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Original Article

Analysis of variations, structures, and phylogenic characteristics of bovine leukocyte antigen DRB3 exon2

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ABSTRACT

Bovine leukocyte antigen (BoLA) DRB3 is a highly polymorphic gene in major histocompatibility complex (MHC) class II that plays a central role in immune responses and production factors**.** As of yet, molecular and evolutionary characteristics of BoLA-DRB3.2* have not been as fully understood as human and mouse. Therefore, we attempted to analyze variability and phylogeny of BoLA-DRB3.2* and illustrate some novel practical evidence on interspecies diversity, the resistance /susceptibility points in cattle breeding, and vaccine design. Initially, BoLA-DRB3.2* alleles and orthologous exons in the selected livestock were retrieved and checked. In the next step, the secondary/tertiary structure of BoLA-DRB3.2*24 gene product was modeled and validated. Then, hypervariable regions (HVRs) of alleles were identified by hybrid approaches. In the last step, interspecies relationship, allele's phylogeny/grouping, and estimate of average evolutionary divergence were explored. Shannon entropy variation analysis showed eight HVRs and three semi-variable regions in BoLA-DRB3.2* alleles. These HVRs were present in all the three sub-structures and dominantly existed in alpha helix. In addition, strong relationships and little diversity were noted in phylogenetic trees of cattle, buffaloes, sheep, and goats. Furthermore, there was some evidence on divergence of DRB3 before speciation among the mentioned species and possibility of cross prediction resistance/susceptibility alleles. Finally, DRB3 alleles were grouped into seven clusters, and older and newer alleles were identified. The results show that similar studies should be done in other animals to better understand the nature of the DRB3 attributes.

Keywords: BoLA-DRB3.2, Cattle, Variation, Modeling, Phylogeny

L'analyse des changements, la structure et les propriétés de Filoujnikagezone 2 de BoLA-DRB3 chez les vaches

Résumé: L'exon 2 de BoLA-DRB3 est un gène lié au MHC classe 2, jouant a un rôle central dans la réponse immunitaire et les facteurs de production. Jusqu'à présent, les caractéristiques moléculaires et évolutives de BoLA-DRB3.2 ainsi que pour des molécules équivalentes comme HLA2-DRB3 chez l'homme et H2-DRB3 chez la souris n'ont pas été suffisamment comprises. A cet effet, des analyses de variabilité, phylogénétiques, structurelles et de séquencage de BoLA-DRB3.2 ont été menées. Cet article présente quelques nouveaux résultats aidant à determiner la variation entre les espèces et la sensibilité / résistance à cette maladie pour la conception d'un nouveau vaccin. En premier lieu, les allèles de BOLA DRB3.2 et les exons orthologues ont été extraits de la base des données afin d'être analysées. Ensuite, les structures secondaire et tertiaire de BoLA-DRB3.2 ont été visualisées et vérifiées. Les régions très variables (HVR) ont été identifiées par des approches hybrides. La dernière étape consistait à étudier les relations interspécifique, comme la phylogénie et le regroupement des allèles ainsi que l'estimation moyenne de la divergence évolutive. Les résultats de ces analyses ont montré huit HVR et trois frontières dans l'analyse de la variabilité de l'entropie de Shannon. Ces HVR ont été détectés dans toutes les sous structures et étaient plus particulièrement localisés au niveau sde l'

alpha hélix. Les analyses phylogéniques montraient une relation évolutionnaire élevée et une faible diversité parmi les bovins, les buffles, les moutons et les chèvres. De plus, ces données suggèrent que DRB3 est exprimé avant la spéciation pouvant, de ce fait, permettre la prédiction et la sélection des allèles responsables de la résistance / sensibilité sur la base de la similitude entre ces espèces. Enfin, DRB3 a été divisé en sept groupes et les classes d'allèles anciennes et plus récentes ont été identifiées.

