

ORIGINAL ARTICLE

Molecular characterization of MHC II DRB3 gene of swamp- and riverine-type water buffaloes

Noraine P. Medina¹, Arren Christian M. De Guia¹, Virginia M. Venturina¹, Claro N. Mingala^{1,2,3}

¹College of Veterinary Science and Medicine, Central Luzon State University, Science City of Munoz, Nueva Ecija, The Philippines

²Biosafety and Environment Section, Philippine Carabao Center National Headquarters and Gene Pool, Nueva Ecija, The Philippines

³Philippine Carabao Center National Headquarters and Gene Pool, Maharlika Highway, Science City of Munoz 3120, Nueva Ecija, The Philippines

ABSTRACT

Objective: Major histocompatibility complex (MHC) is a set of molecular proteins on the surface of antigen presenting cells encoded by a large gene family which are important parts of the immune system. This study was conducted to convey information on the genetic characteristics of the MHC II DRB3 gene in riverine and swamp buffaloes.

Materials and Methods: Characterization of MHC II DRB3 gene was carried out using polymerase chain reaction (PCR)-based assay. Thirty-milliliter milk samples were collected from 10 swamp-type and 10 riverine-type buffaloes. RNA from milk samples were extracted using Trizol and then followed by reverse transcription-PCR (RT-PCR).

Results: The phylogenetic analysis with 1,000 bootstrap replications clearly showed complex parsimony in MHC II DRB3 gene between 10 riverine- and 10 swamp-type but also confirmed that the samples are similar to *Bubalus bubalis*. Aligned sequences of the 20 water buffaloes were compared with three other ruminants (*Bos taurus*, *Ovis aries*, and *Capra hircus*) and non-ruminant (*Sus scrofa*) that serve as an outgroup. MHC sequences from GenBank show that there was an average of 705 identical pairs, with 22 transitional pairs and 30 transversal pairs with a ratio of 0.7.

Conclusion: Based on the molecular data, the current study conforms to other works of literature that this gene is highly polymorphic which can be due to its function in the immune responsiveness and disease resistance. Further study on the immunological response of MHC II DRB3 to infection may elucidate its underlying function and role in the protection against specific disease of animals.

ARTICLE HISTORY

Received May 08, 2019

Revised June 26, 2019

Accepted June 27, 2019

Published July 11, 2019

KEYWORDS

MHC II DRB3; phylogenetic analysis; water buffaloes



This is an Open Access article distributed under the terms of the Creative Commons Attribution 4.0 Licence (<http://creativecommons.org/licenses/by/4.0>)

Introduction

Major histocompatibility complex (MHC) II DRB3 gene is known to be extremely polymorphic and functions in the adaptive immune responsiveness and disease resistance by the animal. The high structural polymorphism of class II molecules is accounted for the differences in the immune response among individuals to infectious agents. Polymorphism studies of MHC class II DRB3 show association with resistance to disease such as Bovine leukemia virus infection, Mastitis and with sheep, resistance to parasitic diseases [1].

In cattle, two alleles of DRB3 of exon 2 were associated with the increased risk with the occurrence of

mastitis [2]. Using sequence-based typing, Yoshida et al. [3] reported two allelic differences of MHC DRB3.2 that are related to the susceptibility of Japanese Holstein cow with mastitis and three alleles were associated with the resistance to the infection. A similar study by Ibrahim et al. [4] in Egyptian cows showed three different alleles of the gene. DRB3.2*11 allele occurring 22% as compared to 47% of DRB3.2*16 and 31% of DRB3.2*24 allele. Animals having the DRB3.2*16 were found to be susceptible to *Staphylococcus aureus* infection while resistant to *Streptococcus agalactiae* infection, whereas DRB3.2*16 individuals were found to be more unaffected by *S. aureus* infection. The overall finding showed that allele *11 was

Correspondence Claro N. Mingala ✉ cnmingala@hotmail.com 📍 Philippine Carabao Center National Headquarters and Gene Pool, Maharlika Highway, Science City of Munoz 3120, Nueva Ecija, The Philippines.

