sawhney *et al*., 2001). The polymorphism study of class-I region of the BOLA is more complicated and less well understood than that of the MHC-II genes (Gowane *et al*., 2013). As the class-I MHC molecule present intracellular peptides to CD8+ T cells, it is highly likely that polymorphism of it has an influence on the immune response (Guzman *et al.*, 2008). Considering the importance of BoLA-A gene in controlling immune response, the present investigation was undertaken to study the polymorphism of exon 2 to 3 in Indian buffalo population for the first time.

MATERIALS AND METHODS

Animals

A total of 102 buffaloes belonging to three breeds viz. Murrah (52), Mehsana (27) and Bhadawari (23) maintained at Cattle and Buffalo Farm, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India; Gujarat Agricultural University, S.K. Nagar, Gujarat, India and Bhadawari Buffalo and Jamunapari Goat Breeding Farm, Etawah, Uttar Pradesh, India respectively were used for the collection of blood samples.

Genomic DNA isolation

Genomic DNA was isolated from 5 ml of venous blood by phenol-chloroform extraction method as described by Sambrook and Russell (2001). The DNA samples were checked for their quality, purity and concentration. The quality of the genomic DNA was checked by submarine agarose gel electrophoresis. DNA samples of good quality, purity and concentration were used for further analysis.

Amplification of exon 2-exon 3

A 714 bp fragment consisting of exon 2 to 3 region of MHC Class I gene was amplified by employing the corresponding forward (5′-GTC CCC ACT CSM TGA GGT ATT-3′) and reverse (5′-TCC AGG TAT CTG CGG AGC-3′) primer pairs as described by Sawhney *et al*., 2001. The forward primer was situated at intron 1 in exon-intron boundary and reverse primer at end of the exon 3.

PCR-RFLP analysis

For each sample, 10 µl of PCR product was digested with five units of restriction enzymes

(TaqI, DdeI and Hinf I) and 5 µl buffer in a final volume of 20 µl. Samples were incubated at 37°C (DdeI and Hinf I) and at 65°C (TaqI) for overnight. The Hinf I digested restriction fragments were resolved on 3% agarose gel electrophoresis at 30 volt for 4 h in 1x TBE buffer and documented through photography under gel documentation system. 50 and 100 bp DNA ladders were used to estimate the size of the fragments. The TaqI and DdeI digested restriction fragments were resolved on 8% polyacrylamide gel and visualized by silver staining.

Cloning and nucleotide sequencing

Selected genotypes were cloned on the basis of PCR-RFLP. The gel extracted purified PCR products were ligated in cloning vector. Cloning was carried out using pTZ57R/T vector. The efficiency of ligation depends on the added nucleotide to the 3'-ends of PCR product. A 20 to 30 minutes extension at 72°C was followed to increase the rate of transformation with extra nucleotides added. This resulted in 3 to 4 folds higher yield of recombinant colonies of host cell E. coli strain DH5α. The inserts were confirmed by colony PCR and restriction digestion of plasmid with 6 units of EcoRI and PstI enzymes having the cutting sites at 30 bases and 18 bases, respectively on either side of the ligation site in the vector. The length of the fragment release on restriction digestion was observed as 762 bp in 2.5% agarose gel electrophoresis. Sequencing of the clones was carried out using M13 forward and M13 reverse primers by automated sequencer (ABI PRISM). The analysis of nucleotide sequences was done by DNASTAR software.

RESULTS AND DISCUSSION

The exon 2 to 3 region in the MHC class I gene was amplified (Figure 1) and a number of alleles were identified by restriction fragment length polymorphism (RFLP). Selected haplotypes of buffaloes were cloned and sequenced to find out the nucleotide changes in coding and non-coding regions.