Mots-clés: BoLA DRB3.2, vache, les changements, la modélisation, la phylogénie

INTRODUCTION

The immune system can detect most invading pathogens affecting the physiological parameters of the body. The presentation of processed pathogen-derived peptides to host lymphocytes is the pivotal initial step of an immune response mediated by major histocompatibility complex (MHC) molecules located on antigen-presenting cells. The potential for detection of pathogens is dependent on the somatic variations and diversity of the MHC genes (Tizard, 2013). To design effective, broad-spectrum vaccines and conceptualize how exactly immune molecules react to pathogens, it is essential to identify characteristics, structure, and variability of BoLA-DRB3 frequent alleles (Ranjbar et al., 2015). MHC is a cluster of genes playing a role in immunological and non-immunological reactions present in all vertebrates (Tizard, 2013). MHCs in buffaloes and cattle are termed buffalo lymphocyte antigen (BuLA/Bubo) and bovine lymphocyte antigen (BoLA), respectively (Tizard, 2013; Ranjbar et al., 2016). Similar to human leukocyte antigen (HLA), in bovidae MHC is composed of three classes including I, II, and III, some of which are expressed as cell surface glycoproteins. The only major difference between HLA and BoLA is the presence of a large gap within the bovine class II region, which splits the BoLA II into two subregions named IIa and IIb. The IIa subregion is composed of two loci of DR and DQ (Tizard, 2013). MHC class II encodes alpha (α) and beta (β) chains on the surface of antigen-presenting cells (APCs) and allows binding of foreign polypeptides (called antigens) (Tizard, 2013). The high degree of genetic polymorphism in MHC II genes was reported in a number of vertebrate species. DR region of MHC class II molecule is highly polymorphic and probably plays an important role in the development of MHC-restricted immune responses (Ranjbar et al., 2016). In cattle, DR consists of two loci known as DRA and DRB, the latter being the only source of DR diversity. Among the three BoLA-DRB genes in bovines, DRB3 was reported to be functional and MHC variations known for other species such as human and mouse were also observed in cattle DRB3 gene polymorphism (Tizard, 2013). BoLA-DRB3 exon 2 (BoLA-DRB3.2*) is a highly polymorphic region in MHC class II, which is homologous to BuLA-DRB3 in buffaloes (De et al., 2002; Ranjbar et al., 2016). Different methods can be adopted for evaluation of DRB3 polymorphism, with sequencing generating more reliable data (Takeshima et al., 2001). Even before immune molecules, especially MHC, were structurally characterized by experimental and *in-silico* methods, it was hypothesized that areas with greater amino acid sequence variability could be considered surrogate indicators of antigen interaction points in the receptor, and this general correlation has since been verified (MacCallum et al., 1996). In a wide range of studies on BoLA-DRB3 polymorphism, some alleles were observed more frequently in Holstein breed (70-89%) (Behl et al., 2012). Furthermore, frequency of BoLA-DRB3.2*24 in different populations was significant (Behl et al., 2012), revealing correlations between this allele and susceptibility to some diseases and production factors (Rupp et al., 2007; Yoshida et al., 2009b). Several quantitative methods were adopted for the measurement of conservation and variability of different sequences of genes and their products, proteins (Johansson and Toh, 2010). Therefore, the

challenge of identification of the cellular processing products directly interacting with antigen in BoLA-DRB3.2 and regions of greater amino acid sequence variability is still a practical approach in the field of host pathogen interactions. For instance, in case of BoLA-DRB3.2, researchers previously found significant association between the existence of glutamic acid at variable position β74 and occurrence of mastitis by *Staphylococcus* species. This motif was reported in BoLA-DRB3.2*22, *23, and *24 alleles (Sharif et al., 2000). Also, arginine or lysine at variable position 13 in *23 and *8 alleles was associated with a high risk of clinical mastitis caused by the same bacteria (Sharif et al., 1999). Recently, a project was implemented on BoLA-DRB3 allelic polymorphism among Holstein cattle and river buffaloes (Nikbakht et al., 2012; Ranjbar et al., 2016). It was found that although BoLA-DRB3 is a crucial gene in animal breeding and resistance/susceptibility to diseases, there is limited knowledge on its variability, structure, interspecies phylogeny, as well as phylogenetic characteristics. Thus, we attempted to characterize BoLA-DRB3.2 allele sequential and structural variations. Furthermore, grouping, interspecies relationship, and allelic evolution were prepared based on BoLA-DRB3.2 allele.

MATERIALS AND METHODS

Sequence retrieval and dataset construction. Protein/nucleotide sequences of exon 2 from BuLA-DRB3 gene in cattle and similar sequences in some large animal species (e.g., water buffalo, sheep, goat, and horse) as well as carnivores (dog and cat) were retrieved from IPD-MHC (http://www.ebi.ac.uk/ipd/mhc/), GenBank (http://ncbi.nlm.nih.gov/), and UniprotKB) databases.