How to cite: Medina NP, De Guia ACM, Venturina VM, Mingala CN. Molecular characterization of MHC II DRB3 gene of swamp- and riverine-type water buffaloes. J Adv Vet Anim Res 2019; 6(3):308–14.

significantly correlated with vulnerability to mastitis, while allele*24 was found to have epidemiological activity in maintaining a source of infection in dairy farm. Dietz et al. [5] reported that DRB3.2*16 allele was associated with the acutely elevated somatic cell count (>500,000 cells) in lactating Holstein cows. Baltian et al. [6], on the other hand, reported allele *23 and *27 as having protective or susceptibility effects, respectively.

A genetic marker is a DNA sequence with a known position on a chromosome and are found to be associated with a particular gene or trait. Most studies on genetic markers are focused on the immunologic gene essential for innate and adaptive immunity. MHC II DRB3 is one of the genes studied in cattle [6–8].

There is inadequate information about the genetic characterization of MHC II DRB3 in dairy buffaloes. Although initial reports have been published in other countries, no specific finding has been mentioned about the Philippine carabao or swamp buffaloes (*Bubalus carabanensis*). It is vital to compare the genetic similarities of the riverine (*Bubalus bubalis*) and swamp buffaloes as most breeding practices promote the upgrading of the Philippine swamp buffaloes. Incidence of subclinical mastitis in riverine buffaloes was reportedly high; hence, there is also a need to identify possible genetic markers that can be associated with this condition which has been vastly studied in dairy cattle.

The objective of this study is to contribute additional information on the genetic characteristics of the MHC II DRB3 gene in riverine and swamp buffaloes. Future identification of genetic polymorphism of these genes can be used to select genetic markers associated with resistance or susceptibility to subclinical mastitis. Establishing the contribution of these genes to the occurrence of resistance or susceptibility in subclinical mastitis can assist farmers in their selection of dairy animals through breeding and selection.

Materials and Methods

Sample collection

For riverine buffalo, RNA samples were extracted from milk of 10 animals on their second month of lactation and above. This prevented collecting high somatic cell count that normally occurs on the first month of lactation [9,10]. The sample animals were manually milked to collect 30 ml of milk sample. The milk samples were collected in a 50-ml conical tube and were placed in a cooler. For the swamp buffaloes, RNA was extracted from blood as these 10 animals were non-lactating. Using anticoagulated vials, 5 ml of blood were collected from the jugular vein of the animals. These were processed a day after the collection.

RNA extraction

RNA from milk and blood samples were extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. One thousand microliters of TRIzol® was used to dissolve the isolated cells then added with 200 µl of chloroform. The resulting mixture was initially centrifuged at 14,000 rpm for 15 min at 4°C. The clear part at the top of the mixture was pipetted and then placed to a 1.5 microcentrifuge tube already containing 500-µl isopropanol. It was followed by incubation for 10 min at room temperature and centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was then discarded then followed by washing with 1 ml 75% ethanol. Final centrifugation at 8,000 rpm was done for 5 min at 4°C. Pellets were dried and reconstituted with 30 µl DEPC treated water. Quality and quantity of RNA were checked using spectrophotometer before reverse transcription-polymerase chain reaction (RT-PCR).

RT-PCR of RNA

Power cDNA Synthesis Kit (iNtRON™, South Korea) was used to synthesize the cDNAs from the total RNA samples extracted. One microliter of dT random primers was mixed with 1 µl dNTP, 5 µl of RNase free water, and 3 µl of RNA template. The solution was incubated at 65°C for 5 min. The prepared RNA primer mix was then mixed with 4 µl 5× buffer, 1 µl reverse transcriptase, 0.5 µl RNase inhibitor, and 4.3 µl RNase free water. It was then subjected to another incubation (segment 1; 10 min for 30°C, segment 2; 45 min for 50°C and segment 3; 5 min at 95°C). The cDNA was then kept in a -20°C freezer before it was subjected to β-actin amplification (using primers 5'-CGCACCACGGCATGCTGAT-3' and 5'-TCCAGGGCCACGTAGCAGAG-3') for verification.

