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Comparitive Genome Sequence Analysis of Bovine Lymphocyte Antigen *BoLA DRB3.2* Alleles in Deoni and Ongole (*Bos indicus*) Cattle Breeds of India

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ABSTRACT

Background: India, a major livestock region of the Asian countries is rich in animal genetic resources having special qualities of hardy nature, resistance to many diseases and adopted to adverse climatic conditions. The cattle MHC, Bovine Lymphocyte Antigen *DRB3* (*BoLA-DRB3*) is considered to be a major gene linked with disease resistance traits of Indian cattle.

Methods: The present study was carried out to sequence the *BoLA-DRB3.2* alleles in Deoni and Ongole breeds of Indian cattle. PCR RFLP analysis of the *BoLA-DRB3.2* alleles in Deoni (n=51) and Ongole (n=60) cattle using three different restriction enzymes *Rsa*I, *BstY*I and *Hae*III to find out the possible restriction pattern. Based on the combined allelic patterns, each sample was further analyzed by PCR- SBT technique to detect the SNP variations present in *BoLA-DRB3*.2 alleles.

Result: The PCR RFLP analysis revealed that the highest frequent alleles are **6* (0.216) and **15* (0.225) in Deoni and Ongole breeds of cattle, respectively. The second-highest frequency was observed for *BoLA* alleles **11* and **6* which were present at a frequency of 0.167 and 0.200 in Deoni and Ongole breeds of cattle, respectively. To get the complete picture of polymorphic pattern of *BoLA-DRB3.2* allele direct sequencing was carried out for each plymorphic pattern. The interesting feature noticed in the Ongole breed was that at position 91 and 133 of the sequence, it had both A and G nucleotides in contrast to *Bos taurus* breed, which had only TT nucleotides. The sequence analysis of *BoLA-DRB3* exon 2 between two breeds revealed that there are numerous variations in exon 2, whatever variation, that lead to different mobility shift and band pattern in gels. Deoni and Ongole breeds of cattle had similar variations at positions 94, 134, 211, 235 and 258noticed due to the unique nature of native breeds.

Key words: *BoLA-DRB3*, Deoni, Diversity, Ongole, Sequence-based typing, Zebu cattle.

INTRODUCTION

There are 50 recognized native breeds of cattle in India having special qualities of hardy nature, resistance to many diseases and adopted to adverse climatic condition. Geneticists and evolutionary biologists have great interest to identify the variation of Bovine Leukocyte Antigen (*BoLA*) because of its variability patterns reflected different evolutionary processes *viz*., adaptation to climate, natural selection and genetic drift (Goszczynski *et al.*, 2014; Takeshima *et al.*, 2014). Several studies have shown that high level of *BoLA* diversity might be responsible for disease resistance especially pathogen recognition and presentation to lymphocyte (Borg *et al.*, 2011; Newhouse and Balakrishnan, 2015). Even though, the *Bos indicus* cattle known for its disease resistance, very few studies have been carried out on the characterization of *BoLA-DRB3* genetic diversity and its structure.

Bovine Lymphocyte Antigen *DRB3* (*BoLA-DRB3*) is a bovine Major Histocompatibility complex (MHC) and its polymorphism highly correlated with many disease resistance traits in Indian cattle breed.*BoLA-DRB3* is the potential genetic markers and up to date more than 136 different alleles have been reported (Van Eijk *et al.*, 1992; Gelhaus *et al.*, 1995; Maillard *et al.*, 1999; do Nascimento *et al.*, 2006; Maccari *et al.*, 2017).Several studies have shown that decreased MHC variability might be caused by population bottlenecks (Mason *et al.*, 2011; Zhang *et al.*, 2016). Bovine leukocyte antigens (*BoLA*) have been used as disease markers and immunological traits in cattle due to their primary role in pathogen recognition by the immune system (David *et al.*,2020).Hence, a study was taken up to characterize *BoLA-DRB3.2* alleles in the native animals of Deoni and Ongole breeds of cattle. These results provide an insight into MHC evolution and contribute toward efforts at describing bovine MHC diversity according to breed and location. Hence, this study was aimed at determining

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variability of *BoLA DRB3* through direct sequencing in Indian Ongole and Deoni cattle breeds and compared to sequence of Taurine breeds.