Alignment and finding the variable regions in BoLA-DRB3 dataset. The obtained sequences were aligned using ClustalW algorithm and analyzed in Bioedit v7.7.9 software (mBio Inc, North Carolina, USA). Shannon entropy analysis, Simpson index, and Wu-Kabat variability coefficient approaches were employed to determine variability (finding the mutational /conservative regions) among sequences.

Structure prediction and energy minimization. The secondary structure of BoLA-DRB3.2*24 allele was predicted using PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/). To predict the tertiary structure, the composite approach of full-length protein structure modeling, implemented in I-TASSER server (http://zhang.bioinformatics.ku.edu/I-TASSER), was used. The model was further subjected to the constraint energy minimization procedure with a harmonic constraint of 100 $kJ.mol^{-1}.Å^{-2}$ applied for all the protein atoms using the steepest descent and conjugate gradient technique with Gromos96 43B1 parameters set, implementation of Swiss-PdbViewer, to eliminate the bad contacts between structural water molecules and protein atoms and improve the stereochemistry of the model.

Models validation. The evaluation of model quality is an essential step in the modeling methodology. The geometric evaluations of the modeled 3D structure (tertiary structure) was performed using the Ramachandran plot. Ramachandran plot is a two-dimensional (2D) scatter plot of φ and ψ torsional angles, which test the stereo-chemical stability of model and the number of outliers. Rampage derives Phi/Psi plots for Gly, Pro, Pre-Pro, and other residues.

Interspecies relationship, allelic phylogenic analysis, and estimates of average evolutionary divergence analysis. To visualize the extent of relatedness among BoLA-DRB3 exon 2 and orthologous exons in other species based on IPD-MHC datasets, MEGA v.7 software package was used to build phylogenetic trees. Interspecies tree was constructed using the neighbor-joining (NJ) algorithm under the global gap removal option and Kimura's two-parameter substitution model. Robustness of phylogenetic analysis was measured by 10,000 bootstrap replications. Grouping and evolutionary trees of BoLA-DRB3.2 were drawn by statistical method of

maximum likelihood using heuristic analysis and were checked by 1000 bootstrap replications. The percentage of replicate trees in which the associated taxa clustered together in bootstrap test is shown next to the branches**.** Estimates of average evolutionary divergence over all sequence pairs and similarity between alleles were also calculated by MEGA v.7 software.

RESULTS

Identified amino acid variable regions in BoLA-DRB3.2. The positions with frequencies higher than H > 2.0 (in Shannon entropy plot), D > 0.6 (in Simpson plot), and variability > 15 (Wu-Kabat) were considered variable, whereas positions with lower frequencies than these thresholds were deemed semi-conserved or conserved regions. Measuring variation plots of BoLA-DRB3.2 for all the known alleles (130 alleles) revealed 8 hypervariable regions (HVRs) (Figure 1a, HVRs are marked by arrows) and 3 borderlines (Figure 1A, marked by circle) for Shannon entropy, while Simpson, and Wu-Kabat plots the numbers of identified HVRs and borderlines were 7 and 5 (Figure 1B) and 3 and 3 (Figure 1C), respectively. Other positions with frequencies lower than the thresholds were supposed as semi-conserved regions (marked as chevron []). Region (position) aa 5 to 7 showed the most inconsistent hypervariability. Furthermore, results of Shannon and Simpson variation plots were comparable and Wu-Kabat plot was less sensitive for the detection of HVRs. As will be discussed later, the results of Shannon plot were more reliable to some extent. Three conserve regions were identified in BoLA-DRB3.2 by evaluation of conserve regions using a window length of 4 residues and considering maximum entropy of 0.2, maximum entropy of 0.2 in each region, and a maximal interval of 2. These regions were amino acids 6 to 17 (NH_2- ECHFFNGTERVR-COOH), amino acids 25 to 28 (NH2-NGEE-COOH), amino acids 31 to 36 (NH2-RFDSDW-COOH), and amino acids 43 to 46 (NH_2-TELG-COOH). The overall mean diversity for all the 135 BoLA-DRB3.2 alleles was calculated to be 0.177±0.026.

