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Genetics of Disease Resistance:

Molecular characterisation of the Major Histocompatibility Complex Class II Genes in *Bos species* of different origin

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A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Master of Science

at

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by

Oyekunle John Oladosu

Lincoln University

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Abstract of a thesis submitted in partial fulfilment of the requirements for the degree of Master of Science.

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by

Oyekunle John Oladosu

Breeding for genetic resilience in farm animals is becoming more important as a consequence of the increasing awareness of pathogen resistance to antibiotics and the need to build more resilient and sustainable agricultural production systems. In this context, the important role the Major Histocompatibility Complex (MHC) proteins play in immune responses would suggest that improved understanding of the MHC genes is vital to understanding and manipulating genetic resilience to infectious diseases.

The genes of MHC are highly polymorphic, and studies have suggested that the large variation existing within the MHC region may be attributed to response to selective pressure from pathogens. It has therefore been suggested that the alleles of MHC genes carried by an individual animal may be influenced by the type of pathogens in its environment. An understanding of the allelic variation in MHC genes across multiple geographic regions and breeds could therefore assist us to better understand the influence of selective forces and to better characterise distinct populations of cattle.

In this study, four breeds of cattle including White Fulani (n =24), Red Bororo (n =5) and Holstein-White x Fulani-cross (n = 11) from Nigeria, and New Zealand Holstein-Friesian x Jersey-cross (n = 40) were genotyped for variation in exon 2 of BoLA-*DQA1*. In addition: Yak (n= 47); 13 breeds of cattle including White Fulani (n = 30), Red Bororo (n= 20), and White Fulani × Holstein-cross (n = 16) from Nigeria; Holstein-Friesian × Jersey Cross (n = 22),

Hereford (n = 20), Murray Grey (n = 20), Red poll (n = 20), Shorthorn (n =22), Angus (n = 20), Simmental (n = 20), Charolais (n = 20), South Devon (n =20) from New Zealand; and Marchigiana (n = 16) from Italy, were genotyped for variation in exon 2 of BoLA-*DRA*.

A Single Strand Conformation Polymorphism approach was used to genotype the animals and selected cattle were subsequently subjected to direct sequencing of the regions of the genes investigated to reveal nucleotide sequence differences. A total of 10 alleles were identified from all samples investigated for BoLA-*DQA1* variation. Allele BoLA-*DQA**001:02 had the highest frequency of occurrence (17.5%) and BoLA-*DQA**010:03 was the least common with a frequency of 6.9%. A total of 12.60% of all nucleotide positions analysed were revealed to be variable. Two novel BoLA-*DQA1* alleles are reported in this study for the first time, but no new sequences were revealed for BoLA-*DRA*. In total, three BoLA-*DRA* allelic variants were observed in the cattle studied, namely 01011, 01013 and 01014. The New Zealand Holstein Friesian x Jersey (HF X J)-cross cattle and the Yak had only two BoLA-*DRA* alleles (BoLA-*DRA**01011 and BoLA-*DRA**01013), while the other breeds studied had the BoLA-*DRA**01011, BoLA-*DRA**01013 and BoLA-*DRA**01014 alleles.

This study is helpful in shaping our understanding of the bovine MHC genes. It may also be relevant to understanding how different breeds respond to pathogen challenges, thus determining their adaptability to their regions. Identifying genomic variation is however, only a first step in developing breeding programmes that enhance livestock genetic resilience to disease.

Keywords: Polymorphism, genetic marker, disease resilience, major histocompatibility complex (MHC), nucleotides, single strand conformation polymorphism, exon, BoLA-*DQA1*, BoLA-*DRA*, bovine, alleles.

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Table of Contents

DECLARATION ii

Abstract iii

Acknowledgement v

Table of Contents vi

List of Figures viii

List of Tables ix

Chapter 1 1

General Introduction 1

1.1 Background of this study 1

Chapter 2 5

Literature Review 5

2.1 The Major Histocompatibility Complex 5

2.1.1 The three-dimensional structure and functions of the Major Histocompatibility Complex protein 5

2.2 Genetics of disease resistance 8

2.2.1 Breeding for natural disease resistance 10

2.2.2 Mechanisms of disease resistance 11

2.3 The bovine Major Histocompatibility Complex 12

2.3.1 Organisation and structure of the bovine MHC 12

2.3.2 The BoLA class I and III regions 13

2.3.3 The BoLA class II region 13

2.3.4 Genetic diversity of the BoLA genes 15

2.3.5 Association of BoLA class II genes with disease resistance and susceptibility 18

2.4 Genotyping methods for MHC genes 20

Chapter 3 24

Investigation of variation in the bovine-leukocyte antigen (BoLA-DQA1) gene 24

3.1 Introduction 24

3.2 Materials and Methods 26

3.2.1 Sample description and DNA extraction 26

3.2.2 Primer design and Polymerase Chain Reaction (PCR) amplification of BoLA-DQA1 26

3.2.3 Single-stranded conformation polymorphism (SSCP) analysis 27

3.2.4 Nucleotide sequencing 28

3.2.5 Sequence analysis 28
3.3 Results 29
3.3.1 Amplification of the BoLA-*DQA1* Exon 2 and SSCP Analysis 29
3.3.2 Distribution of BoLA-*DQA1* Alleles 30
3.3.3 Polymorphism of BoLA-*DQA1* 30
3.3.4 Relationship between the BoLA-*DQA1* alleles 36
3.4 Discussion 38
3.5 Conclusion 41
Chapter 4 42
Genetic variation in bovine leukocyte antigen DRA gene 42
4.1 Introduction 42
4.2 Materials and Methods 43
4.2.1 Sample description and DNA extraction 43
4.2.2 Primer selection and amplification 43
4.2.3 Genotyping 44
4.2.4 Sequence analysis 44
4.3 Results 45
4.3.1 Allele and genotype frequencies of Bovine Leucocyte Antigen BoLA-*DRA* 45
4.4 Discussion 50
4.5 Conclusion 52
Chapter 5 53
General Summary 53
References 55

List of Figures

Figure 2.1. An X-ray crystal structure of a human MHC class I molecule. 6

Figure 2.2. An X-ray crystal structure of a human MHC class II molecules. 7

Figure 2.3 Genetic map showing the location of the three main BoLA-regions on chromosome (Gutiérrez, Esteban, Lützelschwab, & Juliarena, 2017). 13

Figure 3.1. The polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) gel results of the amplified BoLA-DQA1 exon 2 (238 bp) region 29

Figure 3.2 Nucleotide sequence alignments of the BoLA-DQA1 alleles revealed in this study. 31

Figure 3.3 Amino acid sequence alignments of the BoLA-DQA1 alleles revealed in this study. 32

Figure 3.4 Nucleotide Alignment of BoLA-DQA*002:09 with BoLA-DQA*002:02...........32

Figure 3.5 Nucleotide Alignment of BoLA-DQA*010:04 with BoLA-DQA*010:05...........33

Figure 3.6 Predicted protein structure of BoLA-DQA1 genes showing site of difference in the protein structure. 33

Figure 3.7. Graphs of the non-synonymous vs synonymous substitution. 34

Figure 3.8 Neighbour-Joining Tree showing relationship of BoLA-DQA1 alleles with other species. 36

Figure 4.1. BoLA-DRA exon 2 PCR-SSCP gel patterns..44

Figure 4.2 Sequence alignment of the BoLA-DRA alleles...48

Figure 4.3 Phylogenetic tree showing distances of BoLA-DRA alleles with other species...49

List of Tables

Table 3.1 BoLA-DQA1 primers. 26

Table 3.2. Frequencies of BoLA-DQA1 alleles.. 30

Table 3.3: Sequence comparison of BoLA-DQA1 alleles obtained in this study [a]................. 35

Table 4.1 Allele frequencies of BoLA-DRA exon 2 45

Table 4.2 Frequencies of BoLA-DRA genotypes in a variety of breeds 46

Chapter 1

General Introduction

1.1 Background of this study

The study of genetic diversity in farmed animals is important for understanding the variability that exists within a population and to provide a basis for genetic improvement. Such knowledge can be used to improve productivity within specific environments and to facilitate adaptation to evolving breeding objectives (Notter, 1999).

The objectives of traditional breeding programmes in livestock production have been to improve production traits such as growth traits, reproductive performance and milk yield; and product traits such as milk solid content, meat fat content, etc. However, the animals' health can come as a trade-off to achieving these objectives, as some studies have shown negative correlations between disease susceptibility and productivity (Fleischer et al., 2001; Oltenacu & Broom, 2010). Moreover, breeders have concentrated more on improving certain breeds for particular traits, than others. For example, in dairy production, the Holstein breed is the dominant breed globally and has been intensively selected. This raises questions about long term genetic change and maintenance of genetic diversity.

In developing nations where most of the world's livestock breeds are found, livestock genetics is less thoroughly characterised (Notter, 1999). Yet, these breeds have unique characteristics, such as increased thermo-tolerance at the cellular and physiological level (Hansen, 2004), and tick and trypanosome resistance (Mwai et al., 2015). Consequently, livestock breeding has also evolved to focus on conserving livestock genetic resources and improving animal health (Bishop & Woolliams, 2014; FAO, 2019). Researchers are now focusing on the possibility of breeding animals that are resilient or possible totally resistant to infectious diseases. Although

this is not new in the science community, its potential may have been neglected due to the discovery of alternatives (e.g. use of antibiotics) for managing infectious diseases on farms (Adams & Templeton, 1998).

Infectious diseases, while responsible for economic losses on farms and being an ongoing source of public health concern (McElwain & Thumbi, 2017), have in developed economies typically been combated with the use of antibiotics and vaccination. These approaches have proven useful and they have been adopted as a key means of fighting infectious disease. However, there are limitations to these approaches: one is that effective vaccination programs are not available for all diseases, and besides, many strains of vaccine have short shelf lives and are often very specific. This renders them costly to produce on an ongoing basis (Meeusen et al., 2007). The continuous and poorly managed use of antibiotics has also led to the development of microbial resistance to these substances, with this becoming an important health issue for the 21st century (WHO, 2015). There are also food safety concerns among consumers with respect to the presence of antimicrobial residues in food, and these have influenced public perspectives of the livestock industry. For these reasons, a reduction in the usage of antibiotics has been recommended (Sachi et al., 2019), and at the same time the need to find alternatives to fighting infectious disease has become more pronounced.

Breeding for increased disease resilience is now attracting consideration as a method for controlling infectious diseases (Bishop & Woolliams, 2014). Studies have revealed that animals that are resistant to infections, can pass this ability to their offspring, suggesting that ability to repel disease is genetically controlled or heritable (Cameron et al., 1942; Roberts & Card, 1926).

The role of the Major Histocompatibility Complex (MHC) in the immune response makes it vital in the study of resistance to infectious diseases. The MHC encodes proteins that are

expressed on the surface of specific antigen-presenting cells such as macrophages, dendritic cells and B-cells (Brown et al., 1993; Cresswell et al., 2005). The MHC's function is to present peptide antigens to $CD8^+$ Cytotoxic or $CD4^+$ T-helper cells, and through this trigger an immune response including the activation of antibody producing B-cells and plasma cells, and the targeting of macrophages to infected cells (Cresswell et al., 2005).

The MHC is a multigene family, and in mammals the genes are classified into three classes (Class I, II and III) according to their function and structure. The class II genes encode protein heterodimers that present foreign antigenic peptides on the surface of the $CD4^+$ T-cells. The heterodimers consist of an α and β chain, which are tightly linked by non-covalent associations. The genes of the MHC are highly polymorphic, and it is suggested that the nucleotide sequence variation within the MHC region may be a consequence of the selective pressure applied by pathogen responses in populations (Yao et al., 2015).

The class II molecules are encoded by distinct genes including *DRA*, *DRB3*, *DQA* and *DQB* in ruminants (Amills et al., 1998). Over 500 alleles of the bovine MHC class II genes and over 100 alleles of the class I genes have been deposited in the IPD-MHC database. In sheep, goat and buffalo, the allele tally for MHC genes is well over 100 for each species (https://www.ebi.ac.uk/ipd/mhc/group), making the MHC genes the most polymorphic genes described in mammals.

Among the MHC genes, the MHC-*DRA* gene (a class II gene) has been suggested historically to be monomorphic in cattle. More recently it has been revealed to be somewhat polymorphic, and not only in cattle, but in other species (Kalemkerian et al., 2012; Sena et al., 2003; Zhou et al., 2007). Studies of this kind are further shaping our understanding of the MHC genes.

Unlike the *DRA* genes, the MHC-*DQA* genes are highly polymorphic and can be duplicated in some individual cattle and sheep. There are at least five *DQA* loci that have been identified in cattle; hence there is likely a high number of different DQ molecules that can be expressed in an animal. This may increase the capability of the MHC to present antigens (Amills et al., 1998).

Investigating the diversity of MHC-class II genes across geographical regions and breeds can help us to understand the influence of selective forces and to characterise distinct populations of cattle. It may also be relevant to understanding how different breeds respond to pathogen challenges, which can determine their adaptability to a given region.

In the above context, this thesis aimed to investigate genetic variation in the MHC-*DRA* and *DQA1* genes in yak and cattle from Africa, Asia, Oceania and Europe. The following chapter (Chapter 2) is a review of the literature about the major histocompatibility complex, the genetics of disease resilience and resistance, and the commonly used methods of genotyping the polymorphic MHC genes. Chapter 3 and Chapter 4 provide detail of the experimental procedure and analysis used to investigate the variation in bovine MHC-*DQA1* and *DRA* respectively. Overall, the objective of this study could help in the development of a simple genotyping system for these highly polymorphic genes that control the adaptive immune response in livestock. This could facilitate the development of new breeding approaches to enhancing disease resistance in farm animals.