MATERIALS AND METHODS

Genomic DNA was isolated from the whole blood of 51 Deoni and 60 Ongole breeds of cattle as per the method of Miller *et al.* (1988) with slight modifications. Hemi-nested PCR was used for the amplification of the exon 2 (284 bp) of the *BoLA-DRB3* gene, as described by Van Eijk *et al.* (1992) with the primers sequence of HL030 5′- ATC CTC TCT CTG CAG CAC ATT TCC-3′ , HL031 5′ - TTT AAT TCG CGC TCA CCT CGC CGC T-3′ and HL03′ 5′ - TCG CCG CTG CAC AGT GAA ACT CTC-3′. In the first round of PCR, amplification was performed with HLO30 and HLO31 primers with an initial denaturation step of 5 minutes at 94°C followed by 10 cycles of 1minute at 94°C, 2 minutes at 60°C, 1minute at 72°C and a final extension of 5 minutes at 72°C. After the first round, a semi-nested second round PCR reaction was carried out with 1 µl of the first-round PCR product as DNA template, HLO30 and HLO32 primers with the same PCR condition except the annealing temperature 65.5°C for 30s.

In this study, the PCR-RFLP analysis was carried out with three different restriction enzymes *RsaI, BstYI* and *Hae*III in Ongole, and Deoni cattle to find out the possible restriction pattern and restriction patterns were combined to determine the allelic pattern as per the *BoLA* nomenclature (Russell *et al.*, 1997) as reported in the *BoLA* nomenclature homepage (http:// www.projects.roslin.ac.uk/*BoLA*/*DRB3*pcr.htmltable). Results obtained for PCR-RFLP analysis are presented in Table 1. Allele frequencies were determined by Hi =$\sum$ni/N, where H^i is the frequency for allele i, $\sum n_i$ is the sum of alleles in i^{th} population, and N is the total number of alleles in the population. Further, Fishers exact test was performed to verify the difference in frequencies between the breeds. Subsequently the data was subjected to z- test with Bonferroni correction for pair wise comparisons (P<0.05) of proportion (Ryman and Jorde, 2001).

The positive amplified PCR products showing different allelic patterns were further analyzed by PCR- SBT technique to detect the SNP variations present in *BoLA-DRB3*.2 alleles. One sample from each PCR RFLP pattern was selected and sequenced using the forward and reverse primer to detect the variations at the nucleotide level. Sequencing was performed on an automated sequencer (ABI prism) using Sangers's dideoxy chain termination method at SciGenom, Cochin. Clustal Omega is a new Multiple Sequence Alignment (MSA) program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences (Sievers *et al.*, 2011). MSA of *BoLA-DRB3*.2 alleles was done using the DNA star Lasergene MegAlign program (DNASTAR CoreSuit10 software, Inc. USA).

RESULTS AND DISCUSSION

A 284 bp fragment of the *BoLA-DRB3* gene, composed of 10 bp of the 5′ intron, 267 bp of exon 2, and 7 bp of the 3′ intron of chromosme 23, was amplified. PCR-RFLP analysis of the *BoLA-DRB3*.2 alleles in the present study was determined using three restriction pattern of as per *BoLA* nomenclature (Russell *et al.*, 1997). Only 111 alleles were concluded for Deoni (51) and Ongole (60) from established restriction pattern of 150 animals. *BoLA* alleles were unambiguously identified only for 111 animals and the haplotype discriminations were not possible in the remaining animals as they were heterozygous for more than one restriction enzyme and due to the non-availability of production data. Similar findings were reported by Starkenburg *et al.* (1997) and Miretti *et al.* (2001).

The major alleles of *BoLA- DRB3.2*, which had a frequency of 0.05 and above are shown in Table 1 and frequency are significantly higher (P<0.05) based on the χ^2 test.The total number of alleles identified in both breeds was twenty-nine with frequencies ranging from 0.008 to 0.225. Of these total alleles detected, all 29 alleles were similar to those reported in earlier studies (Van Eijk *et al.*, 1992; Gelhaus *et al.*, 1995; http://www.projects.roslin.ac.uk/*BoLA*/*DRB3*pcr.htmltable).