Digestion with DdeI, TaqI and HinfI

A 714 bp fragment, digested with DdeI restriction enzyme, produced two new fragments and also specific fragments for different BoLA-A alleles as reported by Sawhney *et al*. (2001). Two new patterns with fragment sizes as 614, 60, 39 bp and 417, 187, 78, 21, 12 bp in Murrah buffaloes were later confirmed by cloning and sequencing. The digestion with TaqI restriction enzyme also revealed the fragments specific for different

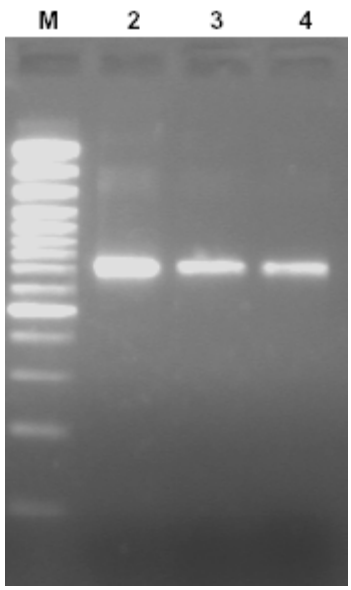

Figure 1. PCR amplification of BuLA-A exon 2-3 (714 bp) of buffaloes.
Lane M: 100 bp DNA ladder

BoLA-A alleles as reported by Sawhney *et al*. (2001). Two new patterns with fragment sizes as 608, 97, 9 bp and 609, 106 bp in Murrah buffaloes were confirmed by cloning and sequencing. Sawhney *et al*. (2001) identified seven BoLA-A types with TaqI and DdeI restriction enzymes. Ellis *et al*. (1999) also confirmed that the A11 haplotype comprised of multiple polymorphic MHC class I genes. Further, they also reported that the DdeI patterns with the largest number of bands suggested that at least five distinct Class I genes were amplified, which included both expressed and non-expressed gene and gene fragments. The genotype frequencies showed wide variations among different breeds of buffaloes (Table 1).

For allele A19, genotype frequencies ranged from 0.037 (Mehsana) to 0.173 (Murrah) in buffaloes, respectively. The genotype frequency of A10 haplotypes in Murrah buffaloes was 0.038. Highest genotype frequency was estimated in Murrah (0.308) followed by Mehsana (0.074) for allele A11. However, the genotype frequencies of allele A18 ranged from 0.259 (Mehsana) to 0.154 (Murrah) and 0.086 (Bhadawari). The results revealed that the genotypes A10/19, A10/31 A11/20 and A20/31 were similar to those reported by Sawhney *et al*. (2001) in cattle. The genotypic frequencies of A18/19 ranged from 0.154 (Murrah) to 0.556 (Mehsana) and 0.652 (Bhadawari). Other alleles (A10/11, A10/19, A10/18, A11/20, A18/31, A19/20 and A18/31) also showed variations in genotype frequencies.

The authors also reported 10 BoLA-A types (A18/31, A10/A19, A10/A31, A18/20, A10/11, A20/A31, A31/A32, A11/A20, A11/A32 and A19/20), out of which A10/A19, A10/A31, A20/A31 A31/A32 A11/A20 and A11/A32 were already recognized in the 5th International Bovine Lymphocyte Antigen (BoLA) workshop and were assigned the workshop ID number as WK5-38, WK5-53, WK5-51, WK5-36, WK5-29 and WK5-48, respectively by Davies *et al*. (1994). Besides, other haplotypes (A18/19 and A19/31) also determined high degree of polymorphism in buffalo population. The digestion of the 714 bp fragment with HinfI restriction enzyme produced fragments

Table1. Genotypes and their frequencies as revealed by PCR-RFLP in buffaloes.

Breed	Genotype	Genotype frequency	Breed	Genotype	Genotype frequency
Murrah	A10	0.038	Mehsana	A11	0.074
	A11	0.308		A18	0.259
	A18	0.154		A19	0.037
	A19	0.173		A10/18	0.037
	A10/11	0.038		A18/19	0.556
	A10/19	0.019		A19/20	0.037
	A10/18	0.038	Bhadawari	A18	0.086
	A11/20	0.058		A32	0.043
	A18/19	0.154		A10/11	0.043
	A18/31	0.019		A10/19	0.130
				A11/20	0.086
				A18/19	0.652

specific to BuLA-A alleles. Five different alleles namely A, B, C, D and E were identified (Figure 2) by cloning and sequencing and it was observed that most of the cattle and buffalo populations were in heterozygous condition. The fragment sizes of alleles A, B, C, D and E were 379, 212, 122 bp; 379, 193, 141 bp; 379, 142, 122, 71 bp; 379, 275, 60 bp and 380, 335 bp respectively.