Three dimensional and secondary structure representations, evaluation of variations, and model validation. For better elucidation of variability and conservation, three-dimensional (3D) and secondary structures were modeled. The degree of variability was illustrated by using different colors from navy blue (to show constant regions) to red (to demonstrate HVRs) in Figure 2. The variable regions play a pivotal role in the interaction between BoLA-DRB3.2 alleles and antigen segments, and the majority of differences between alleles are limited to these regions. As can be noted in Figure 2, variable regions present in the all the three sub-structures of alpha helix, beta strands, and coils. Probably, as shown in Figure 2, antigenic epitope after presenting in exon pocket, majority of bindings occurs with these dark and light red color regions, and then white and with low probability blue regions. These blue regions may acts are framework in pocket. Additionally, most variable regions were in alpha helix structure of exon and functioned as fingers of hand in catching antigen epitopes. Results showed that most variable regions (amino acid 5 to 7) were located in opening of exon pocket, where probably the first contact of epitope with pocket is occurring. Secondary structure of BoLA-DRB3.2 (Figure 2 lower part) showed that *24 had an alpha-helix region, four regions of beta strand with different lengths, and six stretches of coil structure. The predicted 3D model of the BoLA-DRB3.2 protein was validated using Ramachandran plot**.** Assessment of the plot showed that 91.4% (74 amino acids) of residues are in the most favored regions, 8.6% (7 amino acids) in allowed regions, and no residue in the outlier regions. The overall percentage of residues in the favored and allowed regions was 100%. The results obtained for accuracy, quality, and validity of the predicted model in this study were excellent because almost all residues of the protein were located in the most favored and allowed regions of Ramchandran plot.

Interspecies phylogenetic tree and pairwise identities. In phylogenetic tree, based on classification of mammals criteria, carnivores (dog and cat) are

located in relatively distant clade from large animal (Figure 3). Horses show significant divergence from