The PCR RFLP results showed that the highest frequent allele in Deoni and Ongole breeds was *DRB3.2*6* (21.6 per cent) and *DRB3.2*15* (22.5 per cent), respectively. A high degree of polymorphism in exon 2 of *BoLA-DRB3* by the PCR-RFLP technique was also revealed by Van Eijk *et al.* (1992). The second-highest number of allele *BoLA* alleles **11* and *6 which were present at a frequency of 0.167 and 0.200 in Deoni and Ongole breeds of cattle, respectively. Of the 22 identified alleles detected in Deoni, nine alleles (*BoLA-DBB3.2*6, *11, *9, *15, *20 *23, *34, *47* and **51*) represented 78.4 per cent of total allelic frequencies. Whereas in Ongole, 77.5 per cent of allelic frequencies were covered by the seven *BoLA-DRB3.2* (**15, *6, *12, *13, *23, *31* and **47*) alleles. Based on the multiple allelic frequency comparison between two breeds, three allelic frequencies showed a statistically significant (P<0.05) differences, after Bonferroni correction, the results clearly indicate that alleles *DRB3*.2 **9* and **11* are unique to Deoni and the allele *DRB3.2*15* are unique to the Ongole breed of cattle (Table 1).

These two populations studied were not homogenous with respect to their allelic composition and there was a significant difference in their allele frequencies. Alleles **19, *25, *35, *38, *41, *46* and **54* in Deoni and alleles **3, *8, *12, *13, *16, *32* and **42* in Ongole were unique to their respective breeds. Duangjinda *et al.* (2009) revealed that the most frequently detected alleles of Holstein x Zebu were *DRB3*16, *51, *23, *11, *8*and **1* accounting for 61.12 per cent of the alleles. Results of the present study indicate that the *BoLA-DRB3* exon 2 is highly polymorphic both in Deoni and Ongole (*Bos indicus*) breeds of cattle.

Several authors reported that the high degree of polymorphism in the *BoLA-DRB3*.2 in various breeds of cattle. For example, in a study carried out by Sharif *et al.* (1998), the highest frequent allele was *DRB3*.2 **7* among

the six the most common alleles of *BoLA-DRB3.2 *7, *10, *17, *21, *28* and *32* in 66 Jersey cows. But it was not observed by Gilliespie *et al.* (1999) where he detected the *BoLA-DRB3.2 *8, *10, *15, *21, *36* and *ibe* as the most frequent alleles in same Jersey breeds. This is comparable to the observation of our findings in these two indigenous cattle breeds.

Dietz *et al.* (1997) carried out polymorphism studies on the *BoLA-DRB3*.2 locus in a population of 127 Holstein cows. They observed that *BoLA-DRB3.2 *8, *11, *16, *22, *23* and *24* were the six most frequently (70.3 per cent) detected alleles. In another study, on Holstein animals (n = 835), Sharif *et al.* (1998) observed that 7 alleles of *BoLA-DRB3.2 *3, *8, *11, *16, *22, *23* and *24* represented 88.7 per cent of the total alleles. But in contrast to our observations, in Argentine Creole cattle (n = 194), 68 per cent of the gene frequencies were represented by 5 alleles (*DRB 3.2 *15, *18, *20, *24* and *27* (Giovambattista *et al.*, 1996). Approximately 70 per cent of the alleles in the Japanese Shorthorn cattle were accounted for by 6 alleles *BoLA-DRB3.2 *8, *9, *21, *27, *7and *24* (Takeshima *et al.*, 2002). Therefore, it could be observed that differences in allelic frequencies existed among different breeds of cattle. The present study on native breeds of Deoni and Ongole clearly indicates that these were allelic variations in the *BoLA* region which are distinctively different from each other.