Primer design

MHC II DRB3 gene primers were designed using *Bos taurus* MHC DRB3 coding sequence (NM 001012680) downloaded from the National Center for Biotechnology Information (NCBI). Candidate-specific primers for MHC II DRB3 were analyzed using OligoAnalyzer [11] for the presence of hairpins and self-annealing. Thereafter, selected primers were checked using Primer-Blast of the NCBI for its specificity.

Polymerase chain reaction (PCR)

MHCII DRB3 gene having expected amplicon size of 801 bp was amplified using MHC_F (5'-CATTGGTGTGCCTGTATTTC-3') and MHC_R (5'-CTACACTTCAGCCAACAGGG-3') using the following PCR thermal profile: (stage 1) initial denaturation at 94°C for 5 min; (stage 2) denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec, and extension at 72°C for 45 sec (35 cycles); and (stage 3) final elongation at 72°C for 8 min. All PCR assays were performed in 10 µl reaction

volume containing 4.0 µl SdH₂O, 3.0 µl 10X PCR Buffer, 0.5 µl 25 mM MgCl₂, 0.5 µl 25 mM dNTPs, 0.2 µl 10 nM of each primers, 0.1 µl GoTaq™ polymerase (Promega, Madison, USA), and 1.5 µl cDNA template. PCR assay was performed using a thermocycler (SimpliAmp, Amplified Biosystems). After amplification, 3 µl of the PCR product was electrophoresed in 1.5% agarose gel containing 1× TAE (Tris-acetate-EDTA) buffer at 100 V for 30 min and visualized under ultraviolet light using UV transillumination advance imaging system (Flour Chem E by Protein simple). Three microliter 1 kb ladder was placed in the first well of the gel.

Gene sequencing and analysis

Samples for sequencing reactions were sent to first BASE (Malaysia). Sequences assembly and Square matrix based on the number of differences mode were also made using MEGA 7.1 software [8]. The gene sequences generated in this study were compared with the previously reported MHC II DRB3 sequences from *Bubalus bubalis*, *B. taurus*, *Ovis aries*, *Capra hircus*, and *Sus scrofa* (outgroup) from GenBank (Table 1). The estimation of genetic distance and Neighbor-Joining (NJ) analyses from the MHC II DRB3 gene was carried out using the number of differences mode (complete deletion, MEGA 7.1). Nucleotide sequences were submitted at the GenBank under accession numbers LC210724–LC210733. The functional domains of the genes were predicted using the SignalP4.1 (<http://www.cbs.dtu.dk>) server and simple modular architecture analysis tool (SMART) (<http://smart.embl-heidelberg.de/>).

3. Results

Polymerase Chain Reaction

Before using the cDNA template for MHC II DRB3 amplification, samples were subjected to β-actin gene amplification. Fig. 1 shows the agarose gel images positive with

β-actin gene. After checking the quality of the cDNA, samples were then subjected to MHC II DRB3 gene amplification. All 20 samples were successfully amplified.

Sequence analysis of MHC II DRB3

Ten sequences from each type of water buffalo were successfully generated. Amplified MHC II DRB3 gene produced 801-bp (Fig. 2) translating to 201 amino acids. Comparing the aligned sequences of the 20 water buffaloes and four other ruminants' MHC sequences from NCBI show that there was an average of 705 identical pairs, with 22 transitional pairs and 30 transversional pairs with a ratio of 0.7. Comparing riverine from swamp buffalo MHC nucleotide sequences, there was an average of 766 identical pairs, with 17 transitional pairs and 18 transversional pairs with a ratio of 1.0.

Table 1. Nucleotide and amino acid percentage similarity of MHC II DRB3 in ruminant species with reference to swamp and riverine type water buffaloes.

Species	Nucleotide sequences		Amino acid sequence	
	Riverine Swamp	Riverine Swamp	Riverine Swamp	Riverine Swamp
<i>B. bubalis</i> (LOC 102408905, XM006047519.2)	99%	95%	100%	99%
<i>B. Taurus</i> (AY125893.1, D45357.1)	96%	94%	93%	92%
<i>C. hircus</i> (AB008346.1, NM001314217.2)	95%	94%	91%	91%
<i>O. aries</i> (KY860768.1, KY80648.1)	95%	-	-	-
<i>S. scrofa</i> (EU432075.1, AF464034.1)	-	-	-	-