Sequence analysis

Results of sequencing revealed that the sizes of the exon 2 to 3 fragments ranged from 713 to 715 bp in Murrah buffaloes (Accession numbers AY894407, AY894408 and and AY925136). This variation in nucleotide number was due to addition/ deletion/ substitution. The length of the exon 2 was 267 bp, which coded 89 amino acids in case of BuLA-A gene showed large number of amino acid

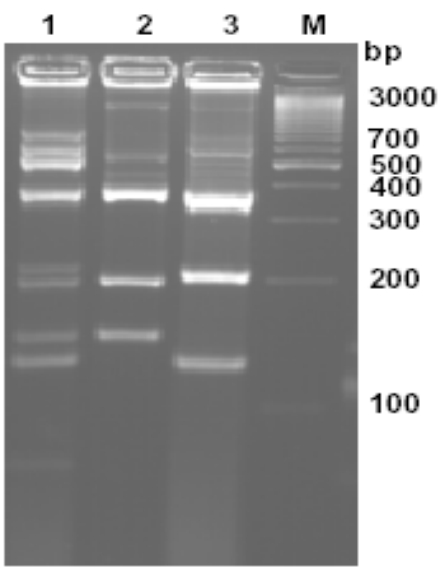

Figure 2. HinfI digestion pattern of BuLA-A exon 2-3
Lane M: 100 bp DNA ladder;
Lane 1: Allele AB;
Lane 2: Allele BB;
Lane 3: Allele AA

changes in this region. Polymorphism in exon 2 occurred mostly due to changes of nucleotide bases at the positions from 62 to 72, 121 to170 and 184 to 237, whereas the part of exon with no change in nucleotides from 238 to 293 bases was the conserved region. Intron 2 followed by exon 2 with sequence GGTGAG had more frequent nucleotide substitutions than exon 2 alone (Figure 3).

In exon 2, nucleotide substitutions from codon CTG to ATG at position 12 changed the amino acid (L to M). Alteration of nucleotides at position 26 changed the amino acid at positions 8 (S to L and V) with respect to the reported sequence of Holstein Friesian (GenBank). Similarly, nucleotide substitutions from CGC to GCC (R to A) at positions 30 to 32 changed the amino acids. A large number of amino acid changes have been identified because of substitution of nucleotides from GAC to GGC (45 to 47), CGG to CGC (60 to 62) and TAC to TTC (63 to 65) at positions 45 to 65, TAC to TTC at positions 63 to 65, CTG to ATC at positions 66 to 68, GAA to TCC at positions 69 to 71 (Figure 4). Similarly, nucleotide substitutions were observed from AAC (N) to GAC (D) at positions 87 and 89. A large number of amino acids changes were seen in this region due to nucleotide substitutions from CGG (R) to CCG (P) at position 120, AAT (N) to GAT (D) at position 123, AGG (R) to AAG (K)(129 to 131), ATG (M) to ATA (132 to 134), GCG to TCG at 144, CGG (P) to CCG(R) at position 147, GTG (V) to ATG (M) (153), CAG (Q) to AAG (K)(159 to 161) and CCG to CCC at position 168 resulted polymorphism.

However, highly polymorphic region was observed from the positions 183 to 200 due to continuous substitutions of nucleotide from CAG to CGG (183); AAC to GAA (186 to 188); ACG to ACC (189 to 191); CGA to CAA (192 to 194); AAC to ATC and AGA (195 to 197) and TCC to GCC and