Chapter 2

Literature Review

2.1 The Major Histocompatibility Complex

The major histocompatibility complex (MHC) genes encode cell surface molecules that are central to the vertebrate's immune defence against pathogens. The genes are part of a large multigene family that is highly polymorphic. This polymorphism may have been the consequences of positive selection for amino acid substitution in the peptide-binding region of the proteins, and the balancing of selection that allows long-term retention of allelic lineages (Klein et al., 2007). The selective factor maintaining those MHC variation lineages is attributed to pathogens, as evidence from various studies reveals associations between individual MHC genotypes and an animal's resistance or susceptibility to infectious diseases (Behl et al., 2012; Brujeni, Ghorbanpour, & Esmailnejad, 2016; Takeshima & Aida, 2006). The genetic variation and evolutionary patterns observed in the MHC, confirm the importance of the study of MHC molecular diversity in adaptive evolution and mammalian behaviour (Edwards & Hedrick, 1998).

2.1.1 The three-dimensional structure and functions of the Major Histocompatibility Complex protein

Generally, MHC proteins have an extracellular component that is composed of two main parts; the immunoglobulin domain-like stalk that anchors the molecule in the surface of the cell, and the receptor which has the shape of a basket, and is called the peptide-binding region or antigen-binding groove (Edwards & Hedrick, 1998). The structure of a human MHC molecule has been determined by X-ray crystallography, and it reveals sites for peptide-binding, and for interaction with the T-cell receptor (Bjorkman et al., 1987; Brown et al., 1993). Based on their structure and function, the MHC molecules are categorised into class I, II and III.

The MHC class I proteins were revealed to be organised into two structural motifs; a membrane-proximal end and a membrane-distal end. At the distal end of the membrane, there is a distinct pairing of two α-helical domains (α1 and α2), which form a groove that can bind peptide antigens (the antigen-binding groove). Another α-helix called α3 is proximal to the cell membrane and is paired with a separate protein, β_2-microglobulin, to form a tertiary structure (Bjorkman et al., 1987; Janeway et al., 2001).

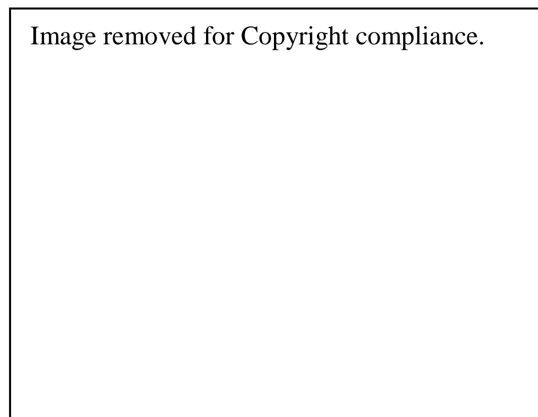

Figure 2.1. An X-ray crystal structure of a human MHC class I molecule.

Shows the proximal and distal membrane end that forms the tertiary structure. The α1 and α2 domains are at the membrane-distal end of the molecule and form the peptide-binding groove. At the membrane-proximal regions are β_2 microglobulin and the α3 domain.

The three-dimensional structure of the MHC class II molecules was found to be similar to that of the class I molecules by X-ray crystallography. The Class II molecules are heterodimers that consist of an α and β transmembrane chains, which are coded by distinct genes. The α1 and α2 chains in class II correspond to the α2 and α3 regions in the class I molecules, but the β chains in class II molecules are not as close structurally to the domain in the class I molecules. The second exons of the class II genes, form the α1 and β1 domains of the protein and produce the peptide-binding pocket, making them the most variable and most often studied region of the class II genes (Brown et al., 1993).

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Figure 2.2. An X-ray crystal structure of a human MHC class II molecules.

The α1 and β1 are the domains that produces the peptide-binding pocket and encoded by exon 2 of the nucleotide sequence.

The MHC molecules have a key role in vertebrate immune responses, but the class I and class II molecules serve different functions. The class I molecules typically present intracellularly-derived peptides to CD8+ cytotoxic T cells (Cresswell, 2005). This can occur when the cell is infected with a virus, and the viral genome is used to produce ‘foreign’ viral peptides. When the cell is infected, the class I molecules adhere to the peptides derived from the pathogens and are presented on the cell surface to cytotoxic T cells. This activates the CD8+ cells against particular MHC/pathogen-peptide combinations (Townsend et al., 1985). The Class 1 molecules also have a variety of other cellular functions, which include a role in cellular transport and regulation of lymphocyte responses to bacterial antigens (Blumberg, 1998).

The molecules of MHC class III do not present antigen, but instead, they code for other proteins such as the complement components (e.g. C2, C4, factor B, etc.), steroid 21-hydroxylase enzymes and tumour necrosis factor (Colten, 1984).

The MHC class II molecules are expressed on the surface of specialised antigen-presenting cells such as macrophages, dendritic cells and B-cells (Brown et al., 1993; Cresswell et al.,

2005). Their function is to present extracellularly derived antigens to CD4+ T-helper cells, which can trigger an immune response such as the activation of antibody production by B-cells and plasma cells, and macrophage activity that can destroy attacked cells (Cresswell et al., 2005). In cattle, Glass and co-workers revealed the ability of MHC class II molecules to present antigen to specific CD4+ cells using both complex and simple antigens and indicated that the antibody response was correlated with the degree of MHC polymorphism (Glass, Oliver, & Spooner, 1991; Glass, Oliver, Collen, et al., 1991).

The interaction that occurs between MHC class II molecules and CD4+ T-helper cells creates a proliferation of antigen-specific effector T-helper and memory T-helper cells. The effector T-helper cells, based on the condition of the inflammatory environment, differentiate and secrete cytokines, chemokines and other immunoregulatory proteins (Jenkins et al., 2001). The cytokines stimulate B-lymphocyte proliferation and differentiation, resulting in the production of antibody-secreting plasma cells and memory B cells, which then initiate the immune response. The cytokines of the CD4+ T-helper cells can also influence the antigen-restricted cell-mediated immune response, which eliminates endogenous pathogens, through the proliferation and activity of CD8+ cytotoxic T cells (Jenkins et al., 2001).

2.2 Genetics of disease resistance

Cattle and cattle products are excellent sources of protein, and they contribute significantly to the world's economy. For instance, about 3% of New Zealand's GDP is accounted for by the dairy industry, and it contributes approximately 25% of the value of New Zealand's merchandisable export trade (Rowarth, 2013).

In South Asia and sub-Sahara African countries, cattle provide a livelihood for small-hold farmers (Enahoro et al., 2019). Accordingly, the economic losses that may come about as a result of disease susceptibility, may have a direct impact on earnings and thus livelihoods.

Sustaining productivity is therefore dependent upon protecting cattle against disease, and this can be at a great cost.

As a consequence of the above, the goal of many breeding programmes in cattle production in developed countries has focused on increasing performance for productivity traits, such as increasing milk yield, while the robustness of cattle is of greater value to the developing nations. Consequently, it has been argued that there has been an increase in disease susceptibility in the cattle from developed countries (Fleischer et al., 2001; Oltenacu & Broom, 2010), and with a potential negative economic impact.

Globally, the use of antibiotics and vaccines to control disease, may well be augmented or supplanted by the alternative approach of utilising the heritability of natural resistance to breed for reduced disease susceptibility (Berry et al., 2011). Research into improving resistance has therefore become of interest to scientists who study animal genetics and health (Bishop et al., 2002; Cameron et al., 1942; Lewin, 1989). Adams & Templeton, (1998) argued that the possibility of breeding for reduced susceptibility to disease was in part ignored due to the discovery of antibiotics and the development of vaccines. These proved very useful and simple to use in combatting both animal and human bacterial infections. Breeding for natural disease resistance in comparison, was much more complicated and could be too slow, thus raising concern that it would impede productivity (Adams & Templeton, 1998). This may in part, explain why genetic studies have focused more on productivity, while the emphasis on animal health has possibly declined.

The ongoing use of antibiotics may have also led to the development of microbial resistance to antibiotics, making this a human health issue for the 21st century (WHO, 2015). Recommendations for reductions in the use of antimicrobials is now driving research for alternative methods of disease control (Park et al., 2012). In hand with a gradual increase in

regulation around reliance on antibiotic usage, pressure has increased on the cattle industry to find alternative methods to improve animal welfare (Bishop & Woolliams, 2014). Breeding for natural resistance to disease should therefore be considered, not as a replacement, but as an additional method to fight against pathogens. Combining breeding for disease resistance with traditional treatment would certainly help reduce animal morbidity and economic losses due to infectious disease.

2.2.1 Breeding for natural disease resistance

Natural disease resistance is defined as the ability of an animal to completely resist the dangers posed by a pathogen, without being formally exposed to or immunised against that pathogen (Hutt, 1958), or the ability of host animal to possess some level of control over the pathogen life cycle, thereby preventing infection (Bishop & Stear, 2003), and leading to the animal being more resistance. Understanding the mechanisms of this resistance will help determine their utility in breeding programmes.

Long-term observations of livestock suggest that disease often doesn't, or rarely appears in all members of a population that are exposed to pathogens (Bishop, 2012). For example, in one study investigating disease susceptibility in pigs, two sows and a possibly related boar were found to be resistant when infected with *Brucella suis*, which typically causes contagious abortion. The authors also found that 77 per cent of the progeny from the boar were resistant to the infection, suggesting that the resistance is heritable (Cameron et al., 1942). Genetic resistance to pullorum infection (*Salmonella pullorum*) in poultry was also reported in an earlier study, which suggests that disease resistance in livestock may be genetically controlled and inherited (Roberts & Card, 1926).

Further research has suggested that disease resilience is often controlled by multiple genes that regulate the different processes of host-pathogen interaction. However, the identification and

characterisation of candidate genes for use as genetic markers, can be used to develop breeding programs for improving natural resilience to infections (Adams & Templeton, 1998).

2.2.2 Mechanisms of disease resistance

There are two broad types of animal resistance to disease. One is manifest through the innate immune system, which prevents the entry and colonisation of pathogens by forming a non-specific barrier. The barrier may occur due to the absence of appropriate receptors for binding and penetration of the cellular membrane, or through the failure of the pathogens to survive or replicate, after entering the host. The host can also develop non-specific defence mechanisms that eliminate the pathogens, including the activity of phagocytes and complement.

The other mechanism enabling resistance or resilience to disease is adaptive or acquired immunity, which encompasses specific lymphocyte-mediated host responses such as the activation of cytotoxic T-cells, T-helper cells, delayed hypersensitivity T cells and B cells; and nonspecific responses including the activation of non-lymphoid cells, natural killer cells and macrophages. The production of cytokines, granulocyte-mediated phagocytosis and inflammation, and humoral-mediated responses, such as antibody production, are also factors that aid the development of immunity to specific pathogens (Adams & Templeton, 1998).

A synergy between the adaptive and innate immune systems is necessary to produce optimal immunity against pathogenic infections, and both systems are genetically controlled (Buschman et al., 1988). The natural non-specific immune response is controlled by non-MHC genes, while the adaptive immune system is typically controlled by MHC genes (Adams & Templeton, 1998).

At very least, the host undergoes three phases of both natural and adaptive immune response to overcome pathogenic infections. The first phase of defence requires the activation of the

innate immune system to provide a physical block at the epithelium to prevent the entry of pathogens. Accordingly, the first step in establishing an infection in a host is that pathogen must attach itself to cell-surface receptors on the epithelium, and often the absence of these receptors will prevent their entry into the host (Wassom, 1989).

In the second phase, natural killer cells, macrophages and neutrophils can lyse the infected cells, and in the last phase there is activation, proliferation and differentiation of the antigen-specific T and B lymphocytes, which is controlled primarily by the class II MHC (Hood et al., 1982). The study of the role of MHC genes in controlling adaptive and innate immune response in vertebrates, can therefore provide insight into improving the health of cattle.

2.3 The bovine Major Histocompatibility Complex

The MHC of both Bos taurus and Bos indicus is termed the Bovine Lymphocyte Antigens (BoLA) (Lewin, Russell, & Glass, 1999). Due to the MHC's important role in immune response, the BoLA genes are deemed to be useful targets in the study of the genetics of disease susceptibility in cattle (Amills, Ramiya, Nonmine, & Lewin, 1998).

2.3.1 Organisation and structure of the bovine MHC

The structure of MHC genes is relatively conserved across mammalian species (Amills et al., 1998). In bovine species, the BoLA genes are divided into three main regions with each region having different sizes, genes and functional roles. The genes have been mapped to bovine autosome 23, whereas in humans and mice the MHC genes are located on chromosomes 6 and 17 respectively (Cunliffe & Trowsdale, 1987; Fries et al., 1993; Péléraux et al., 1996).

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Figure 2.3 Genetic map showing the location of the three main BoLA-regions on chromosome (Gutiérrez, Esteban, Lützelschwab, & Juliarena, 2017).

2.3.2 The BoLA class I and III regions

The BoLA class I region contains approximately 1,550 kilobases (kb) of DNA (Amills et al., 1998). This includes the group of genes that produce transmembrane polypeptide chains with three extracellular domains: α1, α2, and α3, which are characteristic of class I MHC molecules. (Behl et al., 2012). At least ten class I genes have been identified in cattle (Bensaid et al., 1991), and the genes are polymorphic with the alleles identified listed in the MHC-IPD database (see https://www.ebi.ac.uk/ipd/mhc/group/BoLA) for BoLA sequences.