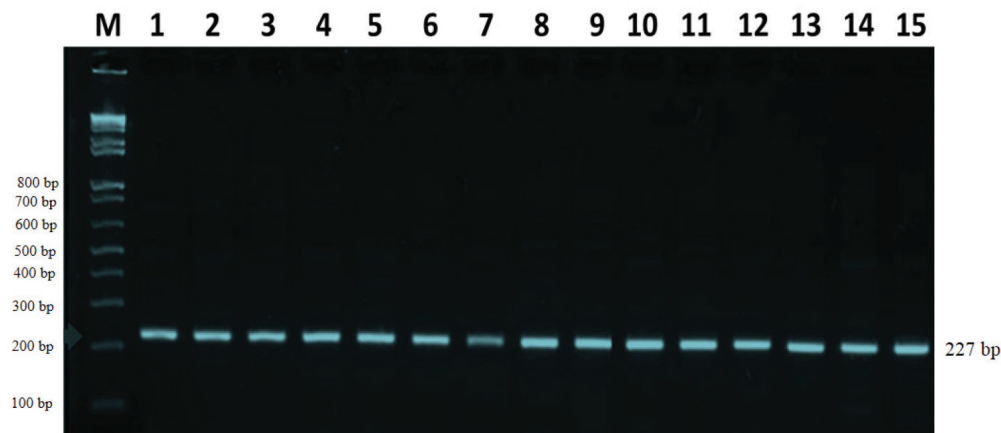


Figure 1. Agarose gel image of β-actin gene amplified from the samples.

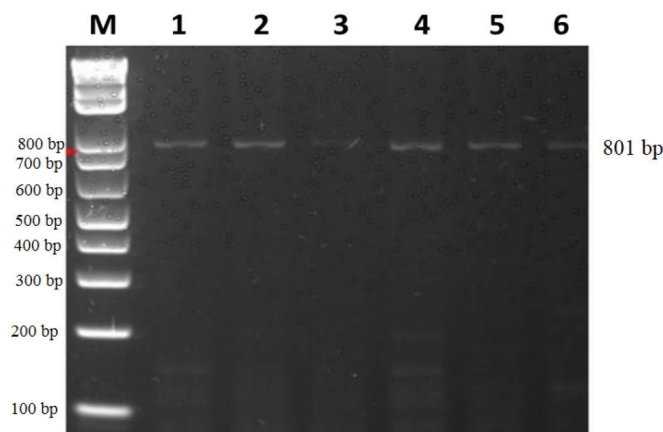


Figure 2. Agarose gel image of MHC II DRB3 gene amplified from the samples.

The nucleotide BLAST of MHC coding sequences of riverine and swamp (Table 1) showed similarity with *B. bubalis* at 99% and 95%, respectively. In general, riverine has higher similarity with other ruminant species than that of the swamp. Furthermore, a lower similarity of nucleotide sequences was seen when riverine samples were compared with *B. taurus*, *O. aries*, and *C. hircus* at 96%, 95%, and 94% similarity, respectively. No data were seen to compare swamp MHC sequence to that of *C. hircus*; however, there was a lower sequence similarity when compared to *B. taurus* (94%) and *O. aries* (94%). Consequently, the lower similarity was seen in the translated amino acid sequence of the water buffaloes as compared to other ruminants' MHC sequences from NCBI. Riverine and swamp buffalo samples had 100% and 99% similarity with *B. bubalis*, respectively. Riverine buffalo MHC sequences were slightly higher in similarity with *B. taurus* at 93%, as compared to the 92% similarity with swamp buffalo samples. Both breeds have 91% similarity of amino acid sequences when compared to *O. aries*. No data were found to compare *C. hircus* with the two water buffaloes.

MHC II DRB3 nucleotide sequences of the samples and other ruminants were subjected to phylogenetic analysis following the NJ algorithm (Fig. 3). The riverine and swamp nucleotide sequences are clustered with the *B. bubalis* samples from GenBank (LOC 102408905, XM006047519.2) with 95% bootstrap value. In Fig. 3, the phylogenetic tree yielded four distinct clades and 13 groups with bootstrap values ranging from 20% to 100%. The roots of the tree showed that riverine and swamp buffaloes may have originated with similar descendants that their similarities in their sequences showed that swamp and riverine can be placed in one clade such that of riverine 3 and swamp 6. The high bootstrap value within the clade represents a conserved sequence of the gene in riverine and swamp buffaloes.

