Genetic Evaluation of South African Indigenous Chickens for Disease Resistance

Nonhlanhla Bridget Nduna

BSc. (*Hons*) Genetics

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College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Westville

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Preface

The research contained in this thesis was done by Nonhlanhla Bridget Nduna based in the Discipline of Genetics, School of Life Sciences, College of Agriculture, Engineering and Science at the University of Kwa-Zulu Natal, South Africa under the supervision of Dr Oliver Zishiri and co-supervision of Dr Meenu Ghai from 2014 to 2015 while based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville campus, South Africa. The research was financially supported by College of Agriculture, Engineering and Science for the first year and NRF-DAAD scholarship for the second year.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Dr Oliver Zishiri (Supervisor)

Date:

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Dr Meenu Ghai (Co-supervisor)

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ABSTRACT

An alternative standard disease control method would be selective breeding in order to increase disease resistance. To set up marker assisted selection programmes, knowledge of the genetic diversity of the chickens is required. To date, indigenous South African chicken lines have received little scientific attention and has never been subjected to purposive selective breeding for any particular trait. The objective of the study was to investigate genetic variation within genes involved in innate and adaptive immune system response. The innate immune system genes which were studied were the Toll Like Receptor-4 (TLR-4), Myeloid Differential protein-2 (MD-2) and Solute Carrier Family 11 member A1 (SCL11A1) genes. The PCR-RFLP method was used to detect single nucleotide polymorphisms in these genes and thereafter, the electrophoretic patterns were analysed and used to compute population genetic analyses. The indigenous chickens were moderately outbred at the TLR-4-*Sau 96I* and INOS-*Alu I* loci as indicated by high observed heterozygosity figures of 0.78 and 0.51 respectively and low fixation indices. The hypothesis of indigenous chicken population having a more genetic variation was supported by the results that showed the high observed homozygosity in commercial chickens at the SLC11A1-*Sac I* locus. The Major Histocompatibility Complex is composed of genes responsible for the adaptive immune system response. The genetic variation in the LEI0258 microsatellite marker within the MHC was investigated in indigenous chicken populations in South Africa and across the 3 populations, 36 alleles were detected and of these, 11 of them were private alleles. Observed heterozygosity levels and high fixation indices suggested a level of inbreeding at this locus. It was concluded that the sampled South African chicken populations were moderately inbred at the MHC locus and that the inbreeding may be due to natural selection of certain alleles in order to acclimate to the natural environment more effectively.

Keywords: Major Histocompatibility Complex; innate immune response; disease resistance; indigenous chickens; heterozygosity

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List of abbreviations

AIV	Avian influenza virus
dH_2O	Distilled water
HWE	Hardy Weinberg Equilibrium
IBV	Infectious bronchitis virus
INOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MD-2	Myeloid differentiation protein 2
MDV	Marek's disease virus
MHC	Major Histocompatibility Complex
PAMPs	Pathogenic associated molecular patterns
PCR	Polymer Chain Reaction
QTL	Quantitative traits loci
RFLP	Restriction Fragment Length Polymorphism
SE	*Salmonella enteritis*
SLC11A1	Solute Carrier Family 11 Member 1
SNP	Single nucleotide polymorphism
TAE	Tris-acetate- EDTA buffer
TLR-4	Toll like receptor 4

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Chapter 1: Introduction

In the animal breeding industry natural selection and selective breeding in conjunction with research on chickens has resulted in a rich collection of phenotypic diversity in the form of breeds or lines with specific characteristics (Weigend and Romanov, 2001). Although in commercial breeding establishments, the breeding practices have resulted in chickens that grow bigger in a shorter time which is important for them economically, there are still issues crippling the poultry industry such as infections that affect the growth, production and welfare of the birds and can also be harmful if pathogens are of a zoonotic nature. The commonly used strategies for combating infections by viral, parasitic and bacterial infections include vaccinations and antibiotics and these have been affective however there is increasing controversy in the use of antibiotics in livestock production (Graham et al., 2007). The excessive use of antibiotics, which is a common practice in animal production, can cause harmful drug resistant bacteria (Lacroix et al., 1996; Poole, 2001; Soto et al., 2003).

An interesting alternative to standard disease control methods would be selective breeding to increase disease resistance in livestock. Genetic resistance to disease involves many facets of the body's defence system and interactions, and is complex. Production traits have negative correlation to disease resistance traits. In addition, disease resistant traits have low heritability, which makes it difficult to use conventional breeding. New opportunities to improve our understanding of the genetic nature of disease resistance have been produced through recent advances in molecular biology, gene mapping, and immunology which makes selection for disease resistance possible. Testing and selection for disease resistance and improved immune responsiveness require knowledge of the genetic correlations between disease resistance and immune responsiveness, and production traits.