The class III genes comprise *CYP21*, *BF*, *HSP70*, and *C4* and they lie telomeric to the class IIa region that contains the *DR* and *DQ* loci (Takeshima & Aida, 2006).

2.3.3 The BoLA class II region

The BoLA class II region lies near the centromere of bovine chromosome 23 and consists of two sub-regions (class IIa and IIb), separated by at least 15 cM (centiMorgans) (Andersson et al., 1988). The class IIa region represents the main class II restriction elements (Takeshima & Aida, 2006) including the DR and DQ loci (figure 2.3).

The BoLA-DR and BoLA-DQ genes are functionally expressed genes and lie in close proximity to both the class III and class I genes (Shin-nosuke Takeshima & Aida, 2006). The class IIb regions contain the *DMA*, *DMB*, *LMP2*, *LMP7* and *TAP* genes (Russell et al., 1997) and they are reported to be involved in antigen processing and transport (Amills et al., 1998). Other class IIb genes are; *DOA*, *DOB*, *DYA* and *DYB*, but the functions of these genes are unknown, and they are not represented in the IPD-MHC database. The BoLA-DR and BoLA-DQ genes have been extensively studied, primarily because they contain the main class II restriction elements (Behl et al., 2012).

The BoLA-DR genes

Cattle have a single BoLA-DRA gene which encodes the α chains of the DR molecules and three BoLA-DRB genes which encode the β chains. BoLA-DRA is conserved across ruminants and was originally thought to be monomorphic, but investigations of DRA polymorphism have revealed nucleotide sequence variation in different ruminants (Ballingall et al., 2010; Zhou et al., 2007).

Unlike BoLA-DRA, the genes encoding the β chains of the DR molecules (BoLA-DRB) are highly polymorphic (Shin-nosuke Takeshima et al., 2018), albeit BoLA-DRB3 appears to be the only functional locus of the three BoLA-DRB loci. BoLA-DRB1 is regarded as a pseudo-gene which has stop codons in the β1-encoding region and transmembrane region, whereas, DRB2 is only expressed at low levels (Burke et al., 1991).

The BoLA-DQ genes

The BoLA DQA and DQB genes comprise the DQ locus and are said to be duplicated, such that the genes of the cluster are tightly linked in most haplotypes ((Andersson et al., 1988). Consequently, when individual cattle carry both copies of DQA and DQB, both molecules may

be expressed (Takeshima & Aida, 2006). At least eight different DQA and DQB genes have been reported. Three of the DQA genes (DQA1-3) are highly polymorphic. Of the five DQBs, DQB1 is the most frequent, while others are found in the duplicated haplotypes (Takeshima & Aida, 2006).

2.3.4 Genetic diversity of the BoLA genes

The polymorphism in BoLA genes is concentrated in the second exon, which encodes the variable portion of the peptide-binding site. This polymorphism may evoke an effective immune response against pathogen-derived peptides that differ in their structural characteristics (Amills et al., 1998). The natural diversity of MHC can, therefore, help us understand the role of MHC in immune responsiveness. Studies of MHC polymorphism have also been useful in understanding the evolution of many species (Edwards & Hedrick, 1998). Given this research, there is an opportunity to learn more about the evolution of bovine species by sequencing populations of cattle from different regions.

In humans, the genetic polymorphism of the human antigen lymphocyte gene has been used to understand differences between ethnic groups in a population (Mizuki et al., 1998). Similarly, several studies have also shown that BoLA class II alleles differ among different cattle breeds. For example, Takeshima et al., (2014) revealed that there were 46 different BoLA-*DRB3* alleles in the ten cattle breeds they studied. Conversely, BoLA-*DRA* has been known historically to be monomorphic (Ellis & Ballingall, 1999), with this perhaps making it escape extensive review. However, more recent research has revealed that variation exists in BoLA-*DRA*, with four unique SSCP patterns being described in 384 New Zealand cattle, with four different nucleotide sequences and three synonymous SNPs detected in the second exon of *DRA* (Zhou et al., 2007).

In other ruminant species, allelic variation has also been described in the MHC-*DRA* genes. Two different alleles have been described in water buffaloes *Bubalus bubalis*; (Sena et al., 2003). Four *DRA* alleles have been described in sheep, and one of the alleles contained 20 substitutions making it far more variable than any other vertebrate species described so far (Ballingall et al., 2010). The authors predicted that the substitution found in the peptide-binding site would have a significant impact on the protein structure and the range of the peptide presented to the CD4+ T cells. Comparison of wild and captive deer also revealed variation in *DRA* within the wild population, which may have offered selective advantage and be responsible for pathogen resistance (Yao et al., 2015). These studies have changed the understanding of the MHC-*DRA* gene and reinforce the possibility of uncovering new variants in a larger population of differing breeds of cattle.

Studying the diversity of BoLA genes is therefore important to our understanding of differences in cattle populations. While many diversity studies have investigated BoLA-*DRB3* from different cattle breeds (De et al., 2011; Nikbakht Brujeni et al., 2016; Oprządek et al., 2012), there is the possibility of identifying new variant of other class II genes within less studied populations of cattle, including African cattle breeds.

One study that did compare the allelic frequencies of BoLA-*DRB3* between some African breeds and some European breeds revealed considerable differences, with this suggesting that BoLA alleles frequencies may have been influenced by selection, genetic drift and bottlenecks in populations (Mikko & Anderson, 1995). Peters et al. (2018) also suggested that there were generally more within-breed differences in BoLA-*DRB3*, than between breeds, in a study that included some African and Asian cattle. They compared African cattle with breeds from America and Asia at BoLA-*DRB3* exon 2 and described 15 haplotypes in the 174 cattle studied. The Sokoto Gudali cattle from West Africa had the greatest number of haplotypes (n =10) with Brangus cows, while the Sahiwal cattle had the smallest number (n = 4). The polymorphism

that existed in the African cattle may provide selective advantage if the haplotype confers resistance towards specific pathogens, and this would provide a basis for the design of a breeding program aimed at selecting cattle with disease resistance. Generally, there are more differences found across the breeds than between the breeds, and three cattle breeds that were studied from the same geographical region of Southern Indian showed genetic diversity (Das et al., 2012). This may imply that the population structure has been preserved, therefore allowing access to unique animal genetic resources.

A study by Peters et al., (2018) also assessed the BoLA-DRB3 genes and reported differences between beef breeds and dairy breeds, with less diversity in the beef breeds than the dairy breeds. This may have been due to their exposure to different pathogens. Dairy cows are more commonly exposed to mastitis, which is caused by multiple pathogens (Watts, 1988), and this may have elicited disparity in the antigen-binding locus when responding to the challenge. The ratio of synonymous and non-synonymous substitution found in this study was greater than one, which also suggests that variation at the antigen-binding sites might be under positive selection and increases the likelihood of cattle responding to diseases.

Characterising cattle breeds for their MHC genes will provide valuable information about diversity and genetic resistance to diseases owing to the key role the MHC has in the immune system (Gowane et al., 2012.; Thompson-Crispi & Mallard, 2012). The *Bos indicus* breeds of cattle are known for several unique features, such as tick and trypanosome resistance (Mwai et al., 2015). They are also distinct from *Bos taurus* breeds, with a higher degree of thermo-tolerance at the cellular and physiological level (Hansen, 2004). In the face of a changing climate, the unique features of these breeds may provide an improved understanding of adaptation to diseases that thrive in higher temperatures and warmer climates. This also reinforces the need to characterise more cattle breeds at the genomic level.

Moreover, as pathogens that cause diseases differ with location, different breeds of cattle at a different location may have evolved over time with resistant alleles that are 'specific' to these locations. Many studies have shown that the ability of a population to respond effectively to infections depends largely on the MHC locus (Carignano et al., 2017; Sun et al., 2013; Zhang et al., 2015). The gene BoLA-*DRA* was one of the seven functional candidate genes that were identified to be highly differentially expressed between healthy cows and cows with mastitis and was found to be significantly enriched in metabolic pathways relevant to inflammatory process (Asselstine et al., 2019). In another study, BoLA-*DRA* was found to display lower expression levels in bovine tuberculosis-infected animals, relative to the uninfected group (Meade 2007). Similarly, BoLA-*DQA1* alleles have been found to be associated with clinical mastitis caused by *Escherichia* and *Streptococci spp.* (Takeshima et al., 2008). Therefore, given its importance, genotyping multiple breeds will be useful for understanding the genetic variation that exists in the BoLA-*DRA* and BoLA-*DQA1* region.

2.3.5 Association of BoLA class II genes with disease resistance and susceptibility

The BoLA genes have been studied for their association with infectious diseases in cattle due to their high diversity and role in controlling immune response. Over the years, fast and reliable typing systems have been developed to make it easy to distinguish different alleles of the highly polymorphic MHC genes. As a result, it is becoming easier to design a breeding scheme for disease resistance in farm animals (Amills et al., 1998).

The BoLA-DRB3 gene has been revealed to be associated with resistance and susceptibility to bovine leukaemia and persistent lymphocytosis (PL) caused by bovine leukaemia virus (BLV) (Nikbakht Brujeni et al., 2016). Bovine leukaemia is the most common neoplastic diseases of cattle. The virus is widely distributed across the world and it causes significant losses as a consequence of the elimination of infected animals (Da et al., 1993). In one study, 190 Iranian cattle were genotyped and sequenced to examine allelic diversity and association of *DRB3*

alleles with BLV infections using multivariate regression analysis. The result suggested that persistent lymphocytosis was associated with three different *DRB* alleles, namely; BoLA-*DRB3.2**0101, *1101 and *4201. Similarly, alleles *1802*, *3202* and *0901 were found to be associated with susceptibility to bovine leukaemia, while *0101 and *1101 were associated with resistance (Nikbakht Brujeni et al., 2016). It was concluded that selection based on MHC genotypes might be helpful in controlling BLV infections, but owing to the influence of MHC on a wide range of disease, it is recommended to identify the association of MHC with other clinically important diseases.

Other studies have revealed that the association with resistance to PL in some individual cattle may be given by the presence of amino acids Glu-Arg at positions 70-71 of the DRB chain (Lewin, 1989). The presence of Glu, Arg and Val at positions 74, 77 and 78 of Bola-*DRB* is also likely to be related to resistance to tumour development (Aida, 2001). In sheep, it was reported that ovine leukocyte antigen (OLA)-*DRB1* alleles encoding the Arg-Lys motif at position 70-71 of the OLA-DRB genes may build resistance to tumour development in BLV infected sheep (Konnai et al., 2003). They experimentally infected the sheep with BLV virus and found that sheep with alleles encoding the Arg-Lys motif released neutralising antibodies against BLV and interferon-γ, which eliminated BLV completely and the sheep did not develop lymphoma.

So-called 'resistant alleles' have been suggested to promote the early development of a subset of T-helper cells that secrete interferon-γ and interleukin-2, which may enhance resistance to infection (Shin-nosuke Takeshima & Aida, 2006). Thus, selection for specific BoLA alleles might be useful to enhance breeding strategies for resistance to infectious diseases.

Mastitis, an economically important disease of dairy cattle is another disease that has been shown to be associated with the polymorphism of BoLA genes. The alleles that were found to

be associated with resistant to BLV infections, were also associated with mastitis resistance (Dietz et al., 1997; Dietz, Cohen, Timms, & Kehrli, 1997). The study of BoLA variation in association with disease occurrence or resistance suggests that BoLA genes can be important genetic markers for disease resistance, hence it is important to reveal more alleles that may exist within the BoLA genes in order to facilitate the development of a breeding scheme that might enhance disease resistance.

2.4 Genotyping methods for MHC genes

Understanding the diversity and significance of the MHC genes relies on an effective method of genotyping to reveal all the alleles present in a population. This process is somewhat difficult, chiefly because the target loci are commonly present in multiple copies, and allele sequences from a single locus can be very divergent (Babik, 2010). Pseudo-genes may also be present with the expressed loci, which makes it difficult to identify functional variants. The failure to identify and eliminate 'phoney' variation that is introduced by the genotyping techniques themselves, poses additional risk in identifying valid nucleotide sequence variation.

Over the years, methods of detecting polymorphism include protein-based techniques such as mixed lymphocyte reactions (Davies & Antczak, 1991a), isoelectric focusing (Joosten et al., 1989), serology (Davies & Antczak, 1991b) and immunoblotting (Sharif, 1999). These methods are often laborious and time-consuming, and they often they lack specificity.

Nowadays, DNA-based methods are used. The first DNA-based method developed for MHC typing was Restriction Fragment Length Polymorphism (RFLP) analyses, which were developed in the 1980s (Babik, 2010). The approach involved the hybridisation of radioactively-labelled probes obtained from MHC clones, to genomic DNA cleaved with a restriction endonuclease and size-fractionated by gel electrophoresis. The challenges of designing an optimal primer are not present in this method because the probe can be hundreds

of base-pairs long. The problem with this typing method is however, that it is not suitable for identifying variation at high resolution, because it can only examine a fraction of the nucleotide positions (those that are variable and in the restriction endonuclease binding-sites). It is also a time-consuming approach, has a low throughput rate and is not suitable for large-scale genotyping (Babik, 2010). Accordingly, RFLP approaches have been surpassed by PCR-based methods.