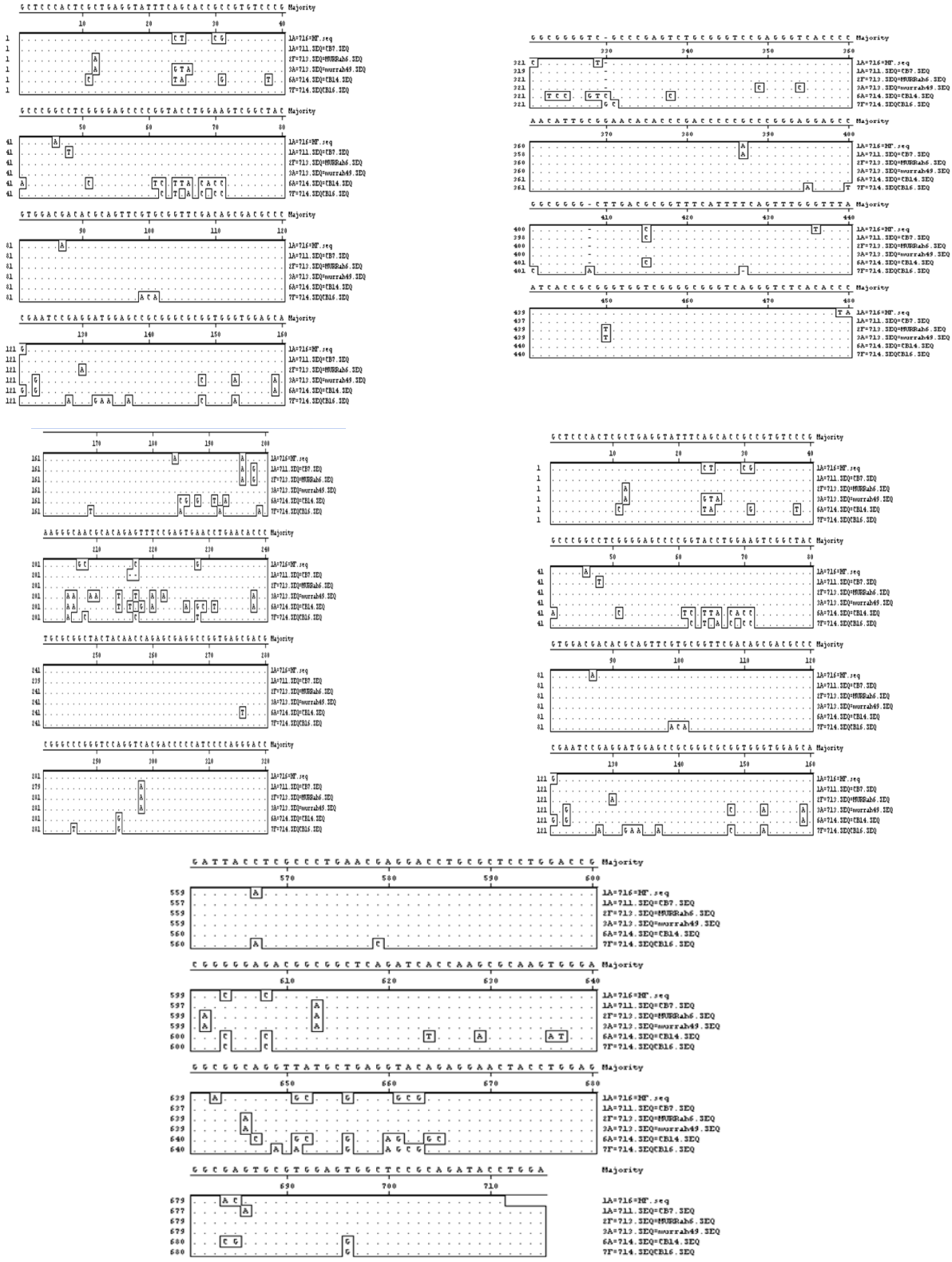

Figure 3. Nucleotide sequences of BuLA-A exon 2-exon 3 alleles.

ACT at positions 198 to 200. Similarly, nucleotide substitutions from GGC to GAC and GAA (204), GCC to ACC, AAC and AAA were observed at position 207 to 209. Nucleotides substitutions from GCA to ACA (210 to 212); CAG to CTG and CAA (213 to 215), ACT to ATT, AGT and TTT at positions 216 to 218, respectively changed amino acids in the region. The codon substitutions from TTC to TAC at position 219, CGA to AGA at 222, GTG to GAG at positions 225 to 227 and GAC to AAC and TAC at position 228 showed change in a number of amino acids. Some more nucleotide substitutions were also noticed at positions 237 to 239 (ACC to AAC). These results revealed a highly polymorphic region end at position 239 in exon 2 and thereafter a highly conserved region was seen from 240 to 269 positions.