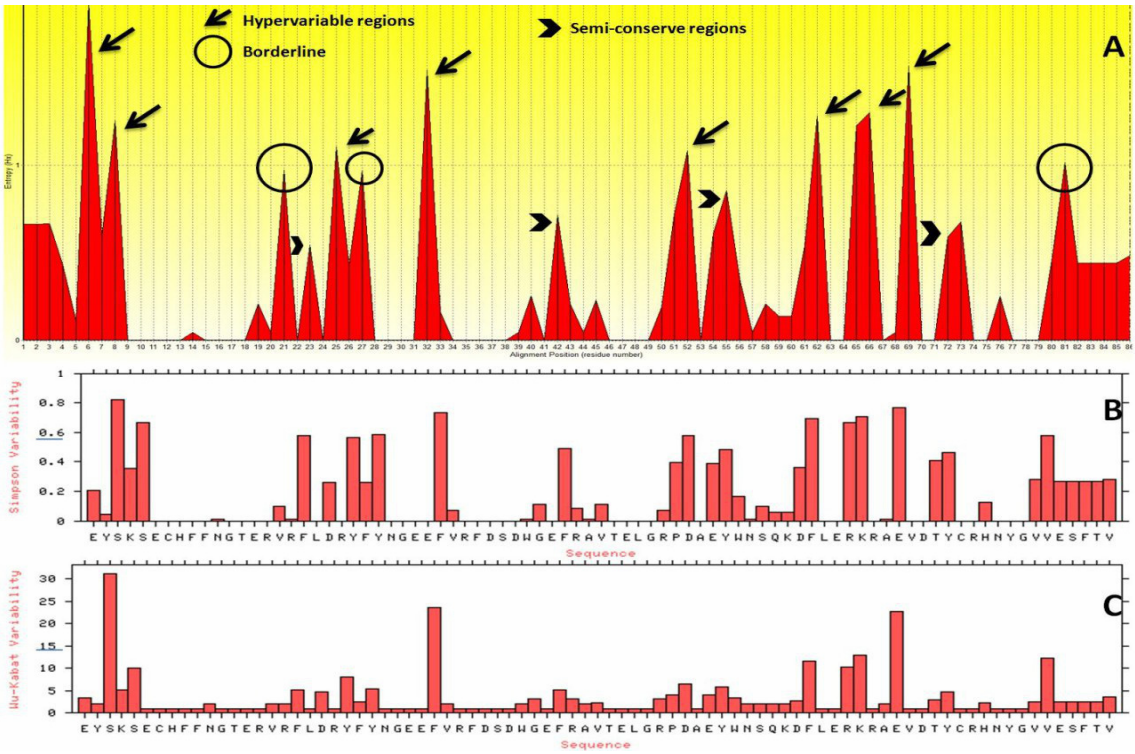

Figure 1. The diversity of the near-complete sequences of BoLA-DRB3.2 alleles measured by the Shanon entropy (A), Simpson (B), and Wu-Kabat (C)
Legend: The values were computed using alignments of allele's sequences. Plots represented variations along protein sequences of 103 haplotypes of BoLA-DRB3.2 alleles. The X-axis shows the probable amino acid on protein sequence positions and the Y-axis is the Shannon, Simpson, and Wu-Kabat computed results. In Shannon plot, the regions with the thresholds higher than 1 are considered hypervariable regions. In Simpson and Wu-Kabat plots, the thresholds were appointed as 0.6 and 15, respectively.

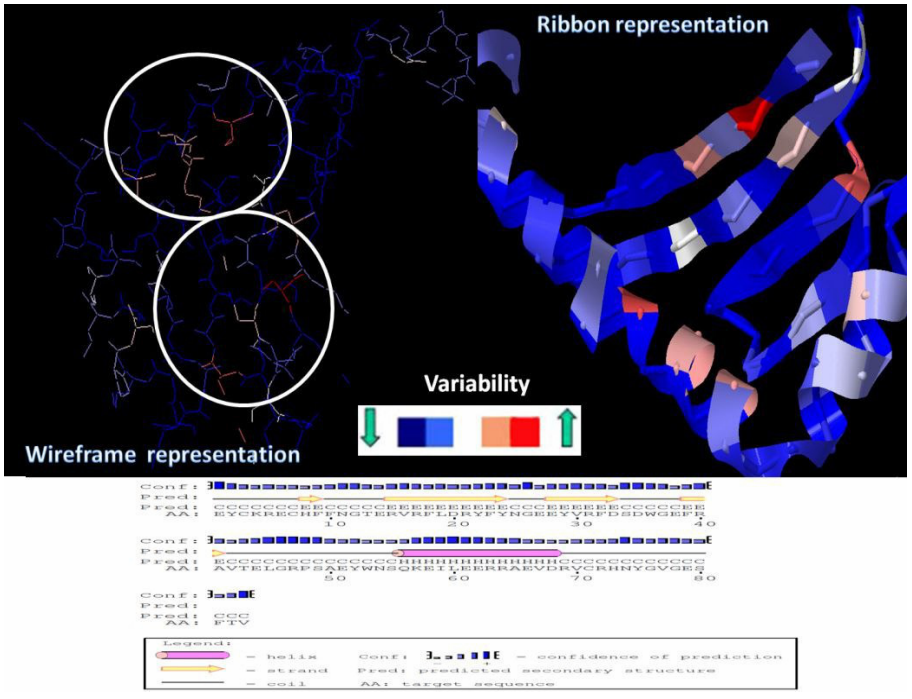

Figure 2. Visualization map of Hyper-Variable Regions (HVRs) by different of representation patterns in 3D (upper) and also secondary (below) structure of) exon 2 of *BoLA-DRB3*.2 allele.
Legend: The picture shows wireframe (upper left), andribbon modeled structure of *BoLA-DRB3*.2 allele. The degree of variability has been illustrated by using different colors. *BoLA-DRB3*.2 allele; as it can be seen in upper part of the figure the hyper variable regions present in all three sub structure of alpha helix, beta strands and coils which has been shown by pink and red parts.

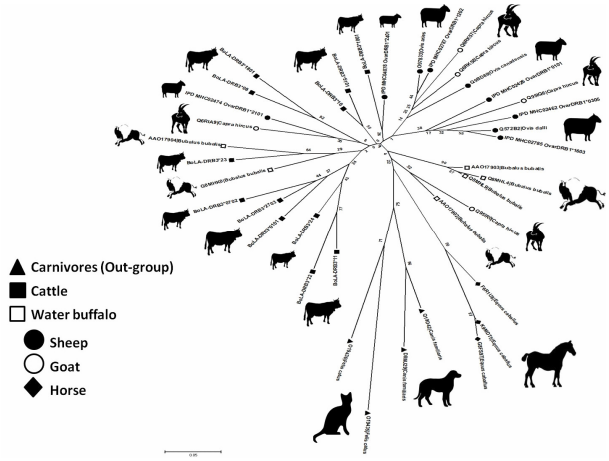

Figure 3 . Phylogenetic tree of cattle exon 2 of *BoLA-DRB3* gen diversity among some livestock (water buffalo, sheep, goat and horse .(The sign for each animal is shown in figure. Dog and cat sequences were selected as out group .For constructing tree we used neighbor joining) NJ (algorithm by 1000 bootstrap.