The PCR-based genotyping methods involve two stages: the design of primers that can amplify the entire region of interest, and the identification of true variants amplified with these primers, and elimination of artefacts. Although a PCR-RFLP method has also been developed and widely used for genotyping MHC genes, this method is said to be limited in precision and once again only detects sites distinguished by its panel of restriction endonucleases (Hayashi, 1992; Sharif, 1999).

The Single-Strand Conformation Polymorphism (SSCP) method is an inexpensive, universally applicable PCR-based method that can detect previously unknown variation at single-base resolution, although new variants have to be specifically characterised via nucleotide sequencing. The analyses are based on the observation that the electrophoretic mobility of single-stranded DNA fragments is conformation-dependent and determined by the DNA sequence (Orita et al., 1989). The method involves denaturing and then the rapid cooling of PCR products, with this allowing the single-stranded molecules to assume a sequence-specific secondary structure or conformation. This is followed by electrophoresis under non-denaturing conditions (i.e. at low temperature in polyacrylamide or synthetic cast gels) (Biedrzycka & Radwan, 2008). The bands from the gels can then be visualised using autoradiography, SYBR Gold staining, or silver staining (Oto et al., 1993). A major advantage of the SSCP approach is that the bands corresponding to individual alleles can be excised, re-amplified, and sequenced

to obtain the nucleotide sequences of the individual alleles (Sommer et al., 2002). Furthermore, SSCP can detect > 90% of all single base substitutions in a 200 bp sequence and > 80% in a 400 bp sequence. It has been specifically adapted for use in typing polymorphic MHC genes (Russell, 1994). The gels produced by SSCP analyses can be difficult to interpret if alleles from multiple loci co-amplify in the sample, not least because the bands may overlap from having similarity in mobility. This can be avoided by designing optimised, target-specific primers and by optimising gel running conditions.

Another method of typing MHC is the use of the Denaturing Gel Electrophoresis & Temperature Gradient Gel Electrophoresis (DGGE) method. One of its advantages is that mutation can be detected in a broad range of fragment sizes, up to 1000 bp (Knapp, 2005). The DGGE approach involves electrophoresing double-stranded molecules through a gel with a denaturing gradient made of formamide and urea. Since double-stranded molecules are believed to exhibit sequence-dependent denaturing characteristics, molecules with differing sequences form discrete bands at various positions in the gel (Fischer & Lerman, 1983). Complete denaturation can be avoided by clamping a GC at the 5' end of the amplification primers. This will help to avoid poor resolution that may be caused by equal mobility with fully denatured DNA fragments of the same length (Myers et al., 1985). The similarity with SSCP is that it produces a single band per allele that can be excised, re-amplified and sequenced (Middleton et al., 2004). However, DGGE can erroneously score a new allele, which may have resulted from the formation of hetero-duplexes that have distinct denaturation profiles, or which have identical mobility to the true alleles (Knapp, 2005).

In recent times, several genotyping methods based on direct sequencing have emerged. Use of these methods for MHC typing has been extensively reviewed (Babik, 2010). One method is sequence-based typing (SBT) technique, where PCR products obtained with specific primers

are directly sequenced to reveal heterozygous chromatogram. These are then interpreted through a comparison with existing allelic combinations from the database (Helmberg et al., 2004). In another sequencing approach, PCR products can be cloned in bacteria, then single molecules from individual colonies are sequenced. Cloning and sequencing procedures can be costly and labour intensive. Additionally, artefacts may be introduced by bacterial mismatch repair systems (Babik et al., 2009). Although using Next Generation Sequencing (NGS) methods for sequencing may be relatively inexpensive for large-scale studies, there is still a high cost of tagging primers and the necessity of developing a refined method for identifying artefacts (Wegner, 2009).

Chapter 3

Investigation of variation in the bovine-leukocyte antigen (BoLA-DQA1) gene

3.1 Introduction

It has been established that the major histocompatibility complex genes are the most diverse in vertebrate genomes (Edwards & Hedrick, 1998). The MHC is crucial in determining an organism's ability to respond to pathogens from the environment, and thus they have been associated with the long-term population fitness. The unprecedented genetic diversity of the MHC genes has been explained by two main mechanisms: the pathogen-driven selection theory and reproductive processes (i.e., MHC-based mating preferences). Preferences for sexual partners bearing different MHCs have been observed in rats, both under laboratory and semi-natural conditions, and they are said to account for most of the genetic diversity observed in *Mus* populations (Potts & Wakeland, 1993).

Pathogen-driven selection favours genetic diversity either through MHC heterozygote advantage (over-dominance) or the preservation of rare MHC genotypes (negative frequency-dependence), or both. In heterozygote forms, the MHC can express twice as many products as homozygotes, giving room for a wide range of antigenic peptides to be presented to the immune system, and with this increasing the relative fitness of heterozygotes compared to homozygotes. Selection also favours the maintenance or preservation of rare MHC genotypes since pathogens are likely to develop mechanisms to avoid immune response initiated by common MHC genotypes (Paterson et al., 1998). In contrast, limited diversity within the MHC can pose a risk of isolation and vulnerability to infectious diseases within populations (Hughes & Yeager, 1998), hence there is motivation to investigate MHC diversity within different animals.

The MHC loci have been well studied in cattle for their potential as a candidate genes for selecting for various bovine diseases and immunological traits (Takeshima & Aida, 2006). Of the several products of the BoLA genes with different functions, the DR and DQ molecules represent the main restriction elements for $CD4^+$ T-helper cells (Amills et al., 1998). Of these, BoLA-*DQ* appears to be duplicated in cattle and both duplicates can be expressed. Duplication and polymorphism of the *DQA* genes can increase variation at the antigen binding site, which contributes to changes in the immune response to antigens (Glass, Oliver, & Russell, 2000). The BoLA-*DQ* region contains five *DQA* loci. The genes *DQA1* and *DQA2* are highly polymorphic in exon 2, with 79 alleles of *DQA1* reported currently (https://www.ebi.ac.uk/ipd/mhc/allele/list/?group=BoLA&organism=BoLA&gene=*DQA*).

The alleles of BoLA-*DQA* have been identified using different typing methods such as restricted fragment length polymorphism (RFLP) analysis (Davies et al., 1994), PCR-RFLP (Ballingall et al., 1997), and sequencing (Takeshima et al., 2008). The RFLP method is however limited in precision and may not identify alleles at high resolution, whereas sequence-based techniques can be too costly and labour intensive. In contrast, SSCP is an inexpensive method that can detect > 90% of all single based substitution in a 200 bp sequence and > 80% in a 400 bp sequence (Russell, 1994). Analysis by SSCP is a PCR-based method and therefore it requires amplification using specific primers.

Designing a locus specific primer for *DQA* genes can be difficult because the 3'-end of exon 2 of the *DQA* genes is conserved across the *DQA1* to *DQA*5 loci (Takeshima et al., 2008). This may result in co-amplification of more than two loci. In this study, a locus-specific primer was designed to amplify a region specific to the *DQA1* locus in cattle. Further, this study also investigated the extent of polymorphism and identified alleles of the BoLA-*DQA1* exon 2 in cattle.

3.2 Materials and Methods

3.2.1 Sample description and DNA extraction

Dried blood samples were punched from FTA cards (Whatman Bioscience, Middlesex, UK) representing a total of 80 cattle from four different breeds from Nigeria and New Zealand, namely White Fulani (n =24), Red Bororo (n =5), Holstein-White x Fulani-cross (n = 11) and New Zealand Holstein-Friesian x Jersey-cross (n = 40). Genomic DNA was extracted and purified from the dried blood following the two-step procedure described by Zhou, Hickford, & Fang, (2006). Briefly, this involved denaturing the cell and protein with 20 mM NaOH at 60 ^{o}C for 30 minutes, followed by washing with 1× TE buffer for 5 minutes.

3.2.2 Primer design and Polymerase Chain Reaction (PCR) amplification of BoLA-DQA1

Polymerase Chain Reaction (PCR) primers were designed to target conserved gene regions of BoLA-DQA1, by aligning exon 2 sequences of the gene that were available from NCBI GenBank. These sequences were aligned against a reference sequence (the BoLA-DQA1*0101 allele; GenBank Accession number AB257103.1) using MEGA version X software (Kumar et al., 2018) and sequences were chosen that were highly conserved. The designed primer pair DQA1Forward and DQA1Reverse are expected to amplify 273-bp containing exon 2 and part of the flanking introns of BoLA-DQA1. These primer sequences were compared to the GenBank database using BLASTN searches to ensure that they are specific to DQA1 and not DQA2 – DQA5. The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

Table 3.1 BoLA-DQA1 primers.

Primer Name	Primer Sequence (5' - 3')	Location[a]	Fragment Size
DQA1-Forward	TTTCCCTTTCTTGCTCCTCAC	44 to 64	273 bp
DQA1-Reverse	AGCAGCAGTAGAGTTGGAC	298 to 316	

[a] Nucleotide position refers to BoLA-*DQA1* *0101 sequence accession number AB257103.1

Bio-Rad S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA) were used for the PCR amplification. Each PCR amplification was performed in a 15 µl reaction volume containing a 1.2-mm disc punched from the sampled FTA cards, 0.25 µM of the *DQA1*-Forward and *DQA1*-Reverse primers, 150 µM of each deoxyribonucleoside triphosphate (dNTP), 3.0 mM Mg^{2+} (Bioline, London, UK), 0.5U of *Taq* DNA polymerase (Qiagen, Hilden, Germany), and 1× reaction buffer supplied with the enzyme. The following thermal cycler temperature conditions were used for the amplification: Initial denaturation at 94 °C for 2 minutes, followed by 34 cycles of denaturation at 94 °C for 30 seconds, annealing at 61 °C for 30 seconds, elongation at 72 °C for 30 seconds and a final extension at 72 °C for 5 minutes.

3.2.3 Single-stranded conformation polymorphism (SSCP) analysis

The SSCP technique was used for genotyping the animals**.** The amplicons were run under different voltages, temperatures and gel compositions until well-separated and clear banding patterns were obtained. A 7-µL aliquot of a loading dye containing 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol, and 98% formamide was added to a 0.7-uL aliquot of the amplicon. The mixture was heated on a hotplate at 95 °C for 5 minutes for denaturation and rapidly cooled on wet ice. The mixture was loaded in a 16 x 18 cm, 14% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels (containing 1% glycerol) for electrophoresis. Protean II xi cells (Bio-Rad) were used for the electrophoresis at a constant 390 V at 3 °C for 21 hours in

0.5× TBE. The SSCP banding patterns were revealed using a silver staining technique (Byun et al., 2009). The staining involved soaking the gels in silver nitrate for 5 minutes, washing with water and subsequent development in a solution containing a mixture of sodium hydroxide and 40% formaldehyde.

3.2.4 Nucleotide sequencing

DNA samples of amplicons that exhibits homozygote SSCP patterns represented by two bands were extracted, amplified and purified for sequencing. When amplicons exhibited heterozygosity (i.e. they had four SSCP banding patterns), the unique bands were excised from the gels, incubated, amplified and purified. Incubation was performed in a shaking rotary incubator for 1 hour at 55 ^{0}C to isolate the DNA. Purification of the samples for DNA sequencing was undertaken using a DNA MinElute purification kit (Qiagen, Hilden, Germany). The purified amplicons were loaded in the SSCP gel alongside amplicons from corresponding genomic DNA for comparison of the banding patterns and for ensuring accuracy. The purified amplicons were then sequenced in both forward and reverse direction at the Lincoln University DNA sequencing facility.

3.2.5 Sequence analysis

MEGA version X (Kumar et al., 2018) was used to carry out sequence alignments, translations and comparison. Adjustments were made to the alignment after visual inspection. Sequence polymorphism analysis was done using DNAsp Version 5 (Librado & Rozas, 2009). Genetic distances for nonsynonymous (dN) and synonymous (dS) substitution were calculated according to the (Nei & Gojobori, 1986) method, using the web-based version of SNAP software (http://www.hiv.lanl.gov) (Korber, 2000). The BLAST algorithm was used to search for the homologous sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/).

A neighbour-joining tree was constructed based on the Jukes-Cantor genetic distance model using Geneious Version 2020.2.4 program. (https://www.geneious.com). The following sequences, together with BoLA-*DQA1* sequences identified in this study, were used in constructing the phylogenetic tree. GenBank accession numbers: MN954679.1 (HLA-*DQA1*), LR797948 (BoLA-*DQA2*), JN798528.1 (BoLA-*DQA*3), JQ864314.1 (BoGr-*DQA1*), NM_001012675.2 (BoLA-*DQA*5), AB257112.1 (BoLA-*DQA1*), AB362377.1 (BoLA-*DQA1*), MG839037.1 (BoLA-*DQA1*), MG571516.1 (BoLA-*DQA1*), AY265308.1 (OLA-*DQA1*).

3.3 Results

3.3.1 Amplification of the BoLA-*DQA1* Exon 2 and SSCP Analysis

A 238 bp fragment of exon 2 was obtained from the cattle genomic DNA using the primers *DQA1* forward and *DQA1* reverse. The primers successfully amplified the exon 2 region in all 80 samples investigated. This amplified region was found to be highly polymorphic upon SSCP analysis. Ten unique SSCP patterns were detected under the established condition (figure 3.1).