Nucleotide substitutions were also observed in intron 2 at positions 294 (A to G), 298 (C to A), 321 (C to G), 329 (T to C), 349 (G to C), 354 (T to C), 363 (C to A), 382 (C to T), 387 (A to G), 391 (GGG to TTT), 397 (A to G), 400 (C to T), 401 (G to C), 415 (C to G), 436 (T to G) and 450 (G to T) respectively with respect to the sequence of Holstein Friesian. Some base pair deletions were also observed in the nucleotide sequences of buffaloes at positions 334 and 408.

The exon 3 initiated at 468 position of nucleotide after intron 2, which had purine rich region conserved sequence CGGGTCA. The results showed that more changes of the bases of this region made it highly polymorphic. Change of nucleotide from ACT to ACC and ACA at position 477 did not make any change in the amino acid. However, nucleotide substitutions at position 480, from ATC to CTC and GTC changed amino acid from I to L and V. A large number of genetic code substitutions were also observed at positions 483 (CAG to CAA), 486 (GCG to TGG and GAG), 492 (TAC to TCC), 501 (GAC to TAC), 510 (CCG to TCG), 516 (GGG TO GGA) 519 (CGT to GGT), 522 (TTC to CTC), 525 (CTC to CGC and CTG), 534 to 536 (TAC to TTC and TAT), 537(AGG to ATG and GAT), 543 (GAC to TAC and TTA), 546 (GCT to GGC), 567 (ATC to CTC), 591 (TCC to TCT), 600 (GCG to GCA), 603 (GCG to GGG), 606 (GAC to GAG), 612 (GCG for GAG), 621 (ATC to GTC), 624 (ACC to ACG), 633 (AAG to

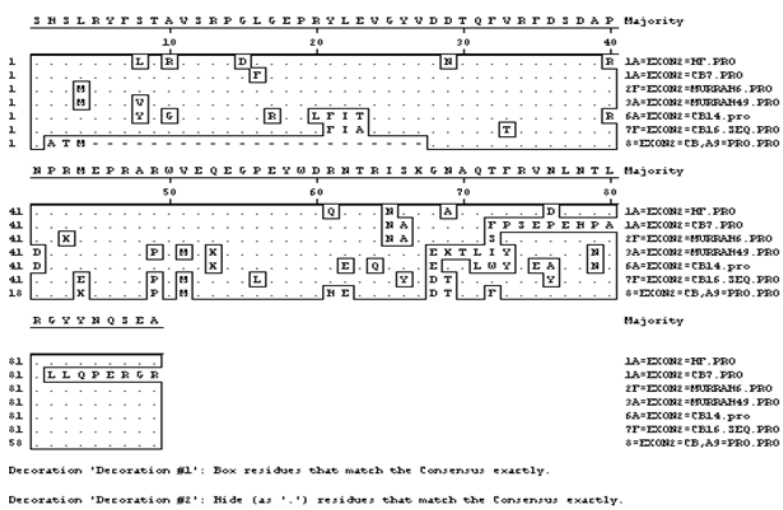

Figure 4. Amino acid sequences of BuLA-A exon 2.

AAT), 636 (TGG to GCT), 642 (GAG to GCG), 645 (GCA to GAA), 651 (GCT to GAT and TAT), 654 (GCG to GCT), 660 (GGC to GTA and CGT), 663 (GAG to GTG and CAG), 669 (AAC to ATC and 684 (ACG to GAG and AAG), which also changed a number of amino acids and caused polymorphism in this region (Figure 5).