Predicted functional domains of MHC II DRB3

The translated amino acid of the MHC II DRB3 nucleotide sequence is shown in Fig. 4. The signal peptide was found to be at 29–30 location, the transmembrane region started at 7 and ended at 29 positions. MHC II beta started at 42 and ended at 116; IGc1 started at 141 and ended at 212, and another transmembrane region started at 228 and ended at 250 positions.

The schematic representation of the predicted domains of the MHC II DRB3 gene is shown in Fig. 5.

Discussion

Genetic characterization of MHC II DRB3 gene was conducted to compare this gene in riverine and swamp buffaloes. MHC II DRB3 presents processed exogenous antigens to CD4⁺T-lymphocytes. This gene is important in innate and adaptive immune responses. Characterization of this gene had provided evidence of their possible role in resistance, tolerance, and susceptibility to diseases of water buffaloes specifically to subclinical mastitis.

Sequence analysis of MHC II DRB3

Several reports about MHC states that this gene is highly polymorphic due to the fact that the protein receptor functions in the detection of various external pathogens [12–14], and it has been imputed for the differences in the resistance to infectious diseases [15]. The phylogenetic tree clearly depicted complex parsimony in the MHC II DRB3 gene between riverine and swamp but shows a high degree of similarity between buffalo and other ruminants. This means that there are conserved regions in the sequences for all the animal species. Consequently, it appears that MHC II DRB3 gene of buffalo had the highest degree of similarity when compared to *B. taurus* (93% and 92% for riverine and swamp, respectively) which coincides with the earlier claims of Abdel-Rahman and Elsayed [16] that there was a high nucleotide similarity between cattle and buffalo and was linked to their molecular markers.

Predicted functional domains of MHC II DRB3

This study showed the transmembrane region, MHC_II beta and IGc1 domains were present in all ruminant sequences except for *O. aries* which had an incomplete sequence. This was in consonance with the findings of [1], who reported the three regions to be conserved among mammalian species. Class II region of the gene was vastly studied as this contained the polymorphic region. Class II genes contained the DR allele. The DR gene is further subdivided into DRA and DRB. DRB encodes the β -chain molecule. Accordingly, only DRB3 was found functional [1,17] and it was found to be highly polymorphic [18].