other large and small animals (cattle, water buffalo, sheep, and goat) and exist in a clade between carnivores and large animals. Cattle, sheep, goat, and water buffalo alleles were significantly similar to each other, while some alleles were more similar to water buffalo and some others to sheep and goat. These four mentioned animals demonstrated high degrees of similarity and relationship in phylogenetic tree, strengthening the discussions of similarity of the immune system in these animals and defining a new field on shared resistance and susceptibility to some diseases. In detail, based on reported alleles in cattle as responsible alleles to resistance or susceptibility, we could predict responsible alleles in sheep for resistance to same diseases or the other way around. This finding helps with minimizing time and costs required for animal breeding. Furthermore, it emphasizes that divergence may occur after speciation in these animals.

In addition, estimates of evolutionary divergence between groups (cattle, goat, and sheep alleles) were 0.219 and 0.200 for cattle-goat and cattle-sheep, respectively. Also, estimate of average evolutionary divergence (and diversity) over all sequence pairs in

Figure 4. Constructing nucleotide phylogenetic tree by Maximum likehood method for BoLA-DRB3 exon 2 alleles . Defined group showed in figure. DLA-DRB1 00101 and 00102 used as Out-group.

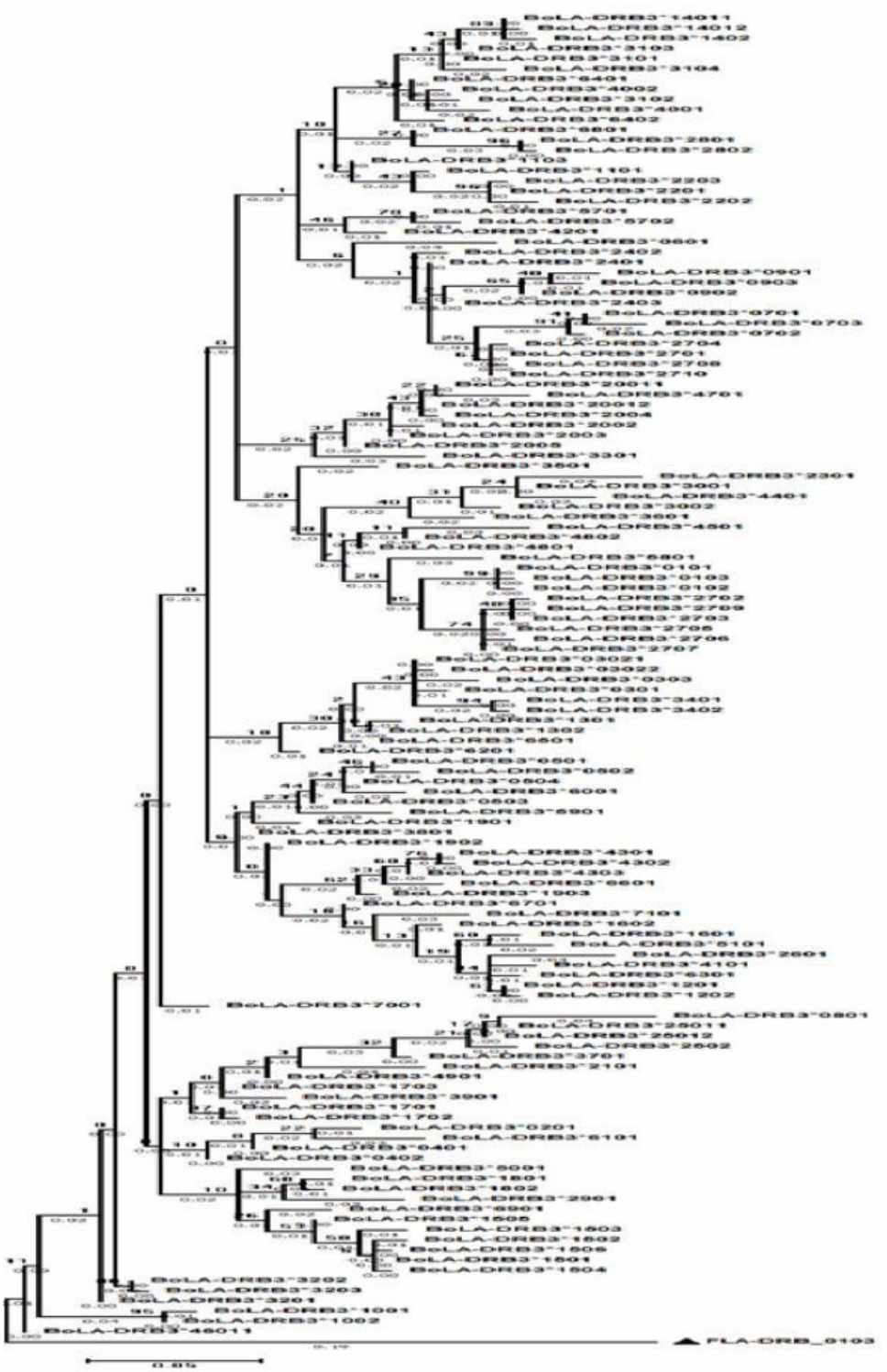

Figure 5. Phylogenetic tree of exon 2 of *BoLA-DRB3* alleles by maximum likehood (ML) using heuristic method.

large (except horse) and small animals was 0.20, reflecting similarity of sequences and low diversity among them. Grouping of BoLA-DRB3 alleles revealed seven clusters including G1 with 33 members, G2 13, G3 14, G5 10, G6 10, and G7 16 (Figures 4 & 5). These allelic groups could help better understand and interpret allele clusters that may be responsible for disease resistance and susceptibility.

DISCUSSION