Phylogenetic tree analysis

The phylogenetic tree analysis was done by aligning the sequences of BuLA-A exon 2 to 3 alleles to identify allelic lineage of characterized alleles with BoLA-A sequences of Holstein Friesian available in the NCBI GenBank. Phylogenetic tree of BuLA-A alleles did not clearly differentiate buffaloes from cattle. Similar results of phylogenetic tree were reported by De (2000) for DRB3 exon 2 in cattle and buffaloes. Exon 2 to 3 nucleotide sequences showed that the two alleles from buffalo have same lineage group close to each other, which were distantly located from Holstein Friesian cattle. The phylogenetic tree based on nucleotide and amino acid sequences of exon 2 to 3 separately showed differences in closeness of the related alleles. In exon 3, alleles of two Murrah buffaloes were similar to each other and the sequence of Holstein Friesian cattle located at most divergent position. The phylogenetic trees predicted changes in nucleotides as well as amino acids of exon 2 to 3, which were of higher degree. The results further revealed that the nucleotide dissimilarity ranged from 3.0 to 14.7% (Table 2). Lack of polyphyletic lineage for the cattle and buffalo alleles does not support the trans-species persistence of allelic lineage in BoLA-A and BuLA-A in the present study. However, Bussche *et al*. (1999) reported trans-species persistence of allelic lineage in DRB alleles. The results of the present study support the finding of Brunsberg *et al*. (1996); De (2000), who have reported characteristic patchwork pattern in DRB alleles, which can be explained as shared ancestral sequences.

The amino acids sequences of different alleles from exon 2 demonstrated differences in the closeness of the related alleles in phylogenetic tree as percent of divergence ranged from 14.9 to 31.7%. The phylogenetic tree based on amino acid sequences revealed the difference in closeness of the related alleles from the exon 3, as some alleles showed closeness to Holstein Friesian. The phylogenetic tree indicated a higher degree of amino acid divergence (0.0 to 41.1%) in this region.

It may be concluded that the exon 2 to 3 BuLA-A gene was found to be highly polymorphic in all breeds of buffaloes. Cloning and sequencing results confirmed that the length of exon 2 to 3 varied from 713 bp to 715 bp, which was due to addition and deletion of bases. Three genotypes of buffaloes were selected on the basis of PCR-RFLP results and these genotypes were cloned and sequenced. The sequences of these genotypes were submitted to the GenBank and allotted the Accession numbers (AY894407 and AY894408 and AY925136).

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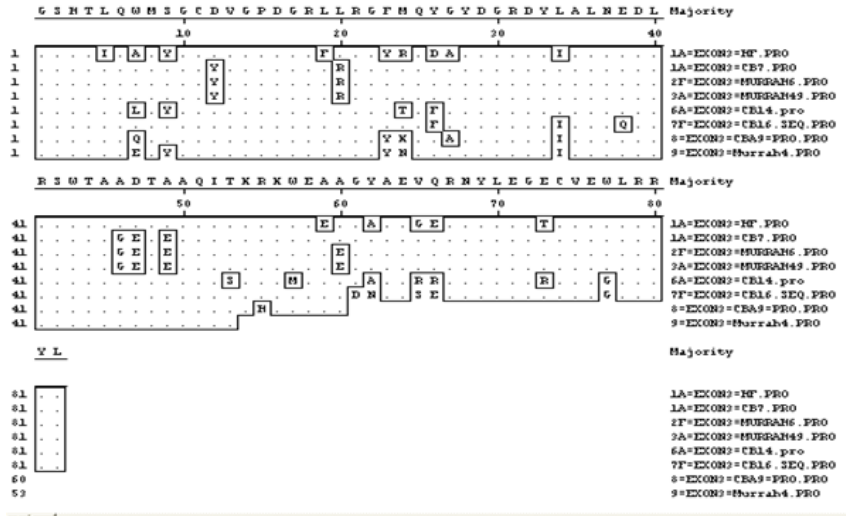

Figure 5. Amino acid sequences of BuLA-A exon 3.

Table 2. Percent similarity and divergence of nucleotide sequences of BuLA-A exon 2-exon 3 alleles.

Percent Similarity

Percent Divergence	1	2	3	4	5	6	
1		93.8	93.0	91.1	89.2	90.7	1
2	6.5		98.7	96.2	89.6	92.4	2
3	7.4	1.3		97.1	89.1	92.1	3
4	9.4	3.9	3.0		89.2	91.0	4
5	11.7	11.2	11.8	11.6		89.1	5
6	10.0	8.0	8.3	9.6	11.8		6
	1	2	3	4	5	6	

1A=716=HF.seq
1A=711.SEQ=CB7.SEQ
2F=713.SEQ=MURRah6.SEQ
3A=713.SEQ=murrah49.SEQ
6A=714.SEQ=CB14.SEQ
7F=714.SEQCB16.SEQ

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