The amplified sequences exon 2 of *BoLA-DRB3* was 284 bp in length in two different breeds. Nucleotide variation of the *Bos indicus* cattle was compared to the *BoLA* reference sequence (NM_001012680) of *Bos taurus* cattle. There are numerous variations in *Bos indicus* cattle breeds when compared to the Taurine cattle breed. The interesting feature noticed in Ongole and Deoni breed was that at positions 92, 134 and 258 base pair level, it had both A, G and C nucleotide in contrast to the *BoLA* reference sequence (NM_001012680) of *Bos taurus* cattle, which had only T nucleotides (Fig. 1). Further, certain nucleotide variation in the position of the 11(C-G), 34 (T-C), 40(G-A), 181(T-C), 183(C-S), 211(C-G), 214(A-G), 260(T-G) and 275(T-G) are unique to Ongole cattle breed, which was absent in both Deoni and *Taurine* animals but they are sharing similar type of sequences. Even though certain nucleotide variations at 201(T-W), 212(A-G), 213 (A-G), 233(G-C), 235 to 237 (GTG -TAC) and 257(G-T) are unique to the Deoni cattle breed when compare to *Bos taurus* cattle, but few Ongole animals also sharing the same variations. The multiple Sequence Alignment sequence variations of *Bos indicus* breeds confirmed the different combinations of RFLP patterns. Therefore, all these variations prove the existence of heterozygosity in *BoLA-DRB3*.2 alleles in Indian cattle breeds. Further, the presence of both nucleotides in the case of Ongole and Deoni may be specific for Indian inheritance.

The major change of T nucleotide variations was confirmed by the Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution model (Table 2). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics. The nucleotide frequencies are 21.27% (A), 18.47% (T/U), 24.43% (C), and 35.83% (G). The transition/ transversion rate ratios are k1 = 0.658 (purines) and k2 = 1.353 (pyrimidines). The overall transition/transversion bias is R = 0.454, where R = [A*G*k1 + T*C*k2]/[(A+G)*(T+C)]. This analysis involved 22 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 287 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar, 2018).

Both the breeds of cattle have similar variations at positions 94, 134, 211, 235 and 258 which is due to the unique nature of native breeds. The variations in nucleotides of *BoLA-DRB3* exon2 were confirmed to similar findings of the ISAG *BoLA* Nomenclature Committee report (Russell *et al.*, 1997).

Therefore this shows the prevalence of nucleotides A, G, G, TAC and C in exon 2 at positions 92, 134, 211, 235 and 258, respectively in native breeds of cattle *viz*., Ongole and Deoni and their respective position and found to be highly conserved in Indian cattle breeds. This is comparable with the report of Rupp *et al.*, (2007) that the lysine encoding allele (AA) is highly prevalent in *Bos indicus* breeds. Similar polymorphic patterns were also detected in 25 *BoLA-DRB3*

Table 1: Test of significance for allele frequencies of *BoLA –DRB3.2* alleles among the cattle.

	breeds studied	
DRB3.2 alleles	Deoni **(N=51)	Ongole **(N=60)
***DRB3.2**6	0.216 (22)	0.200(24)
***DRB3.2**9	0.078(8)^{B}	
***DRB3.2**11	0.167(17)^{B}	
***DRB3.2**12		0.092(11)
***DRB3.2**13		0.108(13)
***DRB3.2**15		0.225 (27)^{A}
***DRB3.2**23	0.059 (6)	0.075 (9)
***DRB3.2**34	0.059 (6)	
***DRB3.2**51	0.059 (6)	
Total	6	6

** P(<0.001) ; Deoni: χ^2 = 28.22, df= 5, p= 0.000033; Ongole: χ^2 = 21.89, df= 4, p= 0.0002.

$^{A, B}$ (P<0.05) z- test with Bonferroni correction .

Table 2: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution.

	A	T	C	G
A	-	6.25	8.26	7.98
T	*7.19*	-	11.18	12.12
C	*7.19*	*8.45*	-	12.12
G	*4.74*	*6.25*	*8.26*	-

Each entry shows the probability of substitution (r) from one base (row) to another base (column).