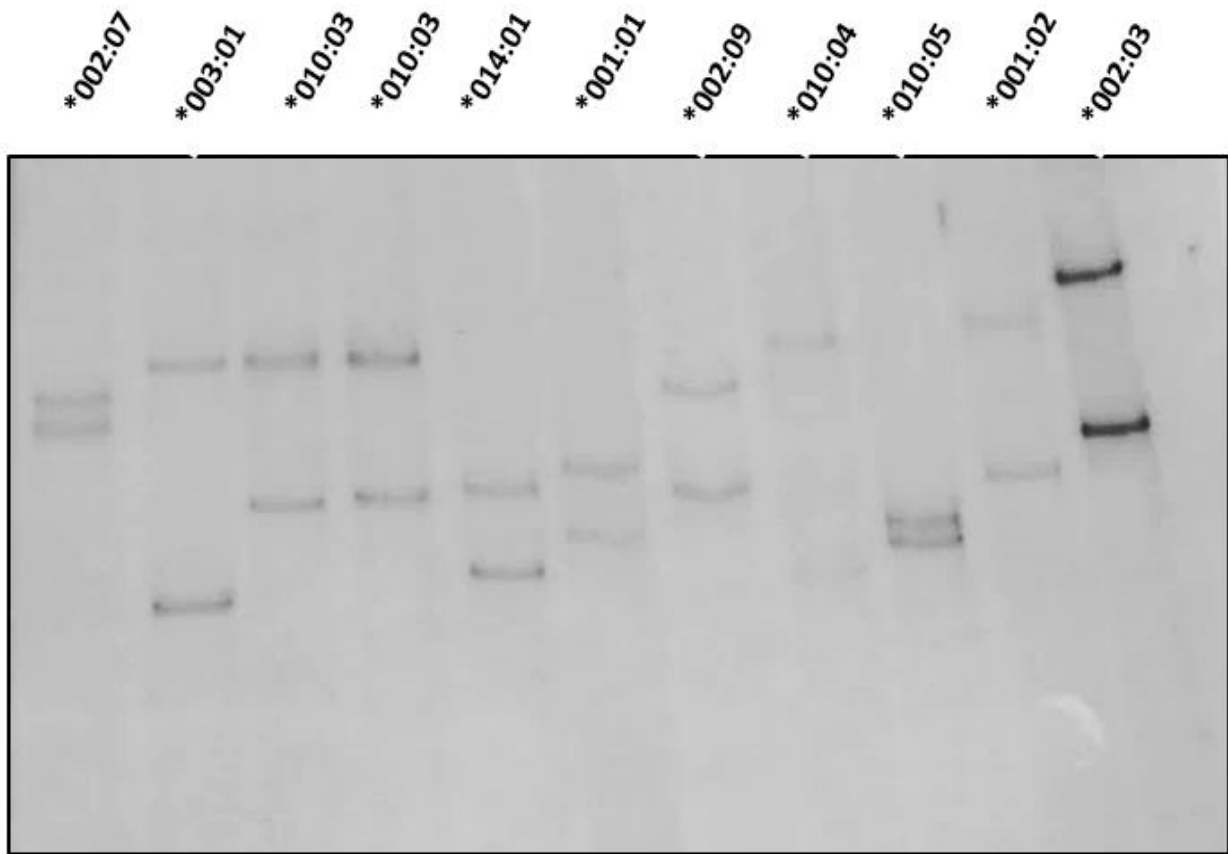

Figure 3.1. The polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) gel results of the amplified BoLA-DQA1 exon 2 (238 bp) region. Names were assigned to the alleles according to the rules proposed by the ISAG BoLA Nomenclature committee Davies et al., (1997)

3.3.2 Distribution of BoLA-*DQA1* Alleles

The frequency of the various BoLA-*DQA1* alleles from 80 animals was calculated and is presented in Table 3.2. Each allele represents a unique band on the SSCP gel. A total of 10 alleles was identified from all the samples investigated. BoLA-*DQA**001:02 had the highest frequency of occurrence (17.5%) and BoLA-*DQA**010:03 was the least common with a frequency of 6.9%.

Table 3.2. Frequencies of BoLA-DQA1 alleles.

Allele	Frequency (%)
BoLA-*DQA**002:07	11.3
BoLA-*DQA**003:01	12.5
BoLA-*DQA**010:03	6.9
BoLA-*DQA**010:04	8.8
BoLA-*DQA**014:01	10.6
BoLA-*DQA**001:01	10.6
BoLA-*DQA**002:09	8.1
BoLA-*DQA**010:05	6.3
BoLA-*DQA**001:02	17.5
BoLA-*DQA**002:03	7.5

3.3.3 Polymorphism of BoLA-*DQA1*

DNA sequencing of the PCR amplicons representing the ten SSCP patterns revealed ten different sequences with 30 polymorphic sites and 32 nucleotide substitutions, which constitutes approximately 12% of all nucleotide positions in the ten BoLA-*DQA1* sequences (Figure 3.2). The sequence comparison of all the alleles identified in this study is presented in Table 3.3. The nucleotide sequences, if expressed, would result in between two to thirteen changes in the predicted amino acid sequence except for alleles BoLA-DQA1 *010:03 and BoLA-DQA1 *010:04, where nucleotide change at position 114 (c.234G>A) would not change the amino acid sequence.

Eight of the ten sequences identified in this study shared 100% homology to previously published bovine sequences from the IPD-MHC database, while the remaining two only shared between 96-99% homology to previously published BoLA-*DQA1* sequences, and are therefore believed to be novel.

```
BoLA-DQA*002:07 TCCGACTCAG CTGACCACAT TGGTGCCTAT GGCATAAACA TCTACCACAC ATATGGTCCC   60
BoLA-DQA*003:01 .......... .......... ...C...... .........G ........T. ..........   60
BoLA-DQA*010:03 ..G....... .......... ...CA..... .......G.. .......... ..........   60
BoLA-DQA*010:04 ..G....... .......... ...CA..... .......G.. .......... ..........   60
BoLA-DQA*014:01 .......... .......... ...C...... .........G ........T. ..........   60
BoLA-DQA*002:09 .......... .......... ...C...... .........G .......... ..........   60
BoLA-DQA*010:05 ..G....... .......... ...CA..... .......G.. .......... ..........   60
BoLA-DQA*001:02 .......... .......... ...C...... .........G .......... ..........   60
BoLA-DQA*001:01 .......... .......... ...C...... .........G .......... ..........   60
BoLA-DQA*002:03 .......... .......... ...C...... .........G .......... ..........   60

BoLA-DQA*002:07 TCTGGCTACT ATACCCATGA ATTTGATGGA GATGAAGAGT TCTACGTGGA CCTGGAAAAG  120
BoLA-DQA*003:01 .......... .......... .......... .......... .......... ..........  120
BoLA-DQA*010:03 .......... .......... .......... .......... .......... ...A......  120
BoLA-DQA*010:04 .......... .......... .......... .......... .......... ..........  120
BoLA-DQA*014:01 .......... .......... .......... .......... .......... ..........  120
BoLA-DQA*002:09 .......... T......... .......... .......... .......... ..........  120
BoLA-DQA*010:05 .......... .......... .......... .......... .......... ..........  120
BoLA-DQA*001:02 .......... .......... .......... .......... .......... ..........  120
BoLA-DQA*001:01 .......... .......... .......... .......... .......... ..........  120
BoLA-DQA*002:03 .......... .......... .......... .......... .......... ..........  120

BoLA-DQA*002:07 AGGGAGACTG TCTGGAATCT GCCTCTGTTT AGTAAATTTA GACGTTTTGA CCCTCAGGGT  180
BoLA-DQA*003:01 .......... .......... .......... .......... .......... ..........  180
BoLA-DQA*010:03 .......... .....CG... ....G..... .......... C.A....... ..........  180
BoLA-DQA*010:04 .......... .....CG... ....G..... .......... C.A....... ..........  180
BoLA-DQA*014:01 .......... .....CG... ....G..... .........G C.A....... ..........  180
BoLA-DQA*002:09 .......... .......... .......... .......... .......... ..........  180
BoLA-DQA*010:05 .......... .......... ....G..... .......... C.A....... ..........  180
BoLA-DQA*001:02 .......... .....CG... ....G..... .......... C.A....... ..........  180
BoLA-DQA*001:01 .......... .....CG... ....G..... .......... C.A....... ..........  180
BoLA-DQA*002:03 .......... .......... .......... .......... .......... ..........  180

BoLA-DQA*002:07 GCGCTGAGAA ACATAGCTAC AGCGAAGCAC AATTTGGAGA TCGTGATTCA AAGGTCCA    238
BoLA-DQA*003:01 .......... .......... GA........ .......... ..A....G.. ........    238
BoLA-DQA*010:03 .......... .........T ..T....... .......... .......... ........    238
BoLA-DQA*010:04 .......... .........T ..T....... .......... .......... ........    238
BoLA-DQA*014:01 .......... ........GT G.G...A.GG .C.......G ..A......G ........    238
BoLA-DQA*002:09 .......... .......... .......... .......... ....A..... ........    238
BoLA-DQA*010:05 .......... .......... .A........ .......... .......G.. ........    238
BoLA-DQA*001:02 .......... .......... ..T....... .......... ..T....... ........    238
BoLA-DQA*001:01 .......... .......... .......... .........G ..T....... ........    238
BoLA-DQA*002:03 .......... .......... ..T....... .......... .......... ........    238
```

Figure 3.2 Nucleotide sequence alignments of the BoLA-DQA1 alleles revealed in this study.

The sequence alignment excludes the primer binding region. Dots (.) denote nucleotide sequence identical to the reference allele (BoLA-DQA*002:07). Sequences unique to this study are in bold.

```
BoLA-DQA*002:07 SDSADHIGAYGINIYHTYGPSGYYTHEFDGDEEFYVDLEKRETVWNLPLFSKFRRFDPQG  60
BoLA-DQA*003:01 .............V..S...........................................  60
BoLA-DQA*010:03 ........T...S................................R..V....TS.....  60
BoLA-DQA*010:04 ........T...S................................R..V....TS.....  60
BoLA-DQA*014:01 .............V..S............................R..V....AS.....  60
BoLA-DQA*002:09 .............V.........F....................................  60
BoLA-DQA*010:05 ........T...S...................................V....TS.....  60
BoLA-DQA*001:02 .............V...............................R..V....TS.....  60
BoLA-DQA*001:01 .............V...............................R..V....TS.....  60
BoLA-DQA*002:03 .............V..............................................  60

BoLA-DQA*002:07 ALRNIATAKHNLEIVIQRS                                          79
BoLA-DQA*003:01 .......T......MM...                                          79
BoLA-DQA*010:03 ......IV...........                                          79
BoLA-DQA*010:04 ......IV...........                                          79
BoLA-DQA*014:01 ......VG.RT..VM.R..                                          79
BoLA-DQA*002:09 ...................                                          79
BoLA-DQA*010:05 .......T.......M...                                          79
BoLA-DQA*001:02 .......V......L....                                          79
BoLA-DQA*001:01 .............VL....                                          79
BoLA-DQA*002:03 .......V...........                                          79
```

Figure 3.3 Amino acid sequence alignments of the BoLA-DQA1 alleles revealed in this study.

The sequence alignment excludes the primer binding region. Dots (.) denote an amino acid that is identical to the reference allele (BoLA-DQA*002:07).

The novel allele BoLA-*DQA**002:09 shared 99.07% homology with a previously identified allele, BoLA-*DQA**002:02 (Ballingall et al., 1997) and differs at the BoLA-*DQA**002:02 positions 181 and 203 (as illustrated in Figure 3.4).

```
BoLA-DQA*002:02 ---------- ---------- ---------- ---------- ---GCGCCTA TGGCATAAAC   17
BoLA-DQA*002:09 TTTCCCTTTC TTGCTCCTCA CTCCGACTCA GCTGACCACA TTG....... ..........   60

BoLA-DQA*002:02 GTCTACCACA CATATGGTCC CTCTGGCTAC TTTACCCATG AATTTGATGG AGATGAAGAG   77
BoLA-DQA*002:09 .......... .......... .......... .......... .......... ..........  120

BoLA-DQA*002:02 TTCTACGTGG ACCTGGAAAA GAGGGAGACT GTCTGGAATC TGCCTCTGTT TAGTAAATTT  137
BoLA-DQA*002:09 .......... .......... .......... .......... .......... ..........  180

BoLA-DQA*002:02 AGACGTTTTG ACCCTCAGGG TGCGCTGAGA AACATAGCTA CAGTGAAGCA CAATTTGGAG  197
BoLA-DQA*002:09 .......... .......... .......... .......... ...C...... ..........  240

BoLA-DQA*002:02 ATCGTGATTC AAAGGTCCAA CTCTACTGCT G                                228
BoLA-DQA*002:09 .....A.... .........- ---------- -                                259
```

Figure 3.4 Nucleotide Alignment of BoLA-DQA*002:09 with BoLA-DQA*002:02

Dots (.) denote nucleotide sequence identical to the reference allele (BoLA-DQA*002:02) and dashes (-) denote missing sequences. Nucleotides in boxes shows the substitution position.