High rate of polymorphism in BoLA-DRB3 alleles and heterozygote advantage in the MHC genes help the immune system to respond with high degree of diversity and to a wide range of pathogen-derived antigens, and in turn, increase fitness of host. More than 20 years has been past from identification of 30 DRB3 cattle alleles using polymerase chain reaction technique. Today, the number of identified alleles has reached to 103 and the association of these alleles with resistance and susceptibility to several diseases has been identified (Takeshima et al., 2003; Yoshida et al., 2009a; Yoshida et al., 2009b). Among the identified alleles, *24 has significant frequency in different cattle populations (Behl et al., 2012; Ranjbar et al., 2016). Furthermore, experimental studies proposed that this allele is responsible for susceptibility to bovine viral leukosis, mastitis, somatic cell count (SCC), and increased milk fat (Yoshida et al., 2009a). In the present study, the modeling of *24 BoLA-DRB3 allele structure and the variability of other alleles with this allele was fulfilled. Shannon entropy analysis method is one of the most useful practices that has frequently been applied for analyzing conserved regions in multiple alignments of protein sequences (Gupta et al., 2011; Gupta et al., 2012). The Simpson index is another variation analysis method for calculation of diversities. The Wu-Kabat variability coefficient is a well-established descriptor of the susceptibility of an amino acid position to evolutionary replacements. Immunologists are more familiar with Wu-Kabat method, especially because they frequently use the method for variation and polymorphism analysis of antibody and MHC molecule (Parham et al., 1989). The Wu-Kabat method has been already used in BoLA research for analyzing variation and polymorphism of different loci (Miyasaka et al., 2011; Gowane et al., 2013). Although this method plays an important role in the study of variation and polymorphism of MHC, its clear interpretation of variation and quantitative studies is not as efficient as Shanon entropy method. In variation analysis by Shanon entropy model, eight regions were identified with high variability and three regions as semi-conserved. Using Simpson and Wu-Kabat methods, respectively seven and three HVRs and five and three semi-conserved regions were identified.