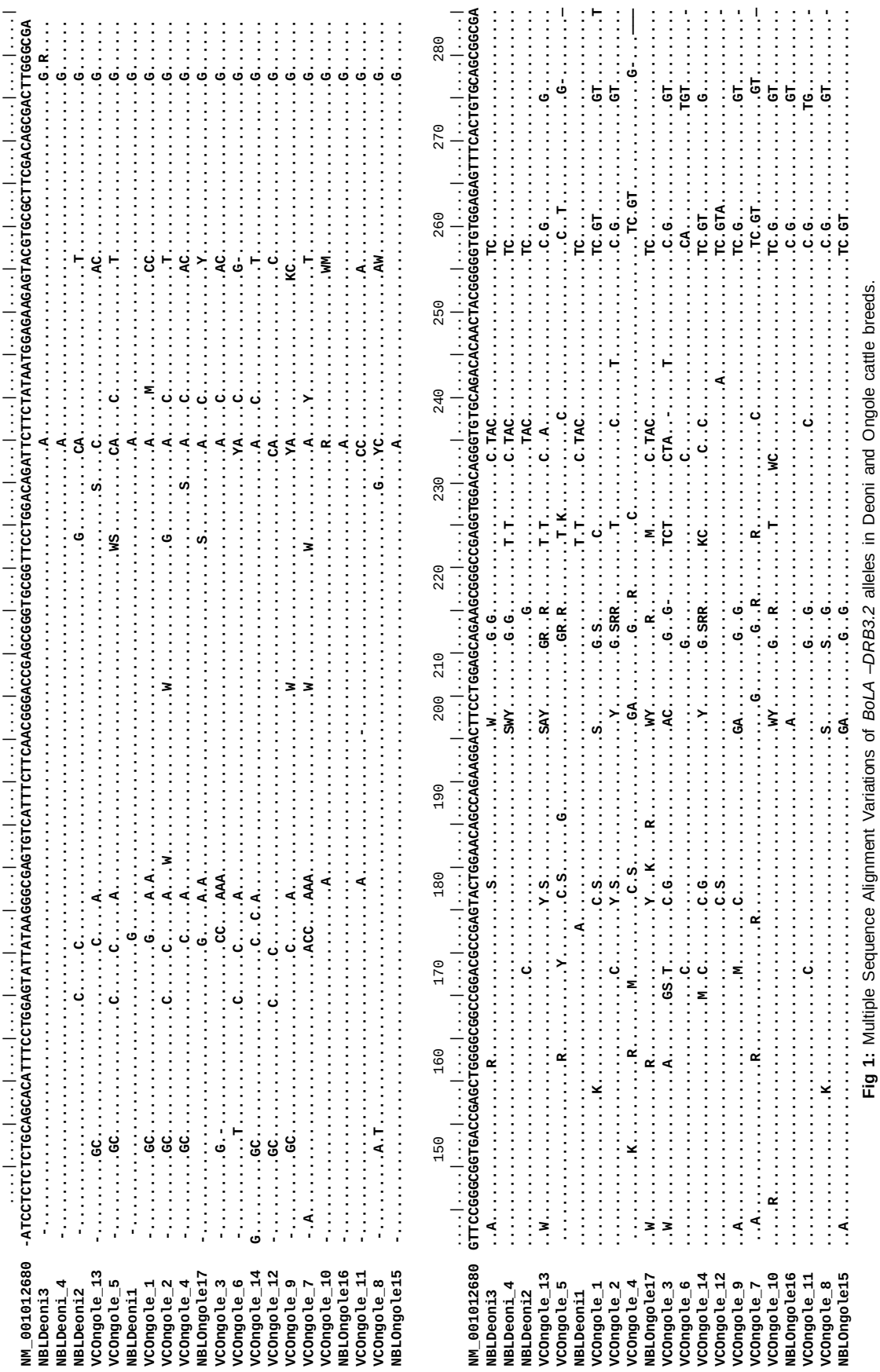

Fig 1: Multiple Sequence Alignment Variations of *BoLA –DRB3.2* alleles in Deoni and Ongole cattle breeds.