Some groups of South African native chickens have survived several disease outbreaks, and are considered to be resistant. Disease resistance traits are related to immunogenetics, in particular, the major histocompatibility complex (MHC) class I and class II molecules (Boonyanuwat et al., 2006). Little research has been put into indigenous South African chickens therefore there is a minimal amount of information on the genetic structure of these populations. Changes in agricultural policies have tried to remedy this by emphasising small-scale production and household food security leading to a greater interest in the roles these chickens play. The indigenous chicken in the developing world has never been subjected to purposive selective breeding for any particular trait. Instead they have been subjected to natural

selection imposed by endemic diseases, climate, nutrition and other stresses. This has created diversity in plumage type and colour, productivity, body size and disease resistance. This immense biodiversity has ensured their survival in diverse ecological zones by naturally being selected for survival fitness. It is well adapted to these conditions such that the size and productivity level match with the environment and feed resources available. Therefore the indigenous chicken populations, probably have a pool of all possible genes found in *Gallus gallus domesticus* unlike the pure breeds and hybrids which have a limited but selected number of economically important genes. There is a general thinking that the rural chicken is resistant to diseases. This thought is founded in the sense that the rural chicken survives in a harsh environment with a host of pathogens and without any veterinary intervention. Against this background, the study was conducted to unpack the genetic basis of disease resistance in South African indigenous chickens.

1.1 Problem identification

Research projects have been directed primarily towards commercial production systems. The indigenous chicken in the developing world has never been subjected to purposive selective breeding for any particular trait. This is due to the genetic resources that these indigenous possess remain unknown as there has been no evaluation of the disease resistance genes in these chickens in South Africa. Instead they have been subjected to natural selection imposed by endemic diseases, climate, nutrition and other stresses. The findings allow for the conservation of these haplotypes for future breeding purposes for better productivity and disease resistance.

Every year approximately 155 000 human deaths occur due to gastroenteritis caused by *Salmonella* infections (Majowicz et al., 2010). It is estimated that 80.3 million of 93.8 million food poisoning cases are due to food borne infection (Majowicz et al., 2010). This poses a large concern in developing as well as developed countries and has resulted in the increased usage of antibiotics. The close proximity in which commercial chickens are housed adds to their susceptibility to disease and decline in health. Antibiotics are generally used for prevention bacterial infections and are also used to promote growth (McEwen and Fedorka-Cray, 2002). Many poultry farms practice metaphylaxis which is the process of mass medicating entire flocks to treat sick individuals while preventing infection of healthy individuals at the same time and it is this over usage of antibiotics that has resulted in the increased resistance of

bacteria to antibiotics (Low et al., 1997). This is due to the antibiotics allowing for selection to take place.

Chickens originated from Asia and from then on different chicken breeds have emerged in different regions. Chickens from a certain region are referred to as indigenous chickens and these are the breeds which are usually used by small holder farmers. These free range chickens are allowed to roam around during the day and are not fed any special feed or medication, however, these chickens are not affected by the same ailments that commercial broilers suffer.

Due to antibiotics loss in popularity due to a number of reasons, other means of preventing losses due to disease are being investigated. One of the disease prevention plans is breeding chickens which are genetically resistant to disease. In the long term, having chicken flocks that are genetically less susceptible disease will serve to be more affordable to farmers and smallholders as the chickens will not require constant monitoring and medication. Indigenous chickens kept under smallholder-low input systems are considered important genetic resources that should be conserved against production threats and replacement with commercial hybrids (Muchadeyi et al., 2005; Muchadeyi et al., 2007; Mtileni et al., 2011). In African countries, indigenous chickens are raised by smallholder farmers with little resources. Characterization of these genetic resources will serve as an essential prerequisite for the identification and effective management and utilization of South African indigenous chickens, which will facilitate their conservation. To accomplish this, genes that affect immune response have to be analysed and one of the most important factors in immune response is the major histocompatibility complex. Candidate genes that have been identified in having an association with disease susceptibility and could also play a role in breeding for disease resistance (Kramer et al., 2003).

In the current study the genetic variation in indigenous chicken populations and one commercial population are investigated. It was hypothesized that the indigenous chicken populations will have a moderate to high genetic diversity in the innate and adaptive immunity genes. It was also hypothesised that the indigenous chickens would be more genetically diverse for these genes than their commercial counterparts due to natural selection occurring to acclimate the indigenous chickens to the natural environment.

1.2 Research Aims and Objectives

The objectives of this study were to:

1. Detect SNP's through genotyping the candidate genes that are responsible for resistance to several diseases in chickens and to examine the genetic diversity of these genes.
2. Examine the genetic variation of the adaptive immune system in South African indigenous chickens using the LEI0258 marker.

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Chapter 2: Literature Review

2.1 Introduction

In this literature review I will be briefly covering the origin of the different *Gallus gallus domesticus* breeds that are now available today and how we are in danger of losing some of these breeds due to various reasons. Previous research that has been carried out on the relationship between the chicken Major Histocompatibility Complex and disease resistance and susceptibility will be reviewed as well as the candidate genes (inducible nitric oxide synthase, solute carrier family 1, toll like receptor 4 and myeloid differentiation protein 2 genes). There are reports of marker assisted selection being successful in the farming industry (Muhammad et al., 2008) and to do this we need to assess the genetic variability of these genes hence this current study. The methods used to detect and gage genetic variability will be reviewed along with similar studies. There are various studies on the genetic diversity of commercial chickens and a few indigenous chickens worldwide however there is a gap in this research in Indigenous chickens is South Africa.