Studies on MHC class II showed that these regions are associated with susceptibility and resistance to diseases (Sharif et al., 2000; Takeshima et al., 2009). HVRs and semi-conserved regions in peptide-binding groove contribute to establishing connections with epitope, in humans defined as antigen recognition site (ARS). In animals, the structure of DRB3 is not as well-studied as in human and mouse (Tizard, 2013). A similar study by Wang et al. in 2008, which sequenced 15 new BoLA-DRB3.2 alleles, identified only four HVRs. These highly variable regions were HV1 (9-13), HV2 (26-38), HV3 (56-61), and HV4 (66-78). These results were not consistent with our findings, which identified eight HVRs. One reason for this difference can be the limited data on alleles at the time of Wang's (2008). Here, we performed a comprehensive analysis of variations in exon 2 of BoLA-DRB3 based on the IPD-MHC dataset, which provides a centralized repository for sequences of MHC from a number of different cattle species. Protein function is dependent on corresponding tertiary (3D) structure, and therefore, information on tertiary structure is a prerequisite for understanding protein actions, developing effective vaccines, and *in-silico* evaluation of amino acids related to disease susceptibility and resistance and dockings studies (Marti-Renom et al., 2000; Tramontano et al., 2001; Paital et al., 2011). Structural characterization of proteins requires methods such as X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR), and

cryo-electron microscopy; however, due to the labor intensiveness and technical difficulties of these methods, computational techniques are frequently used to model the protein molecules whose structure is not available in the Protein Data Bank. The computational modeling could cover gaps between the accumulated protein sequences data and structural information and can reduce time and expenses (Berman et al., 2000). In human and mouse, MHC 3D structure is well-defined (Behl et al., 2012), while data on other animal species is not as complete. Accordingly, the present study on prediction of secondary and tertiary structures of BoLADRB3 molecule could be groundbreaking. The phylogenetic tree represented high similarity among cattle, water buffalo, sheep, and goat in this orthologous exon. Besides their evolutionary relationships, similarities between the immune systems of these species, and the overlapping range of pathogens that infect these four livestock species may support our hypothesis that disease associations discovered for the BoLA system can have similar applications in other closely related species and vice versa (Burt, 2009), which helps to save time and costs in animal breeding and improve the quality of designing effective and broad-spectrum vaccines for diseases affecting all cloven-hoofed animals, in particular cattle, water buffaloes, sheep, and goats. Given the similarities in antigen recognition and representation, when there is limited access to alleles of a species, alternative alleles of another related species can be utilized for prediction of binding peptide (Nielsen et al., 2010; Tomar and De, 2010). On the other hand, some alleles in cattle are associated with production factors and body condition scores. Therefore, by closest similarity evolutions of DRB3 alleles in cattle, water buffalo, sheep, and goat one could cross predict responsible alleles for immune response and production factors, if they presented before in experimental correlation studies in an animal species. As described in previous studies, notably by De et al. (2002), these four species (cattle, buffalo, goat, and sheep) are located in one group and there were no differences between cattle and water buffaloes in BoLA-DRB3 alleles. The justification for their conclusion was diversion of this gene before speciation in two animal species. In the present study, we hypothesized that sheep and goat could be added to this finding, as well. Similar results (Takeshima et al., 2001; Jeong et al., 2007) in evaluation of variation among different cattle breeds such as Holstein, Jersey, Japanese Shorthorn, Japanese black, and Hanwoo showed high similarity and no distinct clade in phylogenetic tree. We found more probable ancient allelic lineages in cattle.Also, in comparison between cattle and water buffalo, some studies revealed that BuLA-DRB3 (Bubu-DRB) recombination and/or gene conversion is lower than cattle alleles ,since it may be more ancient than BoLA-DRB3 alleles (Sena et al., 2003). Some other articles also indicated that particular ancient allelic lineages were preserved, and clearly, most major lineages in the gene tree in some large animal species diverged well before the family level diversification. It seems that DRB3 alleles may have evolved through multiple lineages (Russell et al., 2000). It can be concluded that the BoLA-DRB exon 2 genes have wide variations across their alleles, and this exon represented high degree of similarity between cattle, buffaloes, sheep, and goats. Computational approaches have been successfully used by many scientists, supporting the potential of immuneinformatics as a powerful tool for investigating the characteristics of molecules and immune system structures. The present article can help with further analysis of BoLA-DRB3.2 alleles for designing effective broad-spectrum vaccines for cattle, buffaloes, and sheep based on T-helper lymphocytes. It can also be used in optimization of breeding programs, as well as finding alleles associated with resistance/susceptibility to infections, fertility, growth, and milk production parameters.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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