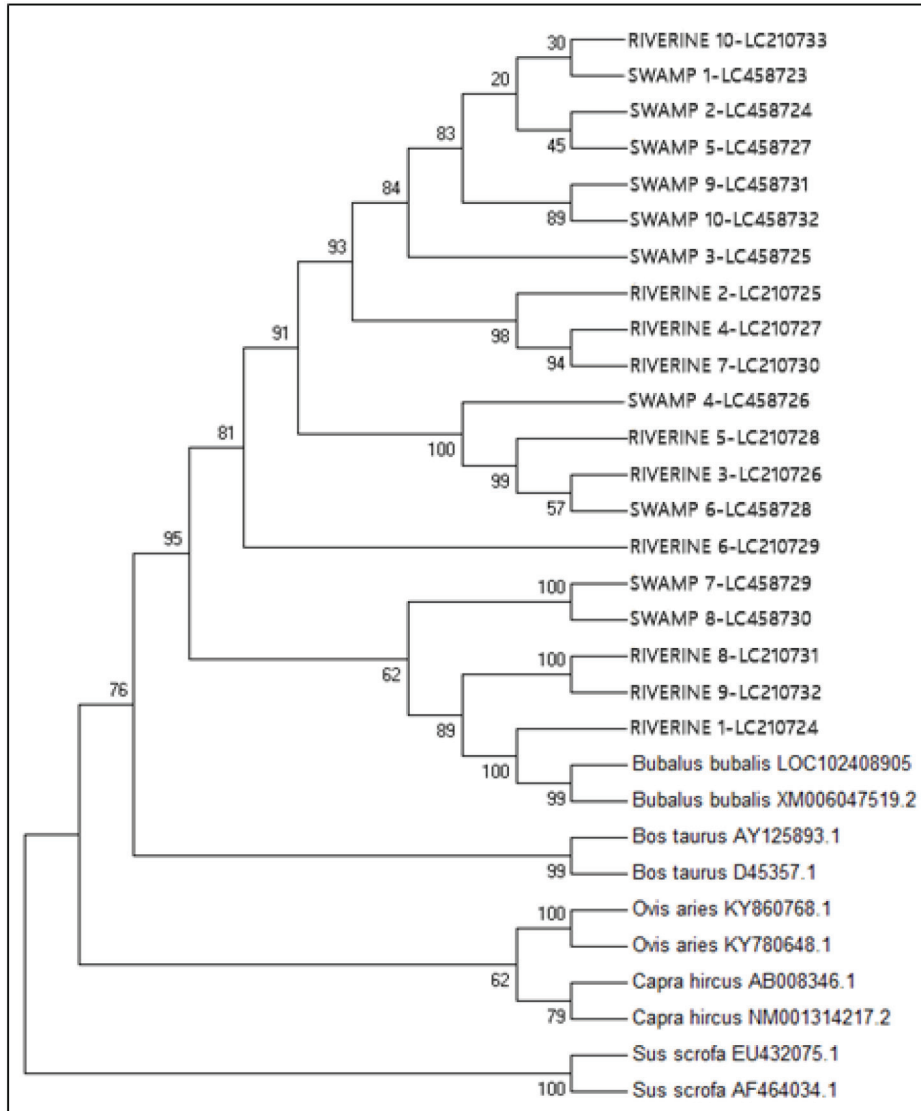


Figure 3. Phylogenetic tree showing the evolutionary relationship of MHC II DRB3 of water buffaloes and other ruminant species.

MHC II beta domain is an important site for peptide binding while IGc1 is responsible for cell-to-cell recognition and cell-surface receptors [19]. Both have been found to be variable in sequence in this study which can possibly be attributed to its function in the immune system. Likewise, De et al. [15] also found high variability in amino acid sequence in Indian cattle and buffalo at the peptide-binding site. He also explained that high heterozygosities in DRB alleles in cattle, sheep, and goat accounted for the great genetic variability among these animals. This inferred their better adaptableness in different geographical regions. Similarly, this study showed riverine and

swamp buffaloes MHC II DRB3 sequences were clustered in various clades inferring to the highly polymorphic property of the target gene in isolated buffalo samples.

MHC is an essential component of the immune system of all animals. Defining the structures and diversity of the system is important in understanding the immune response in an individual. Characterization of DRB3 locus had been thoroughly studied in cattle and the polymorphism identified in this region had been related to the differences of immune responsiveness of different animals to infectious agents.