Similarly, allele BoLA-*DQA**010:05 differed from BoLA-*DQA**010:04 at the positions 104, 126, 127, 190, 192, 193, 218 (Figure 3.5).

```
BoLA-DQA*010:04 ---------- ---------- ---------- -CTGACCACA TTGGCACCTA TGGCATAAGC   29
BoLA-DQA*010:05 TTTCCCTTTC TTGCTCCTCA CTCGGACTCA G......... .......... ..........   60

BoLA-DQA*010:04 ATCTACCACA CATATGGTCC CTCTGGCTAC TATACCCATG AATTTGATGG AGATGAAGAG   89
BoLA-DQA*010:05 .......... .......... .......... .......... .......... ..........  120

BoLA-DQA*010:04 TTCTACGTGG ACCTGGAAAA GAGGGAGACT GTCTGGCGTC TGCCTGTGTT TAGTAAATTT  149
BoLA-DQA*010:05 .......... .......... .......... ......AA.. .......... ..........  180

BoLA-DQA*010:04 ACAAGTTTTG ACCCTCAGGG TGCGCTGAGA AACATAGCTA TAGTGAAGCA CAATTTGGAG  209
BoLA-DQA*010:05 .......... .......... .......... .......... C.AC...... ..........  240

BoLA-DQA*010:04 ATCGTGATTC AAAGGTCCAA CTCTACTGCT GCTACCAACA                        249
BoLA-DQA*010:05 ........G. .........- ---------- ----------                        259
```

Figure 3.5 Nucleotide Alignment of BoLA-DQA*010:04 with BoLA-DQA*010:05

Dots (.) denote nucleotide sequence identical to the reference allele (BoLA-DQA*010:05) and dashes (-) denote missing sequences.

The Amino Acid sequence was used to predict the structure of the two new BoLA-*DQA1* alleles. The substitution potentially would lead to a slight difference in the structure of the protein obtained (Figure 3.6).

Figure 3.6 Predicted protein structure of BoLA-DQA1 genes showing site of difference in the protein structure.

Finally, computation of the average distance revealed that the total number of non-synonymous substitution was higher than synonymous substitution as shown in Figure 3.7 (Averages of all pairwise comparisons: ds = 0.0331, dn = 0.0495, dn/ds = 1.4954).

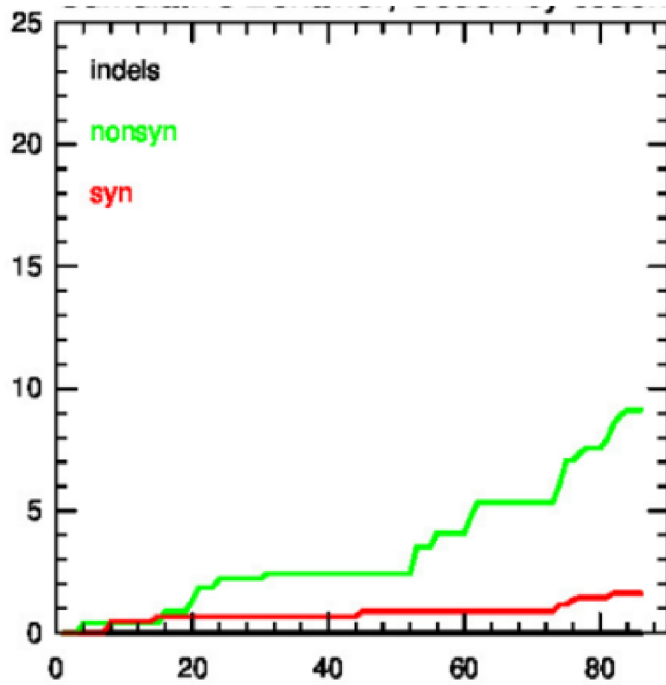

Figure 3.7. Graphs of the non-synonymous vs synonymous substitution.

Table 3.3: Sequence comparison of BoLA-DQA1 alleles obtained in this study [a]

	*002:07	*003:01	*010:03	*010:04	*014:01	*002:09	*010:05	*001:02	*001:01	*002:03
*002:07		97.1	95.0	95.4	91.6	98.3	96.2	96.2	96.2	98.7
*003:01	93.7		92.9	93.3	92.9	97.1	95.8	95.4	95.4	97.5
*010:03	89.9	84.8		99.6	92.9	94.1	97.1	97.1	96.2	95.4
*010:04	89.9	84.8	100		93.3	94.5	97.5	97.5	96.6	95.8
*014:01	83.5	86.1	84.8	84.8		91.6	91.2	94.5	95.0	92.4
*002:09	97.5	93.7	87.3	87.3	83.5		95.4	96.2	96.2	98.7
*010:05	91.1	89.9	94.9	94.9	82.3	88.6		95.8	95.8	95.8
*001:02	91.1	89.9	93.7	93.7	88.6	91.1	91.1		99.2	97.5
*001:01	91.1	88.6	91.1	91.1	89.9	91.1	89.9	97.5		96.6
*002:03	97.5	94.9	89.9	89.9	84.8	97.5	89.9	93.7	91.1	

[a]Nucleotide sequence identities are shown above the diagonal and predicted AA identities are shown below the diagonal. All sequences compared exclude the PCR primer binding regions, and the similarities are shown in percentages

3.3.4 Relationship between the BoLA-*DQA1* alleles

Between 91.6 and 99.6% homology was observed in pairwise comparisons between the 238 nucleotide sequences (excluding the primer binding region) of BoLA-*DQA1* exon 2 sequences, which corresponded to 82.3 to 97.5% similarity in the predicted amino acid sequences (Table 3.3). These sequences with published BoLA-*DQA2*, BoLA-*DQA*5 and BoLA-*DQA*3 together with selected *DQA1* sequences of other ruminants (ovine, caprine), swine and human were used to construct a neighbour-joining phylogenetic tree. A human HLA-*DQA1* sequence was used to root the tree. There was close relationship between the BoLA-*DQA1* alleles and other ruminants, when compared with other non-ruminants. This tree also confirmed that the BoLA-*DQA1* sequences found in this study are more specific to the *DQA1* locus than to other BoLA-*DQA* loci (Figure 3.8).

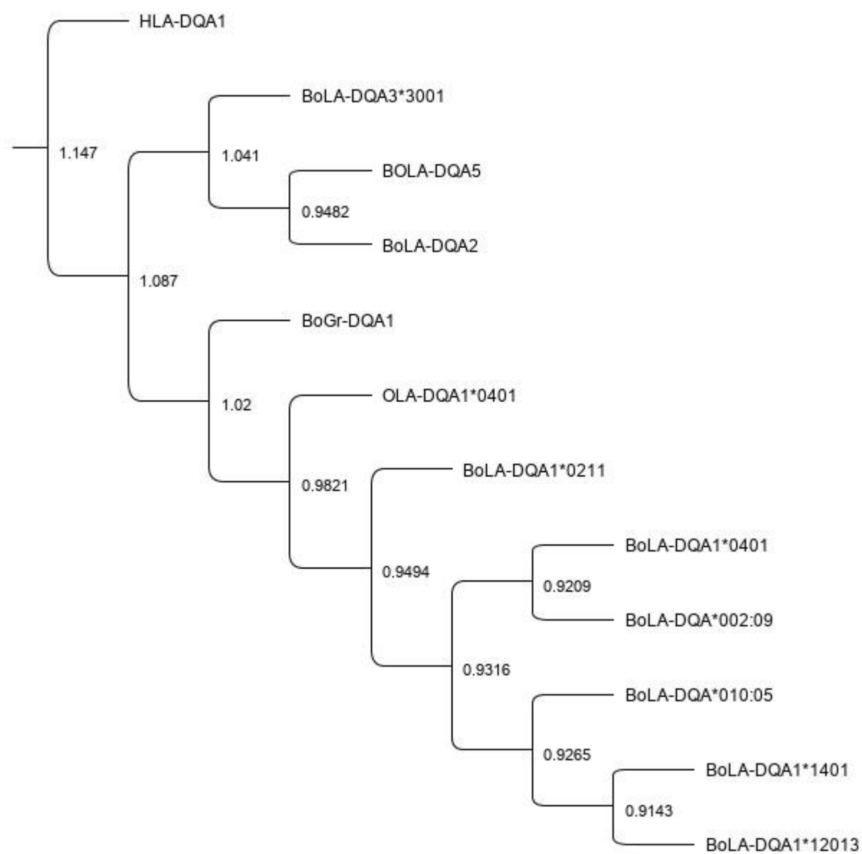

Figure 3.8 Neighbour-Joining Tree showing relationship of BoLA-DQA1 alleles with other species.

Sequences included in the construction are; MN954679.1 (HLA-DQA1), LR797948 (BoLA-DQA2), JN798528.1 (BoLA-DQA3), JQ864314.1 (BoGr-DQA1), NM_001012675.2 (BoLA-DQA5), AB257112.1 (BoLA-DQA1*002:09), AB362377.1 (BoLA-DQA1*010:05), MG839037.1 (BoLA-DQA1*1401), MG571516.1 (BoLA-DQA1*12013), AY265308.1 (OLA-DQA1*0401)

3.4 Discussion

The ability of a population to respond to pathogenic infestation may be dependent on the level of diversity at the MHC loci (Sun et al., 2013). In this study, nucleotide sequence variation in the exon 2 of the BoLA-DQA1 gene was examined. Primers were used to amplify a 238 bp fragment of exon 2 of the BoLA-DQA1 gene in eighty (80) cattle of different breeds from New Zealand and Nigeria. As expected, we found a similar MHC class II diversity to previous studies. There were ten alleles with variable nucleotide sequences present in the 80 samples. Two alleles are reported here for the first time, confirming that BoLA-*DQA1* is polymorphic and that new alleles can still be discovered.

Allele uniqueness was confirmed by them having distinct SSCP patterns, as shown in Figure 3.1. All the sequences have a similar conserved and variable region with previously identified alleles selected from the IPD-MHC database and GenBank, upon alignment; with this suggesting they were all derived from the BoLA-*DQA1* locus. The alleles were named following the proposed MHC nomenclature (Davies et al., 1997).

In addition, all the alleles found in this study were also confirmed to be unique following sequence comparison analysis, as indicated in Table 3.3 for nucleotide variation and amino acid distances. The sequences shared up to 99.6% homology with previously published BoLA-*DQA1* alleles. Allele BoLA-*DQA**010:03 and BoLA-*DQA**010:04 were the most similar and only differed at one nucleotide position. This would if expressed result in similar amino acid sequences, while the other sequences differed by between 3 and 21 nucleotides, and these substitutions would likely affect the amino acid sequences.

This study has demonstrated that SSCP analysis using the primers designed in this study can discriminate polymorphic alleles of BoLA-*DQA1*. This could provide a cost-effective way for future studies to investigate the role of BoLA-*DQA1* in immune response to pathogens.

Some of the findings in this study are consistent with previous studies of the BoLA-*DQA1* locus. Sahoo et al., (2019) reported 30 polymorphic sites with 29 mutations and one insertion from a 249 bp fragment of BoLA-*DQA1* exon 2 from Tharparkar cattle. In total, 32 mutations were found in this study within 30 polymorphic sites, but no insertion/deletion event. Similarly, using sequence-based technics, Kul Aj, Pokorska, Ormian, & Dusza, (2015) reported 14 *DQA1* alleles from 60 individuals. Miyasaka et al., (2011) reported 13 alleles of BoLA-*DQA1*, while Sahoo et al., (2019) reported 14 in 50 animals, compared with the ten alleles identified in 80 cattle using SSCP and direct sequencing technique described here. This range is also similar to the variation of ovine *DQA1* found in three hundred sheep, where 14 sequences were identified using SSCP analysis (Zhou & Hickford, 2004).

Regardless of the methods used, the MHC-*DQA* region has been demonstrated to be highly polymorphic in ruminants. However, this variation is not as extensive as has been reported for BoLA-*DRB3*, which is the most polymorphic mammalian gene (Amills et al., 1998). Currently, the IPD database lists 370 alleles of BoLA-*DRB3*, while there are 77 alleles of BoLA-*DQA* (https://www.ebi.ac.uk/ipd/mhc/group/BoLA/) reported.

Peters et al., (2018) reported that the high polymorphism in MHC loci would result in increased population fitness as it may be an indication that the population can respond to a wider range of pathogens. Moreover, high variation in the MHC genes has been suggested to be responsible for the recognition of bacteria, which may result in balancing selection (Niskanen et al., 2014).

The greater than one ratio of non-synonymous to synonymous substitutions observed in this study, suggests that the population undergoes balancing selection. This is because the dN/dS ratio is regarded as a good indicator of selective pressure (Sahoo et al., 2019).

The alleles found in this study have frequencies that ranged between 6 -17%. Alleles BoLA-*DQA**001:01, 014:01 and 001:02 were the most common alleles identified (Table 3.2). These alleles were also found to be common in other populations of different cattle breeds (Kul Aj et al., 2015; S. Takeshima et al., 2008). In contrast, allele *010:01:01 had a high occurrence in other studies (Schwab 2009, Miyasaka 2011,) but was not found in this study. This suggests that the distribution of BoLA-*DQA1* alleles may be dependent on the population studied. Allele *DQA**001:02 was the most common allele in this present study, but was the least common elsewhere (Kul Aj et al., 2015).

Following the negative frequency-dependence hypothesis, it may be suggested that common alleles may confer a selective advantage. The hypothesis proposes that the alleles with selective advantage tend to have higher frequencies within a population (Slade & McCallum, 1992). The homozygous form of the frequently occurring BoLA-*DQA1**001:01 was found to be associated with susceptibility to clinical mastitis caused by *Streptococci* and *Escherichia*. However, in a heterozygote form, this same allele was associated with resistance to the pathogens (Takeshima et al., 2008). This suggests that increasing heterozygosity of the most common allele may provide more advantage than being homozygous for that same allele. Such an idea can be supported by the heterozygote advantage hypothesis, which states that heterozygosity in MHC genes may enhance defence against infectious diseases (Takeshima et al., 2008).