2.2 The History of *Gallus gallus* and *Gallus gallus domesticus* breeds

Gallus gallus or the Red Jungle fowl is the common ancestor to all the poultry breeds that we have available to us today due to domestication which is said to have occurred as early as 3 200 BC (Zeuner, 1963). The Red jungle fowl was originally from Southeast Asia. These birds were then disseminated all over the world which is where various processes led to the divarication into different breeds. Once domestication in the different countries had occurred, the birds then underwent selective breeding programmes, mutations as well as adaption to the environment which is what has brought to over sixty chicken breeds (Stevens, 1991; Peterson and Brisbin, 1998) which can be separated depending on the birds uses such as birds used for meat, egg layers, game type or bantam type (Moiseyeva et al., 2003). As such, there has been a decrease in the number of breeds being used as the chickens being bred in mass has been limited to layers, broilers and turkey (Hoffmann, 2009). As there are only a few breeds being utilized, no attention is being paid to the breeding of the non-commercial breeds and this is causing an increase in the loss of genetic diversity (Frankham, 1995). There are 938 avian breeds of the chicken, duck, Muscovy duck, goose and turkey species and half of these have been classified as being at risk hence the need for the conservation of indigenous breeds (Weigend and Romanov, 2001). This is occurring in South

Africa where chickens are an important and common form of livestock for smallholder and commercial farmers (Ahlers et al., 2009). There should be great importance placed on assessing the genetic diversity of the indigenous breeds in order for their conservation as well as the use of this information for breeding programmes. Against this background, this study aimed to explore genetic diversity of indigenous chicken breeds in South Africa with a special focus on genes related to disease resistance.

2.3 Tools used to assess genetic diversity

Molecular markers are used to assess genetic diversity in organisms and there are many DNA technologies or methods that have been developed to detect these markers. These methods include Polymer Chain Reaction (PCR), Sequencing, the Random Amplified Polymorphic DNA (RAPD) method and the Restriction Fragment Length Polymorphism (RFLP). All these methods are each suited to certain applications with advantages and disadvantages.

2.3.1 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) is one of the methods used to assess the genetic variability available in populations. Welsh and McClelland (1990) describe RAPD as a simple and rapid method able to offer detailed fingerprinting of the genomic composition of the organism (Welsh and McClelland, 1990). This technique was developed in 1990 and similar to other methods, it makes use of primers of lengths 8-12bp that are used in one PCR reaction. Since the primers anneal randomly there is no need for prior knowledge of the genome. These primers are random sequences which means that when amplification occurs, the primers amplify both coding and non-coding regions situated all over the genome (Lynch and Milligan, 1994).

Zhang et al. (2002) reported that the RAPD method resulted in a 43 polymorphic band profile. However, only three different polymorphisms were found (Zhang et al., 2002). This showcases one of the advantages of using RAPD as this method is able to sample different loci within the genome more randomly than other methods. There are however disadvantages of using RAPD. One being that the molecular weight of the PCR product from one locus could be very similar to the molecular weight in another locus. Another issue is that from an agarose gel it cannot be determined whether an individual is a homozygote or a heterozygote (Sharma et al., 2001). This disadvantage is due to the fact that in RAPD, when a locus has two copies of an allele then amplification occurs and this shows up on an agarose

gel. When the locus has one copy of allele, then no amplification occurs therefore, no band is visualized on a an agarose gel and this is the reason why it is not beneficial to use this method in a candidate gene application because knowledge of specific polymorphisms, whether homozygotes or heterozygotes, is required to assign their respective chickens genotypes which may have an effect on the resistance or susceptibility to disease. Another issue is that the results from a RAPD profile are not reproducible.

2.3.2 Restriction Fragment Length Polymorphism

Unlike RAPD, the PCR-RFLP method does require knowledge of the sequence in order to design the correct set of primers. The running cost of RFLP is more expensive but more precise genotypes are produced. For this study RFLP was the preferred method for the genotyping of the candidate genes. This is because we were looking at specific single nucleotide polymorphisms that are causing changes in the immune response to infections in chickens rather than the general genetic diversity or structure which would be more suited for RAPD. Jin et al. (2010) used the RFLP technique to determine the haplotype that each of the Marek's disease infected chickens belonged to. The study required exact understanding of which of the chickens were homozygous or heterozygous for the BLB2/BF2 gene in order to assign the MHC haplotypes and make the correct comparisons to previously reported haplotypes (Jin et al., 2010).

2.4 Microsatellite markers

Microsatellite markers are highly polymorphic stretches of base pair repeats that are within all the known organisms (Epplen et al., 1993). The number of repeats may vary from 1 bp (mononucleotide), 2 bp (dinucleotide) to 4 bp (tetranucleotide) repeats which is what results in the abundant genetic variability in these molecular markers (Weber and May, 1989). These markers are distributed throughout the genome and can be found within coding regions as well as in non-coding regions. At the moment there are approximately 651 microsatellite markers that have been mapped in the chicken genome (McConnell et al., 1999). The bigger the nucleotide repeats the easier it is to score and visualize the alleles (Santos et al., 1993). The highly polymorphic nature of microsatellites is the feature that allows them to be used in evolution and genetic variation studies. Minisatellites are similar to microsatellites in that they are they are also variable number tandem repeats (Tautz, 1993). They differ from microsatellites in that they are composed of 15-100 bp motifs and these repeats can span a length of several kilobases (Weigend and Romanov, 2001).