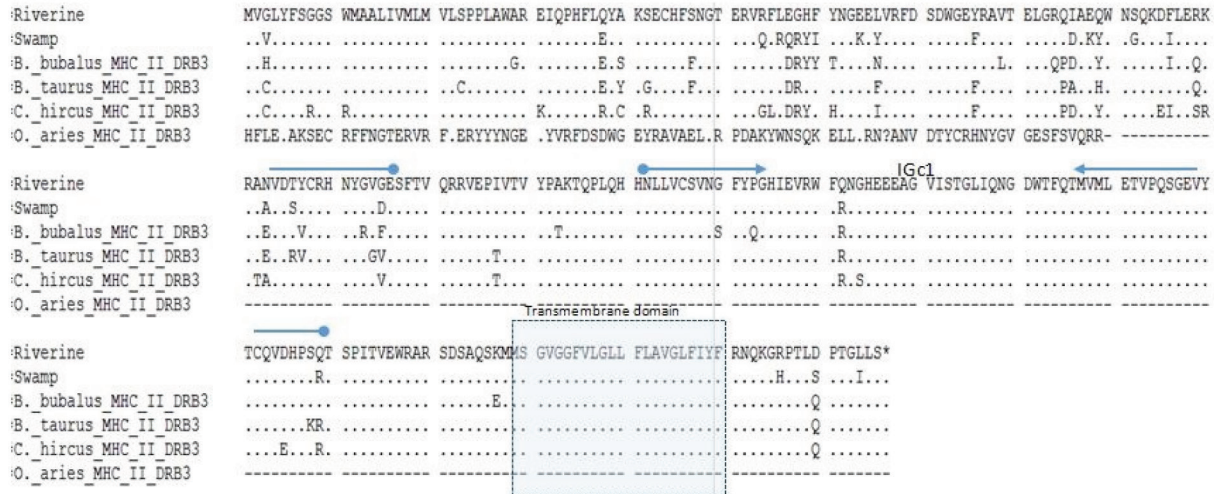


Figure 4. Predicted functional domains in MHC II DRB3 of riverine and swamp buffaloes. Vertical bars represent TM domain, and horizontal dark gray bars represent a simple sequence. MHC_II beta- Class II histocompatibility antigen, beta domain; IGc1- Immunoglobulin C-Type.

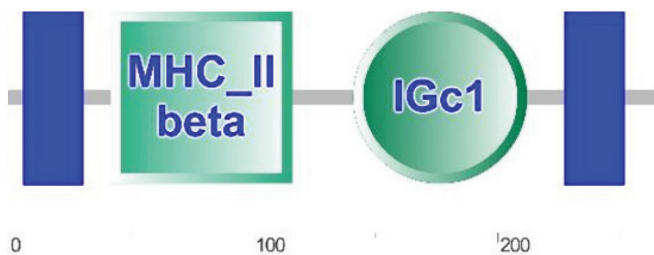


Figure 5. Predicted functional domains in MHC II DRB3 of riverine and swamp buffaloes. Vertical bars represent TM domains, and horizontal dark gray bars represent a simple sequence. MHC_II beta- Class II histocompatibility antigen, beta domain; IGc1- Immunoglobulin C-type.

Conclusion

Characterization of riverine and swamp buffalo MHC II DRB3 provided inferences on the variation of the two types of buffaloes as well as in comparison with other ruminants. And this also explained the variability of immune responsiveness between the two types of water buffaloes. These findings have potential application in the possible selection of genetic marker and study the possible association of such in the disease resistance of a particular animal. The polymorphic nature of the genes merits further investigation for other nucleotide loci and their significance to diseases resistance or susceptibility. Expression analysis can also help in the understanding of how the gene progresses in disease protection. Since subclinical mastitis is considered as a multifactorial disease, identification of microbial cause can also be associated with the genotype of the genes.

Acknowledgments

The authors would like to thank DA-BIOTECH for providing financial assistance to this research project. The authors would also like to thank the Philippine Carabao Center for their support in this study. Moreover, due credits to the Biosafety and Environment Section staff or their technical assistance that we are greatly indebted to.

Conflict of interest

The authors declare that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Author's contribution

Dr. Noraine P. Medina is the main proponent of the project. Mr. Arren Christian M. de Guia is the research assistant, while Drs. Claro N. Mingala and Virginia M. Venturina were the co-researchers involved in checking the veracity of the protocols.

References

- [1] Amills M, Ramiya V, Norimine J, Lewin HA. The major histocompatibility complex of ruminants. *Rev Sci Tech Off Int Epiz* 1998; 7(1):108–20; <https://doi.org/10.20506/rst.17.1.1092>
- [2] Ledwidge SA, Mallard BA, Gibson JP, Jansen GB, Jiang ZH. Multi-primer target PCR for rapid identification of bovine DRB3 alleles. *Anim Genet* 2011; 32:219–21; <https://doi.org/10.1046/j.1365-2052.2001.00761.x>
- [3] Yoshida T, Furuta H, Kondo Y. Association of BoLA-DRB3 alleles with mastitis resistance and susceptibility in Japanese Holstein cows. *Anim Sci J* 2012; 83(5):359–66; <https://doi.org/10.1111/j.1740-0929.2011.00972.x>