Phylogenetic tree construction suggested that the newly found BoLA-*DQA1* sequences were closer to previous *DQA1* sequences obtained for other ruminants (Sheep and Yak), than to BoLA-*DQA2*, BoLA-*DQA*3, BoLA-*DQA4* and BoLA-*DQA*5 (Figure 3.8), and seems to

confirm the specificity of the primers used in this study. This relationship may be explained by what is called the ‘trans-species polymorphism phenomenon’, which suggests that the alleles of a locus in one species are more similar to the alleles of the same locus in a closely related species, than to alleles of other loci of the same species (Hughes & Yeager, 1998). Additionally, Zhou & Hickford (2004) suggested that the similarities in the allelic sequence between OLA_*DQA1* and BoLA *DQA1* may have risen from the need for a specific immune response to a common pathogen.

3.5 Conclusion

The major histocompatibility complex is highly polymorphic. Although, the samples studied were limited in number, this study could demonstrate that new alleles are still identifiable at the BoLA-*DQA1* locus and they can be identified using PCR-SSCP analysis. It has been established that specific primers *DQA1*-Forward and *DQA1*-Reverse can be used to amplify the BoLA-*DQA1* locus. Studying the associations of all BoLA-DQA1 variants with immune response traits could improve our understanding of the role of this gene in disease resistance.

Chapter 4

Genetic variation in bovine leukocyte antigen DRA gene

4.1 Introduction

The bovine leukocyte antigen (BoLA) class II genes are well known for their polymorphism and role in immune responsiveness. They encode glycoproteins consisting of α and β polypeptide chains that are expressed mainly on the B lymphocytes and macrophages (van der Poel et al., 1990). The *DR* and *DQ* loci are the two main expressed groups producing the BoLA class II peptides. The *DQ* loci appears to have five genes and extensive polymorphism, which can be created by duplication (Glass et al., 2000). There are two DR chains, the DR α chain and the DR β chain. Only one gene has been identified for the α-chain (DRA) and *DRB3* is the only functional gene of the three β-chain genes. The BoLA-DRB3 gene is the most polymorphic of the MHC genes in cattle, with over 300 alleles listed in the IPD-MHC database (https://www.ebi.ac.uk/ipd/mhc/group/BoLA/).

Among all the BoLA genes, BoLA-*DRA* is the least studied, perhaps because it was earlier described as being monomorphic (Ellis & Ballingall, 1999). Although *DRA* has been shown to be conserved in mammals, sequence variation has been reported (Zhou et al., 2007). Polymorphism has also been reported in sheep, (Ballingall et al., 2010), deer (Yao et al., 2015), donkey (Arbanasič et al., 2013), and swine (Gao et al., 2014). Studies have also shown that MHC-*DRA* genotypes are associated with susceptibility and resistance to diseases (Huang et al., 2016; Yang et al., 2015), which suggests that *DRA* variation also plays a role in immune response.

To better understand the role of BoLA-*DRA* in immune responsiveness, it is crucial to study more breeds of cattle to understand the extent of its polymorphism. The aim of this study was

to survey the genetic variation in the MHC-*DRA* gene in different *Bos* species and across multiple breeds and geographical locations.

4.2 Materials and Methods

4.2.1 Sample description and DNA extraction

Genomic DNA from two hundred and sixty-six (n = 266) cattle from 13 breeds and forty-seven yak (n = 47) was extracted and purified from dried blood samples on FTA cards using a two-step procedure (Zhou, Hickford, & Fang, 2006). The breeds of cattle used are from Nigeria, New Zealand and Italy and they include: White Fulani (n = 30), Red Bororo (n= 20), White Fulani × Holstein-cross (n = 16) from Nigeria; Holstein-Friesian × Jersey Cross (n = 22), Hereford (n = 20), Murray Grey (n = 20), Red poll (n = 20), Shorthorn (n =22), Angus (n = 20), Simmental (n = 20), Charolais (n = 20), South Devon (n =20) from New Zealand; and Marchigiana (n = 16) from Italy.

4.2.2 Primer selection and amplification

Two primers, *DRA-up* (5′-TCTTCCTCTCCTGGTTCCCAC-3′) and *DRA-dn* (5’-GCTACAATCCCAAGTCTAGG-3’) described by Zhou, Hickford, Fang, & Byun, (2007) were used to amplify the entire exon 2 sequence of BoLA-*DRA*. The primers were designed according to published bovine *DRA* sequence available in GenBank (Accession no M30120).

Amplification was undertaken in Bio-Rad S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA) Each PCR amplification was performed in a 15-ul reaction volume containing 1.2 mm disc of the sampled FTA cards, 0.25 uM of the *DRA-up* and *DRA-dn* primers, 150 uM of each deoxyribonucleoside triphosphate (dNTP), 3.0 mM Mg^{2+} (Bioline, London, UK), 0.5U of Taq DNA polymerase (Qiagen, Hilden, Germany), and 1× reaction buffer supplied with the

enzyme. The following thermal cycling profile was used for 34 cycles after an initial denaturation of 94 ^{o}C for 2 minutes; denaturation at 94 ^{o}C for 30 seconds, annealing at 60 ^{o}C for 30 seconds, elongation at 72 ^{o}C for 30 seconds, and a final extension at 72 ^{o}C for 5 minutes.

4.2.3 Genotyping

Allelic variants of the amplicons were screened by Polymerase Chain Reaction – Single Stranded Conformation Polymorphism (PCR-SSCP) analysis. A 0.7-µL aliquot of the amplicons was added to 7 µL of loading dye, containing 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol, and 98% formamide. The samples were denatured by placing them in a 95° C hot plate for 5 minutes, followed by snap chilling on wet ice. They were loaded onto 16 cm x 18 cm, acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was carried out using Protean II xi cells (Bio-Rad) using 14 % polyacrylamide gels, at a room temperature of 15 ^{o}C. A silver staining techniques was used to reveal the SSCP banding patterns (Byun, Fang, Zhou, & Hickford, 2009).

4.2.4 Sequence analysis

Homozygous samples with unique banding pattern were subjected to direct sequencing, while unique bands in heterozygous samples were excised from the wet gel, incubated in water at 51 ^{o}C for 1 hour, amplified and then sequenced. The DNA sequences were aligned using MEGA X software and the CLUSTALW algorithm to enable identification of the position of the single nucleotide substitutions. Translation, sequence comparison and phylogenetic analysis was done using Geneious Version 2020.2.4. The following sequences were used to construct the tree together with the nucleotide sequences obtained in this study. Human-HLA-*DRA* (NC_000006.12:32439887-32445046 Homo sapiens chromosome 6, GRCh3), *Moschus berezovskii* Deer (Chinese forest musk deer)-*DRA* (KJ938306.1), Equine-ELA-*DRA* (L47172.1), Ovine-OLA-*DRA* (Z11600.1:101-346), and Swine-SLA-*DRA* (KP324808.1).

4.3 Results

4.3.1 Allele and genotype frequencies of Bovine Leucocyte Antigen BoLA-*DRA*

Nucleotide variation in exon 2 region of BoLA-*DRA* was examined. Primer pairs developed by Zhou, Hickford, Fang, & Byun, (2007) were used to amplify 333 bp of the *DRA* region, and then screened using PCR-SSCP followed by DNA sequencing. A total of three unique SSCP banding patterns representing three allelic variants were found (Figure 4.1).

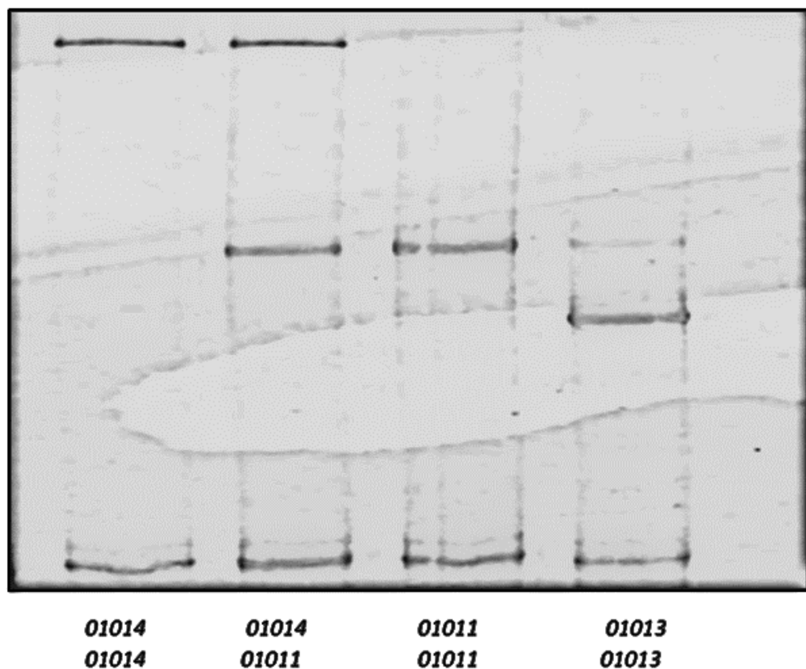

Figure 4.1. BoLA-DRA exon 2 PCR-SSCP gel patterns

The New Zealand Holstein Friesian x Jersey (HF X J)-cross cattle and the Yak had only two alleles (BoLA-*DRA**01011 and BoLA-*DRA**01013), while the other breeds studied had the BoLA-*DRA**01011, BoLA-*DRA**01013 and BoLA-*DRA**01014 alleles. The frequency of each allele is presented in Table 4.1. There were relatively high frequencies of BoLA-*DRA**01011 (> 65%) in all of the breeds studied and the distribution of all alleles were skewed except in the Italian Marchigiana breeds. The Italian breed had the highest frequencies of BoLA-*DRA**01013 allele, while the BoLA-*DRA**01014 allele had a low frequency of occurrence in all breeds studied.

Table 4.1 Allele frequencies of BoLA-DRA exon 2

Breed	N	BoLA-*DRA* exon 2 variants and frequencies (%)		
		01011	*01013*	*01014*
Nigerian breeds	66	65.57	19.67	14.75
New Zealand beef breeds	162	80.43	17.39	2.17
NZ HF x J dairy cattle	22	82.92	17.07	0
Italian Marchigiana	16	40.62	43.75	15.62
Yak	47	91.21	8.79	0

The genotypic frequencies of BoLA-*DRA* alleles found in yak and 13 breeds of cattle are given in Table 4.2.

Table 4.2 Frequencies of BoLA-DRA genotypes in a variety of breeds

Breed	n	BoLA-*DRA* Variant Genotypes (%)					
		01011/ 01011	01011/ 01013	01011/ 01014	01013/ 01013	01013/ 01014	01014/ 01014
White Fulani	30	60.0	13.3	20.0	6.7	-	-
Red Bororo	20	35.0	20.0	15.0	20.0	-	10.0
White Fulani × Holstein	16	56.3	18.8	6.3	6.3	-	12.5
Holstein-Friesian × Jersey	22	63.6	27.3	-	9.1	-	-
Hereford	20	80.0	15.0	-	5.0	-	-
Murray grey	20	50.0	30.0	5.0	10.0	-	5
Red Poll	20	50.0	30.0	-	15.0	-	5
Shorthorn	22	59.1	18.2	4.5	18.2	-	-
Angus	20	75.0	25.0	-	-	-	-
Simmental	20	70.0	30.0	-	-	-	-
Charolais	20	75.0	20.0	5.0	-	-	-
South Devon	20	80.0	20.0	-	-	-	-
Marchigiana	16	25.0	18.8	12.5	31.3	6.3	6.3
Yak	47	85.1	6.4	-	-	-	-
Total	**313**						

Genotypes 01011/01011 and 01011/01013 were the most common in all the breeds. The Marchigiana cattle were the only breed with the genotype 01013/01014 (6.3%) in all the cattle

studied, and the breed has the highest frequency of 01013/01013 genotypes (Table 4.2). The White Fulani breed is the only breed from Nigeria without the genotype 01014/01014.

4.3.2 Sequence analysis of BoLA-*DRA*

Amplicons of the three unique SSCP patterns of BoLA-*DRA* were sequenced. The result revealed three different sequences with three single nucleotide polymorphisms (Figure 4.2). All the alleles found in this study have been previously reported, and no new allele was identified.

```
BoLA-DRA*01011 TCCCACCCTG ACCCCCTTTC TTGTCTTTTC AGAGAATCAT GTGATCATCC AAGCTGAGTT   60
BoLA-DRA*01013 .......... .......... .......... .......... .......... ..........   60
BoLA-DRA*01014 .......... .......... .......... .......... .......... ..........   60

BoLA-DRA*01011 CTATCTGAAA CCTGAGGAAT CAGCCGAGTT TATGTTTGAC TTTGATGGTG ATGAGATTTT  120
BoLA-DRA*01013 .......... .......... .......... .......... .......... ..........  120
BoLA-DRA*01014 .......... .......... .......... .......... .......... ..........  120

BoLA-DRA*01011 CCACGTGGAT ATGGGGAAGA AGGAGACGGT GTGGCGGCTT CCAGAATTTG GACATTTTGC  180
BoLA-DRA*01013 .......... .......... .......... .......... .......... ..........  180
BoLA-DRA*01014 .......... .......... .......A.. .......... .......... ..........  180

BoLA-DRA*01011 CAGCTTTGAG GCTCAGGGTG CCCTGGCCAA TATGGCTGTG ATGAAAGCCA ACCTGGACAT  240
BoLA-DRA*01013 .......... .......... .......... .......... ........T. ..........  240
BoLA-DRA*01014 .......... .......... .......... .......... .......... ..........  240

BoLA-DRA*01011 CATGATAAAG CGCTCCAACA ACACCCCAAA CACC                               274
BoLA-DRA*01013 .......... .......... .......... ....                               274
BoLA-DRA*01014 .......... .......... .......... ....                               274
```

Figure 4.2 Sequence alignment of the BoLA-DRA alleles

Dots (.) represent similarity to the reference sequence BoLA-DRA*01011.