Microsatellites were thought to be merely evolutionary neutral DNA markers (Schlötterer and Wiehe, 1999) and have no use biologically. As more microsatellites are discovered and more association studies are being conducted it is becoming clear that some these microsatellites have functional roles even though it is rare to find them in coding regions (Metzgar et al., 2000; Morgante et al., 2002). An example is a species of Japanese puffer fish, *Fugu rubtripes*, where of the 6042 microsatellites in the entire genome, only 11.6% were present in protein coding regions and this seems to be a common recurrence not only in plants across mammals such as primates as well as smaller organisms such as *Saccharomyces cerevisae* (Edwards et al., 1991; Metzgar et al., 2000; Morgante et al., 2002). However there are studies that support that microsatellites do have a function in eukaryotes such as aiding in the packaging of eukaryotic chromosomes (Stallings et al., 1991). Previously microsatellites were thought to follow the stepwise mutation model of Ohta and Kimura (SMM). However upon studying microsatellites over many generations it was discovered that the infinite allele model (IAM) (Kimura and Crow, 1964) was more suited to this type of marker as it does not evolve purely on SMM however this does vary from marker to marker. Microsatellites with a larger repeat motif generally follow the SMM however when it comes to the 1 or 2bp repeat motifs, they tend to mutate according to the IAM (Shriver et al., 1993; Di Rienzo et al., 1994). The IAM model states that a mutation results in a new allele which is not currently in the population, leading an infinite amount of alleles as seen in most VNTR cases (Shriver et al., 1993).

2.5 The Avian Immune system

The immune system can be split up into two mechanisms: innate immune response and adaptive immune response. The innate immune response is the dominant immune response to infection and is the first line of defence when the host is under threat. This type of immunity is often referred to as ‘inborn’ or ‘genetic’ factor of the immune system due the innate immunity system using defence mechanisms that can be available immediately or within hours of infection such as physical barrier (skin) or cellular components (cytokines and macrophages) (Figure 2.1) (Culty et al., 1994). Innate immunity is specific to pathogens by recognizing pathogen associated molecular patterns (PAMPs) and then trying to resist infection.

Adaptive immune response uses more complex mechanisms in fighting infection and this is why it takes a substantially longer period to be activated than the innate immune system. Instead of using 'ín born' mechanisms, the adaptive immune response adapts to the infection to combat it and build up a resistance to it by being antigen specific (Ohshima and Hiramatsu, 2000).

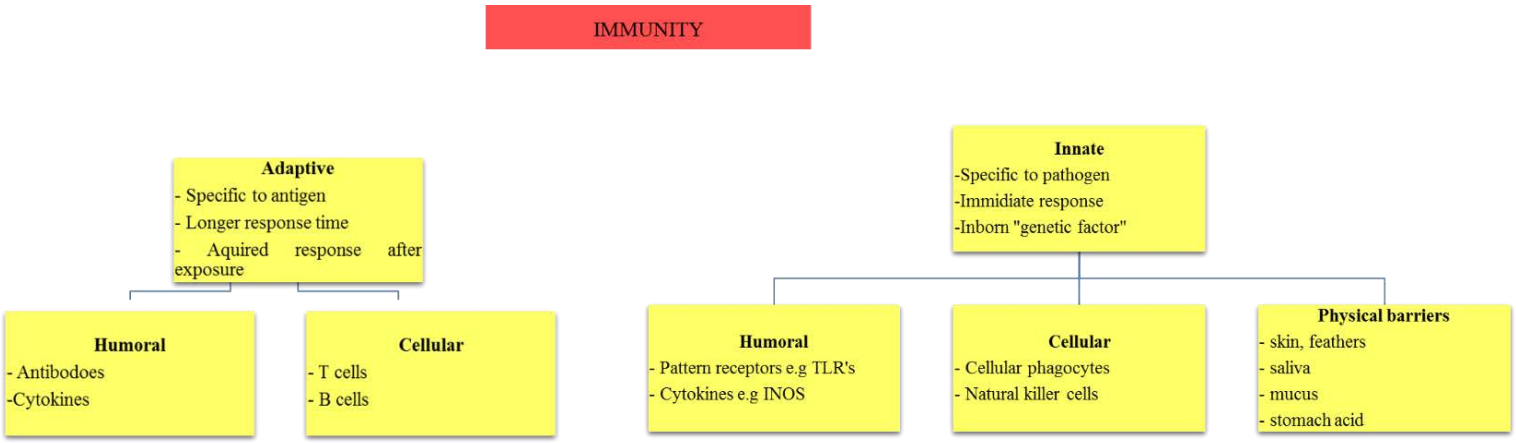

Figure 2.1: The Adaptive and Innate response mechanisms.

The Salmonella and candidate genes association studies generally gage the chicken's severity of infection by inspecting bacterial load in the caecum or more commonly bacterial load in the spleen. The bacteria can enter the chickens' gastrointestinal tract through the M cells before being engulfed by macrophages (Cuellar-Mata et al., 2002). Once the process of phagocytosis has occurred the macrophages carry the contents to the spleen and it is in this process that some bacteria are able replicate within the macrophages (Cuellar-Mata et al., 2002). This in conjunction with dendritic cells (antigen presenting cells) carrying bacteria to the spleen (Rescigno et al., 2001) are the reason why the spleen all round indicator of the chickens response to infection.