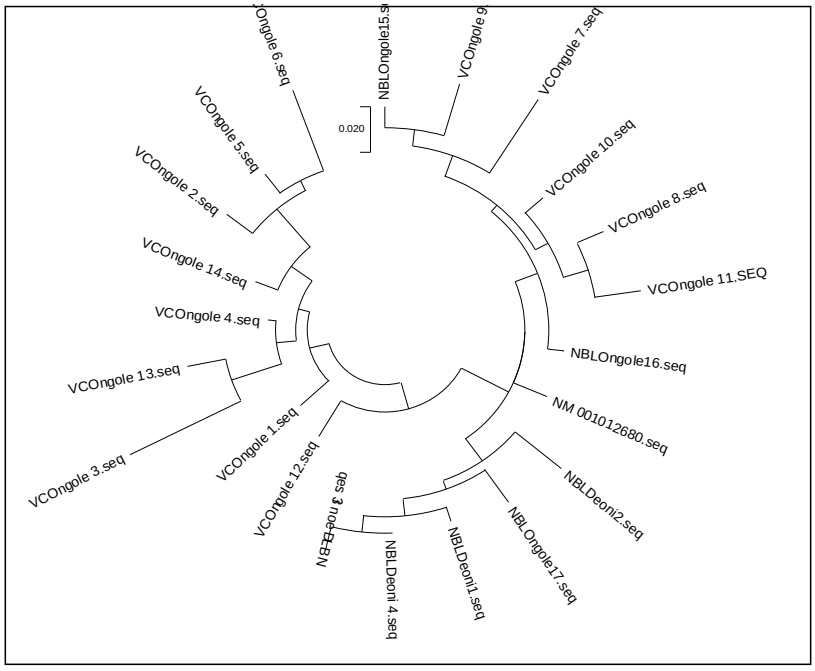

Fig 2: Phylogenetic tree of *BoLA –DRB3.2* sequences of Deoni and Ongole cattle breeds.

alleles including 2 deletions in five Indian cattle breeds (De and Singh, 2006).

Further, the *BoLA –DRB3*.2 sequences of Deoni and Ongole cattle are showing the variation in their origin reflects through the groups of different clades of the phylogenetic tree (Fig 2). Phylogenetic tree analysis revealed that there are three major clusters each cluster indicates the three different nucleotide variations of breeds. Further, the observed sequences of *Bos taurus* and *Bos indicus* are showing the difference between different clads. All the Ongole nucleotides are almost similar in nature clustered in a single clad branched exclusively from the *Taurine* origin. The nucleotide variations present between Ongole and Deoni reflect in two separate clades of the phylogenetic tree, but the two clades branched from the *Bos taurus* cattle breed which indicates that the *BoLA* sequence of Indicus is different from Taurine origin. The other clusters contain both Deoni and Ongole cattle sequences which show that both breeds have a certain sequence of common nucleotides. The different branches of the clad clearly indicate that the *Bos indicus* sequence is different from Taurine sequences.

The aligned nucleotide sequences have confirmed the uniqueness of aligned sequences with other sequences available in the NCBI gene bank database. The Multiple alignments of finally selected and trimmed sequences along with the *BoLA* Consensus sequence defined by the *BoLA* Nomenclature Committee was obtained using ClustalW2 from EMBL for determining the amino acid variations between the protein sequences of the two breeds mentioned above. The alignment of final protein sequences of Deoni and Ongole breeds along with the *BoLA* Consensus sequence is depicted in Fig 2. A similar polymorphism was also observed by Takeshima *et al.* (2002), who analyzed PCR-SBT in a total of 176 Japanese Shorthorn cattle and identified 21 distinct alleles with different amino acid sequences in the translated *BoLA-DRB3* alleles. The aligned amino acid sequences were in accordance with the detailed DNA sequence as reported by Russell *et al.* (2004). In a PCR based typing method, Takeshima *et al.* (2018) identified that South American Zebu cattle breeds had a gene diversity score and a nucleotide diversity score higher than 0.86 and 0.06, respectively. Further, the authors observed that the historical divergence between Taurine and Zebu cattle breeds which may be due to their respective origin, selection and adaptation. The results could clearly explain that these Zebu and Taurine types had differences in *BoLA-DRB3* gene exhibiting diversity.

CONCLUSION

PCR-RFLP and PCR-SBT are powerful techniques to detect polymorphism in the exon 2 of the *BoLA-DRB3* gene. The amplified fragment of *BoLA-DRB3*.2 alleles was found to be highly polymorphic as revealed by the PCR-RFLP variant data. The number of RFLP variants and types of DNA sequences indicates the involvement of more than one allele in PCR amplified genomic DNA. In Deoni and Ongole breeds of cattle *BoLA DRB3*.2*6 (0.216) and *15 (0.225) are the most common alleles with the highest frequency. The amplified fragment of *BoLA-DRB3.2* alleles was found to be highly polymorphic in both Deoni and Ongole (*Bos indicus*) breeds of cattle as revealed by the PCR-RFLP variant data. Further, exploring the inheritance pattern of the *BoLA-DRB3* gene with large number of samples form this two breeds may further reveal the crucial role of the *BoLA-DRB3* gene in providing disease resistance. The observed alleleic varition may also be studied for its

association with the disease resistance in indigenous cattle by incorpoarting large sample size and from differnt indigenous cattle breeds to draw coclusion for the role played by BoLA DRB3 genes in combating the disease occurance.

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