- [4] Ibrahim EA, Allam NA, Kotb EZ, El-Rafey GA, Alaa-El-Deenand MM, Fadlallah MG. Sequence-based typing on the relationship between subclinical mastitis and BoLA-DRB3.2 allelic polymorphism in Egyptian Cows. *Global Vet* 2012; 9(1):8–22.
- [5] Dietz AB, Cohen ND, Timms L, Kehrl J. ME. Bovine lymphocytes antigen class II alleles as risk factors for high somatic cell counts in milk of lactating dairy cows. *J Dairy Sci* 1997; 80:406–12; [https://doi.org/10.3168/jds.S0022-0302\(97\)75951-4](https://doi.org/10.3168/jds.S0022-0302(97)75951-4)
- [6] Baltian LR, Ripoli MV, Sanfilippo S, Takeshima SN, Aida Y, Giovambattista G. Association between BoLA-DRB3 and somatic cell count in Holstein cattle from Argentina. *Mol Biol Rep* 2012; 39:7215–20; <https://doi.org/10.1007/s11033-012-1526-y>
- [7] Hameed KGA, Sender G, Kossakowska AK. Association of BoLA allele DRB3.2*16 and DRB3.2*23 with occurrence of mastitis caused by different bacterial species in two herds of dairy cows. *Anim Sci Pap Rep* 2008; 26(1):37–48.
- [8] Kumar S, Sangwan ML, Rupender. Polymorphism in DRB3 exon 2 by PCR-RFLP and its association with mastitis in Nili-Ravi breed. *Indian J Biotechnol* 2008; 7:398–400.
- [9] Salvador RT, Beltran JM, Abes NS, Gutierrez CA, Mingala CN. Prevalence and risk factors of subclinical mastitis as determined by California Mastitis test in water buffaloes (*Bubalus bubalis*) in Nueva Ecija, Philippines. *J Dairy Sci* 2012; 95:1363–6; <https://doi.org/10.3168/jds.2011-4503>
- [10] Khan Z, Khan A. Basic facts of mastitis in dairy animals: a review. *Pak Vet J* 2006; 26(4):204–8.
- [11] Owczarzy R, Tataurov AV, Wu Y, Manthey JA, McQuisten KA, Almabrazi HG, et al. IDT SciTools: a suite for analysis and design of nucleic acid oligomers. *Nucleic Acids Res* 2008; 36(Web Server issue):W163–9; <https://doi.org/10.1093/nar/gkn198>
- [12] Apanius V, Penn D, Slev PR, Ruff LR, Potts WK. The nature of selection on the major histocompatibility complex. *Crit Rev Immunol* 1997; 17:179–224;
- [13] Hedrick PW, Kim TJ. Genetics of complex polymorphisms: parasites and maintenance of MHC variation. In: Singh R, Krimbas C (eds.). *Evolutionary genetics: from molecules to morphology*, Harvard University Press, Cambridge, MA, pp 204–34, 2000.
- [14] Hameed KGA, Sender G, Mayntz M. Major histocompatibility complex polymorphism and mastitis resistance—a review. *Anim Sci Pap Rep* 2006; 24(1):11–25.
- [15] De S, Singh RK, Brahma B. Allelic diversity of major histocompatibility complex class II DRB gene in Indian cattle and buffalo. *Mol Biol Int* 2011; 2011:120176; <https://doi.org/10.4061/2011/120176>
- [16] Abdel-Rahman M, Elsayed H. Genetic similarity among the three Egyptian water buffalo populations using RAPD-PCR and PCR-RFLP techniques. *Res J Agric Biol Sci* 2007; 3:351–55.
- [17] Murphy K, Travers P, Walport M, Janeway's Immunobiology. Garland Science, Taylor and Francis Group, LLC, New York, USA, 2008 ; <https://doi.org/10.1007/978-3-8274-2219-4>
- [18] Behl JD, Verma NK, Tyagi N, Mishra P, Behl R, Joshi BK. The major histocompatibility complex in bovines: a review. *ISRN Vet Sci* 2012; 2012:872710; <https://doi.org/10.5402/2012/872710>
- [19] Sena L, Schneider MP, Brenig B, Honeycutt RL, Womack JE, Skow LC. Polymorphisms in MHC-DRA and -DRB alleles of water buffalo (*Bubalus bubalis*) reveal different features from cattle DR alleles. *Anim Genet* 2003; 34(1):1–10; <https://doi.org/10.1046/j.1365-2052.2003.00920.x>