In poultry, the lung is most likely to become infected by viruses or bacteria. To combat this issue, avian species have developed a highly organized lung associated immune system which is unique to the avian species (Reese et al., 2006). Within the lung structure is the bronchus associated lymphoid tissue (BALT). In a matured chickens, this structure harbours a considerable number of lymphocytes which also play a role in dealing with infection as a part of the innate immune system (Fagerland and Arp, 1993) (Figure 2.1) . Macrophages and dendritic cells which are also involved in innate immune response as well T cell zones and B cell follicles which are involved in adaptive immune response, were found throughout the BALT (Fagerland and Arp, 1992; Fagerland and Arp, 1993).

2.6 Chicken Major Histocompatibility Complex haplotypes and their effect on disease susceptibility

Over the years, more robust methods for identifying quantitative trait loci (QTL) have evolved (Hocking, 2005). These are areas of DNA that are linked to DNA that controls quantitative traits.

The chicken major histocompatibility complex (MHC), located on microchromosome 16 is the first line of defence against infection by pathogens (Izadi et al., 2011). In chickens, the MHC is referred to as the B complex and has been described as a highly polymorphic erythrocyte antigen or blood group system (Briles et al., 1950). The chicken B complex is very compact as it is only about 30 to 100kb long compared to the 4Mbp MHC in humans (Kaufman and Wallny, 1996). The avian B complex is separated into the BF/BL (class I/class II) (Figure 2.2) regions which encode for proteins involved in communication upon infection (Delany et al., 2009) as well as the BG region (class IV). This region codes for erythrocyte antigens (Delany et al., 2009). The proteins which are encoded for by different regions of the MHC facilitate the cellular communication which occurs in immune response indicate the importance of the chicken MHC in disease resistance and susceptibility.

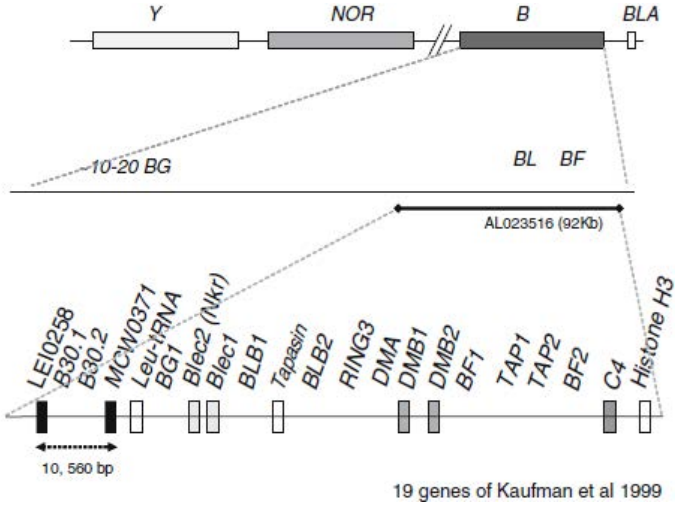

Figure 2.2: A map of the chicken major histocompatibility complex showing the position of the LEI0258 and MCW0371 markers (Fulton et al., 2006).

The expression level of cell surface class I MHC molecules varies among different MHC haplotypes in chickens whereas in humans the expression level of cell surface class I MHC molecules is fairly consistent among the different haplotypes (Kaufman, 2000).

With the increase and improvement of molecular diagnostic techniques, the development of animals with genetic resistance is possible. It is now possible to map genetic regions within the MHC with certain traits such as immune response to various diseases like Marek's disease (Briles et al., 1977a), Fowl cholera (Lamont et al., 1987) Avian leucosis virus (Yoo and Sheldon, 1992) and Avian infectious bronchitis amongst others.

The LEI0258 and MCW0371 are the markers of choice in determining MHC allelic variation in chicken populations. These markers were identified in 1999 and were first described in chicken populations (McConnell et al., 1999; Fulton et al., 2006). As these are well studied markers, they have been used by other scientists for genetic diversity studies in different chicken populations as seen in a study based on indigenous chickens (*Gallus gallus domesticus*) in Kenya was conducted in order to determine the genetic diversity with the chicken MHC using LEI0258 and MCW0371 (Ngeno et al., 2014). Out of the 15 MHC LEI0258 haplotypes reported in Fulton et al. (2006), only 3 (B2, BW3 and B15) LEI0258 haplotypes from the Kenyan population corresponded with these while the remaining 12 have not been reported in any other studies (Ngeno et al., 2014).

The LEI0258 microsatellite marker which is situated in the non-coding region in the MHC between the B-F/B-L and BG loci (Figure 2.2) (Fulton et al., 2006). LEI0258 is variable number tandem repeat made up of 12 and 13bp conserved repeats and there are currently 26 alleles (Fulton et al., 2006). These two microsatellites have been used extensively to determine the amount of allelic diversity within the chicken MHC. There have been associations that have been made between this marker and susceptibility to infections caused by microorganisms such as *Salmonella enteritidis*, *Pasteurella multocida* and the parasitic worm *Ascaridia galli*. Schou et al. (2010) report on how the indigenous Vietnamese Ri chicken and commercial Luong Phuong chicken lines were infected with *Salmonella enteritidis*, *Pasteurella multocida* and the parasitic worm *Ascaridia galli.* The group chickens with LEI0258 allele sizes of 195 bp, 207 bp, 219 bp and 294 bp represented the group that was found to have increased immune response when infected with *S. enteritidis*. Similar findings have also been presented in studies by (Liu et al. 2002; Zhou and Lamont 2003) where there were correlations between MHC haplotypes and antibody response to *S. enteritidis* (Zhou and Lamont, 2003). Increased antibody response and decreased cecum bacterial load in response to *S. enteritidis* infection was based on the chickens genetic potential (Setta et al., 2012). Therefore it is possible that certain MHC haplotypes that are associated with high antibody response can be less susceptible to infection (Liu et al., 2002). The antibody response to *S. Enteritidis* infection was higher in the indigenous