The three alleles found in this study shared 99% homology for their nucleotide sequences and 98% for the amino acid sequence Table 4.3. The nucleotide sequences were used to construct a neighbour-joining tree with *DRA* sequences from sheep, deer, dog, equine, human, and pig. Human *DRA* was used to root the tree. The tree suggested that all BoLA-*DRA* sequences were closest to other ruminants, than to non-ruminants (Figure 4.3).

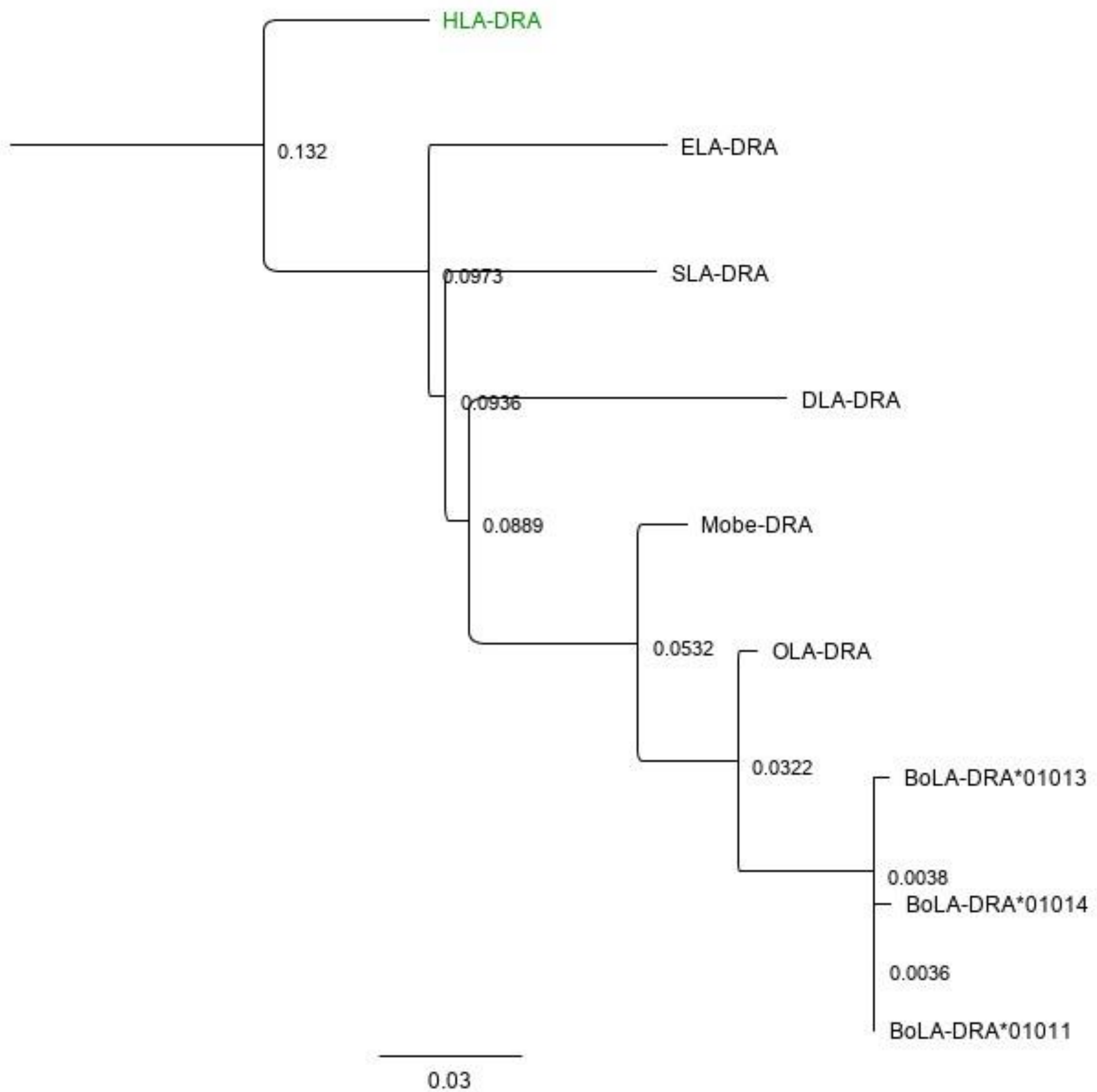

Figure 4.3 Phylogenetic tree showing distances of BoLA-DRA alleles with other species.

Species included in the tree include; Human-HLA-DRA (GenBank: NC_000006.12:32439887-32445046 Homo sapiens chromosome 6, GRCh3), Chinese Forest Musk Deer Moschus berezovskii-DRA (GenBank: KJ938306.1), Equine-ELA-DRA (GenBank: L47172.1), Ovine-OLA-DRA (GenBank: Z11600.1:101-346), Swine-SLA-DRA (GenBank: KP324808.1)

4.4 Discussion

The MHC class II genes present foreign peptides on the surface of the immune cells, making them an important part of the immune response process in mammals. The *DRA* gene has been described as being the most conserved MHC class II gene in many vertebrate species, and BoLA-*DRA* was originally suggested to be monomorphic (Ellis & Ballingall, 1999). However, Zhou, Hickford, Fang, & Byun, (2007) revealed three SNPs in the cattle gene and polymorphism have also been reported in related species (Plasil et al., 2016; Yao et al., 2015).

In this study, three alleles of BoLA-*DRA* were identified confirming that MHC-*DRA* is not monomorphic in cattle. However, there was no new variation found, despite multiple breeds from different geographical locations being investigated. To date, only four alleles of BoLA-*DRA* have been published in the GenBank database. The three alleles found in this study from 313 cattle, were present at different frequencies in different breeds. Allele 01012, which had been identified previously, was not found. No breed had a unique allele, but the BoLA-*DRA**01014 allele was not found in the New Zealand Holstein-Friesian x Jersey-cross (HF x J) and Yak populations in this study. Although (Zhou et al., 2007) found the presence of the BoLA-*DRA**01014 allele in their Holstein Friesian x Jersey-cross population, this may be because they studied more samples (n = 389) from the breed. In spite of this, the BoLA-*DRA**01014 allele had the lowest frequency (0.1) in their study, which is consistent with what was found in this study.

When comparing with the Nigerian and European Breeds, the New Zealand cattle generally had a very low frequency of BoLA-*DRA**01014. In total, the New Zealand beef breeds had a 2% frequency for the BoLA-*DRA**01014 allele, although this allele was not found in some beef breeds including the; Hereford, South Devon, Angus, and Simmental breeds. New Zealand

beef breeds that had the allele, included Murray Grey, Red Poll, Shorthorn and Charolais, albeit at a very low frequency relative to other breeds that where studied.

Conversely, the Nigerian indigenous and Italian Marchigiana breeds studied had the highest frequency of the BoLA-*DRA**01014 allele, but lower than the other two alleles. The rare allelic variant has been suggested to be as a result of adaptation to a changing environment (Huang et al., 2016). On the other hand, the high frequency of the BoLA-*DRA**01011 may also be important for pathogen resistance in all breeds of cattle, if we consider the negative frequency-dependence hypothesis, which states that the alleles with selective advantage may occur at higher frequencies (Slade & McCallum, 1992). Perhaps selecting this allele will be advantageous to improving livestock health.

The concern when selecting animals for resistance to specific disease resistance is whether there will be an association with other diseases and or performance. While this link has not been adequately studied for MHC genes, Bishop, (2012) reported that sheep's ability to develop resistance to nematode infection does not impair its performance. Livestock productivity depends on several other factors such as disease management, housing, better nutrition; hence, the genetic relationship between resistance and productivity is complex and dependent on the environment. Conversely, Juliarena et al., (2009) indicated that cattle carrying BoLA alleles associated with BLV resistance were not susceptible to other bovine virus including; foot and mouth disease virus, bovine viral diarrhoea virus, or bovine herpesvirus type 1. Nonetheless, it is not widely studied as to whether selecting for resistance to a specific disease will predispose animals to other disease. This missing link requires further study.

Further studies of the association between *DRA* and disease resistance are also warranted to understand if the allelic variation in BoLA-*DRA* has any functional implication in cattle. In swine, some homozygote genotypes of SLA-*DRA* were found to be associated with

susceptibility to diarrheal diseases (Huang et al., 2016), while some alleles in heterozygous genotypes were associated with resistance to immune-related diseases (Gao et al., 2014; Le et al., 2012). Consequently, heterozygous MHC genotypes are considered to confer an advantage over homozygous genotypes in immune responsiveness.

In ruminants and other studied mammals, the MHC-*DRA* gene is considered to be the least polymorphic of all the MHC class II genes. Li et al., (2013) studied 82 samples of forest musk deer and found only four alleles of Mobe-*DRA* compared with 10 alleles in 46 samples for Mobe-*DRB3*. Sun et al., (2014) revealed a similar trend. They found only three-nucleotide polymorphisms in the *DRA* locus of Yak that are nearly identical to those of cattle. The conservation in the *DRA* locus across ruminants may be explained by identification of the specific antigens presented by pathogens that may be common to species (Huang et al., 2016; Zhou & Hickford, 2004) and may also suggest that *DRA* gene remained conserved even after species divergence. The phylogenetic tree confirms that Bovine *DRA* is generally closer to ruminants than non-ruminants, but somewhat surprisingly closer to Dog *DRA* than to Equine *DRA*.

4.5 Conclusion

In this study, the BoLA-*DRA* gene was characterized in Chinese yak and 13 different breeds of cattle from Nigeria, Italy and New Zealand. It was revealed that BoLA-*DRA* is less polymorphic compared with other DR and DQ molecules and studying more breeds from different parts of the world, did not reveal any new alleles. Future studies could examine a larger number of samples that can more accurately represent the population than in this study; and investigate the relationship between the different alleles of BoLA-*DRA* and immune responsiveness to infection.

Chapter 5

General Summary

The livestock industry globally faces economic costs as a result of infectious diseases. The treatment of infectious disease also raises concern for food safety and can influence public perception of the livestock industries. The use of antibiotics in livestock is considered to be a driver of antibiotic resistance in microbes. A future challenge to the livestock industry would be to find an alternative or supplementary method to managing diseases as Governments begin to reduce or stop the use of antibiotics in farming. As an alternative to antibiotic use, there is promising research into identifying host genes that can be used as genetic markers in breeding schemes that enhance resistance to disease without impairing productivity.

This thesis examined genetic variation within two class II genes of the bovine major histocompatibility complex; BoLA-*DRA* and BoLA-*DQA1*. These genes were studied because of their previously reported role in immune response.

The BoLA-DQA1 gene was found to be highly polymorphic and this is not surprising because there are 79 alleles of the gene described in the IPD-MHC database (Chapter 3). In this thesis, two novel nucleotide sequences that shared up to 98% homology to other *DQA1* sequences was revealed. These new sequences would if expressed likely change the predicted amino acid sequence of the gene. The functional implication of these allelic differences were however not studied in this research, but the typing mechanism employed and results obtained, could serve as basis for future research to investigate associations between BoLA-*DQA1* and disease resistance or susceptibility. It was also confirmed in this study that specific primers can be used to amplify the BoLA-*DQA1* gene. The gene BoLA-*DQA* is usually duplicated in cattle and 5 copies have been described, but these genes are tightly linked and share similar sequences making it difficult to amplify a specific locus. However, the technique used in this study

appears to have successfully amplified only BoLA-*DQA1*, meaning it could possibly be used for future research.

In chapter 4, a BoLA-*DRA* exon 2 fragment was revealed to be far less polymorphic, reinforcing the idea that it is the least polymorphic of the MHC class IIa genes. Only three single nucleotide polymorphisms, which have been reported previously, were identified. More breeds than had been studied previously were examined, but no further genetic variation was found, suggesting that the BoLA-*DRA* region has not changed much since the evolutionary divergence of the *Bos* genera.

This study used the polymerase chain reaction coupled with single stranded conformation polymorphism (PCR-SSCP) to examine the nucleotide variation within the MHC class II genes. The SSCP analysis is based on the observation that the electrophoretic mobility of single-stranded DNA fragments is conformation-dependent and determined by the DNA sequence. Major advantages of using the SSCP approach is that it is an inexpensive method and the bands corresponding to individual alleles can be excised, re-amplified, and sequenced to obtain the direct sequences of specific region. It also enables the rapid typing of large numbers of animals very quickly. However, PCR-SSCP may become problematic if alleles from multiple loci co-amplify. Interpreting gels from such cases can be difficult due to the overlapping of bands representing various alleles with similar mobility. It is therefore possible that some alleles are missed or are falsely reported. In addition, excising heterozygote bands requires high precision to avoid the mixing up of alleles. Selecting a locus-specific primer in this study helped to minimise the possibility of co-amplification of the MHC alleles and this was achieved by designing the primer sequences from the most conserved region of the genes of interest.

Identification of nucleotide variation using an inexpensive approach may provide a better means to select animals that are resistant to diseases, but it is only the first step in identifying

gene markers. Future research needs to be undertaken to differentiate alleles that appear to increase the ability of an animal to respond to wide varieties of pathogens, from those that don't. It would be important for such research to examine specific pathogens that may be endemic to a particular geographic location.

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