Ri chicken compared to antibody response in the commercial chickens (Schou et al., 2010). In another study, the BESS-T method, which is used to detect point mutations, repeats, deletions and insertions (Hawkins and Hoffman, 1997) was used revealed an A to T substitution of the coding MHC class I α_2 region which is highly involved in peptide binding therefore it was hypothesized that this might change the 3D protein structure and hence its ability to bind as well as to present antigens (Liu et al., 2002). This substitution resulted in an amino acid change from Lysine^{148} to Methionine^{148} which in turn changed a polar side chain into a non-polar side chain (Liu et al., 2002). The chickens used the latter study were of the Leghorn breed, Fayoumi and Spanish lines. Four outbred broiler breeder sires were mated to three to six dams of three inbred. The authors were able find that the Lysine^{148} to Methionine^{148} polymorphism was associated with the spleen bacterial load and also that individuals with the A allele seemed to be more susceptible to *S. enteritidis* bacterial colonization in the spleen (Liu et al., 2002) adding to the hypothesis of the genetic link between disease susceptibility and the chickens haplotype.

2.7 MHC and viruses

2.7.1 MHC haplotypes and resistance to Marek's disease

In addition to the immune defence role the MHC plays in defending against bacteria and parasites, the MHC is also a defence mechanism against viral infections. The relationship between Marek's disease virus (MDV) and chicken MHC is becoming more and more understood due to the amount of research based on this topic (Yonash et al., 1999; Hunt et al., 2001). Marek's disease is a virus that is responsible for a substantial economic loss each year in the poultry industry in the US alone (Yonash et al., 1999). The initial infection stage the T lymphocytes carry the MDV genome through the blood stream to further infecting organs, nerves and feather follicles (Baigent et al., 2006).This viral infection results in tumours throughout various tissues in the body and eventually death (Briles et al., 1977a). The virus can then be disseminated through entire flocks through contact with infected individuals, droppings and feathers.

What also has to be considered is that at times different genes may work together in order to fight off infection or correct the symptoms. For example, the expression of MHC class

I was discovered to be decreased in chickens infected with MDV (Levy et al., 2003). Upon further investigation it was observed that the up regulation of the interferon gene (IFN) resulted in the alleviation of MD effects (Levy et al., 2003). There is agreement in different literature on haplotypes that have been identified as highly susceptible to MDV infection such as the B19 haplotype while the B21 haplotype is highly resistant (Briles et al., 1977b; Bacon et al., 2001). Koch et al. (2007) did research on the B21 haplotype and found that the B21 haplotype has an MHC Class I molecule that rearranges its binding site in order to detect a more diverse array of peptides from viruses. This is what is thought to be the cause for the haplotype's heightened resistance to MD (Koch et al., 2007).

2.7.2 MHC haplotypes and resistance to Avian Coronavirus (Infectious bronchitis virus)

Unlike the other diseases mentioned previously, Infectious bronchitis virus (IBV) is a non-zoonotic virus therefore it does not pose the threat of being passed onto human through transmission by ingestion of chicken meat (Jackwood, 2006). However IBV infections in are a large issue in the poultry industry (Williams et al., 1992; Cavanagh et al., 2002). This 27.6kb single stranded RNA virus was first described in the 1930s and is to be passed easily and efficiently among animals by aerosol or through contact with an infected chicken's faeces (Cavanagh and Naqi, 2003). IBV can be a devastating virus to an animal as besides the infecting the respiratory tract, the virus is also able to replicate efficiently in other tissues such as kidneys as well as reproductive tracts which can all lead to several complications (Cavanagh and Naqi, 2003). These complications can range from bronchitis to hepatitis, nephritis, encephalitis amongst others (Cavanagh, 2007). There are vaccines that have been developed in order to combat the spreading of IBV infections however the virus is forever evolving and giving rise to various genetically different strains and although the differences rendering some of the IBV vaccines ineffective (Figure 2.3) (Cavanagh and Naqi, 2003). The presence of several phenotypes makes cross-protection difficult.

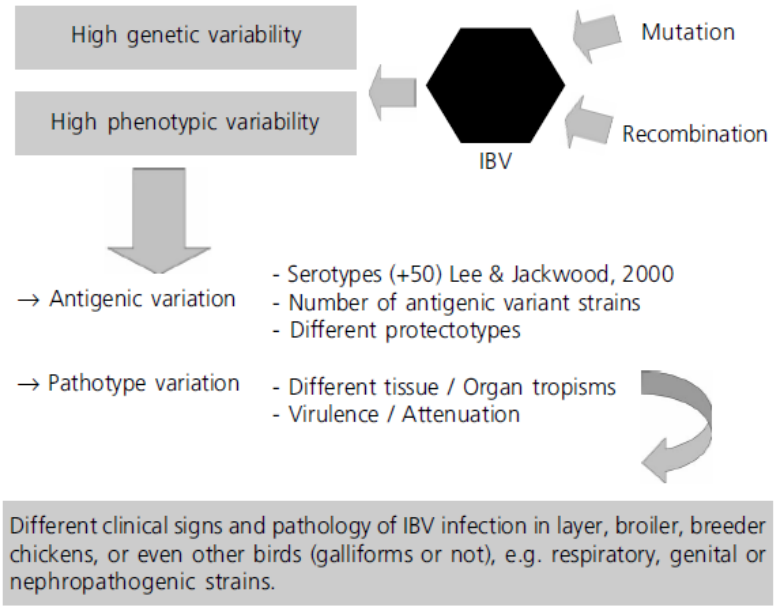

Figure 2.3: Image depicting the mechanisms that IBV undergoes in order to evolve phenotypically and genetically (Montassier, 2010).

The virus's ability to genetically evolve is linked to the surface spike glycoprotein gene which is responsible for antigenic changes such as point mutations, deletions, insertions and recombination (Cavanagh and Naqi, 2003). This gene codes for the spike glycoprotein (S) which is cleaved into subunits S1 which is 92-kDa and S2 which is 84-kDa (Cavanagh, 1983). These two subunits are not cleaved in a mature virion and anchor each other to the virion membrane (Cavanagh, 2007). The main functions of this protein are to attach the virus to host cell receptors as well as to facilitate the fusion of the virion and host cell membranes to allow for the viral genome to be inserted into the host cell (Cavanagh, 2007). Although the S glycoproteins of different isolates of IBV only differ by 2 to 6 % at amino acid level (Williams et al., 1992), Cavanagh and colleagues found that it does not take a large amount of S protein differences to have a damaging effect on cross-protection (Cavanagh and Naqi, 2003).

Bacon et al. (2004) report on 1-day old broiler and layer chicks from B2, B13, B21 and B15 MHC haplotypes being mistakenly vaccinated with an IBV vaccine that is intended for 4 week old chicks which have been previously vaccinated and this resulted in mortality of the chicks. When this mistake was discovered, chicks from hatches 3 and 4 were vaccinated with the more appropriate vaccine B which resulted in reduced chick mortality. Results demonstrated that of the chicks which were vaccinated with the lowly attenuated IB vaccine (hatches 1 and 2) only

12% of the B15 haplotype, which have a LEI0258 allele size of 261bp, died after vaccination whereas haplotypes 7_1, B13 and B21 (205bp and 357bp) had mortality rates of 67%, 47% and 48% respectively (Bacon et al., 2004). These mortality rates occurred repeatedly in correspondence to the MHC haplotypes. Chicks with the B15 haplotype lived longer than the other haplotypes however when ANOVA was performed it revealed that the days to death were not significantly different among the different lines used in the study (Bacon et al., 2004). The chicks which were properly vaccinated with the highly attenuated vaccine were able to have higher survival rates compared the chicks from hatches 1 and 2. These findings indicate that the B15 haplotype chicks were more resistant to the development of IBV especially when compared to the 7_1 chicken line (Bacon et al., 2004). This is in agreement with findings from (Bumstead et al., 1989). It was concluded that the B15 haplotype is the more resistant haplotype when it comes to IBV there would be issues in selecting for solely the B15 allele alone. As Figure 2.3 shows, the IB virus is forever undergoing recombination and giving rise to new strains of the virus which perhaps the B15 haplotype would be susceptible to. However these findings did give insight on the relationship between the chicken B-complex haplotypes and IBV severity.

Although most of these studies focus on individuals that are homozygous at the LEI0258 locus, there have also been cases of what seems as dominance of specific LEI0258 alleles over others in IBV infected heterozygous chickens. Chickens of the B2, B5, B8, B12, B19 and B2/B12 (261 bp, 295 bp, 405 bp, 487 bp, 539 bp and 261/539 bp) haplotypes were vaccinated with the Gray IBV strain and thereafter were monitored for signs of illness (Banat et al., 2013).

Figure 2.4 shows how from early on, the chickens with the B12 and B19 haplotypes showed the highest degree of illness than the others. The B2 and B5 haplotypes showed less signs of illness. The haplotype B2/B12 which was a combination of the more susceptible haplotype (B12) and less susceptible haplotype (B2) resulted in what seems to be a haplotype which is less susceptible to IBV infection (Banat et al., 2013). This is what led to the suggestion that the B2 allele is dominant over the other allele. This was reflected in another study on IBV infection where the observed illness in B2/B15 and B2/B21 birds was not significantly different from one another (Joiner et al., 2007). It was concluded that the B2, B5 and B8 haplotypes had potential to decrease IB severity as well as other repertory pathogens.

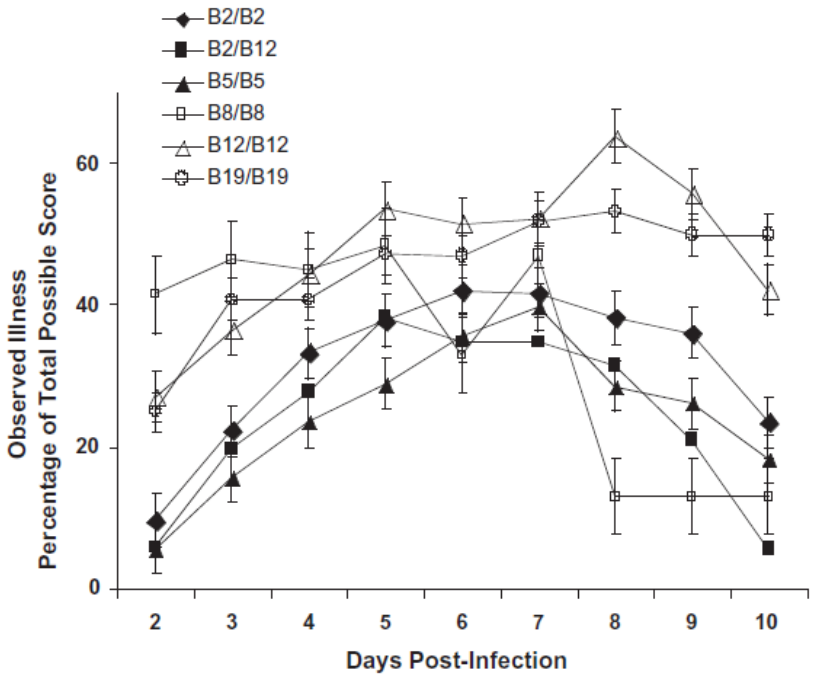

Figure 2.4: Observed illness in chickens of different B haplotypes, following vaccination with IBV (Banat et al., 2013).

2.7.3 MHC haplotypes and Newcastle Disease

Newcastle disease virus (NDV) is another disease that can be crippling to the poultry farming industry due to its severity. It is estimated that at least 60% of birds infected with the disease can die from the symptoms (Alexander, 2001). The symptoms of this disease include twisting of the neck, sneezing, diarrhoea and a complete drop in egg production. The traditional approach for prevention of this disease has been to vaccinate the chickens but these prevention methods can be improved even further by including MHC genetic information in the selection of chickens for breeding stocks. Due to the MHC's large role in immunity, there are studies that have investigated the relationships between various MHC haplotypes and Newcastle disease. There are various methods that can be used to do this and usually the gene mRNA expression of the NDV infected chickens is measured to identify changes in expression in different haplotypes. The other method that is commonly used in immunity is to measure the antigen specific T cell proliferation using flow cytometry (Norup et al., 2011). An example of the latter is a study where inbred MHC chickens of the B12, B13, B130 and B201 haplotypes were vaccinated with a strain of the NDV and thereafter had the antigen specific T cell, namely CD4 and CD8, proliferation was measured. These haplotypes refer to individuals with the

following LEI0258 allele sizes respectively: 487bp, 205bp, 357bp and 357bp (Fulton et al., 2006).

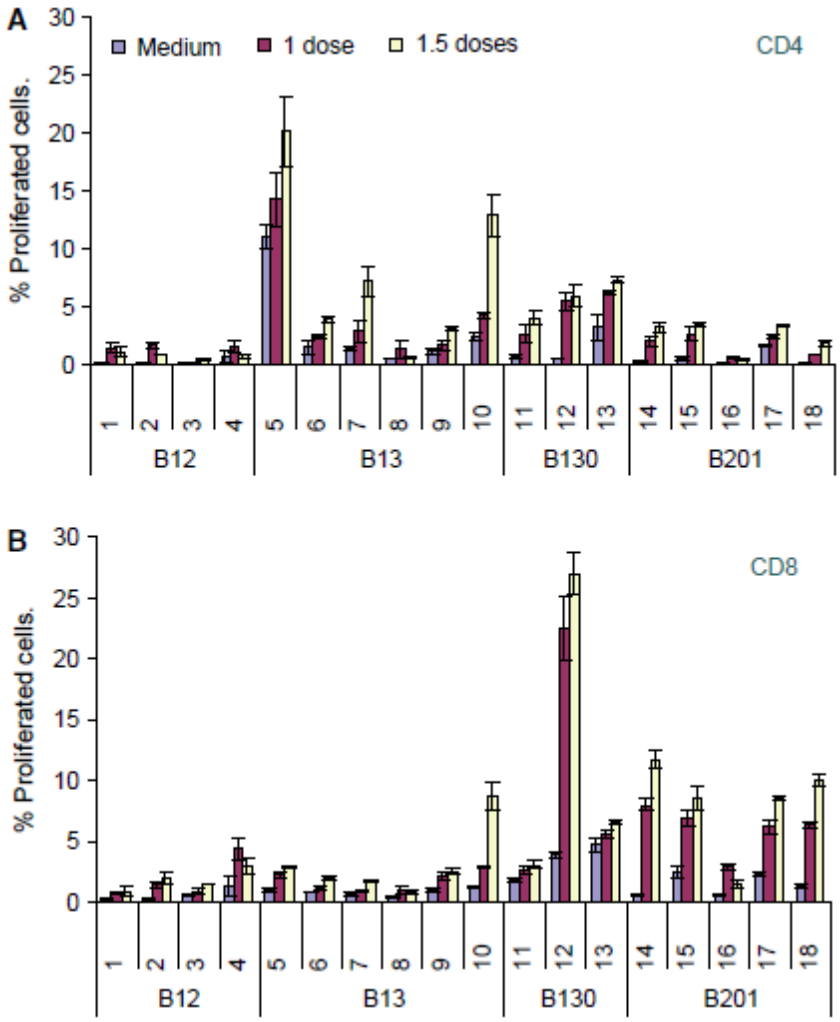

Figure 2.5: Percentages of proliferated T cells of chickens from different MHC haplotypes that have been vaccinated with an NDV strain (Norup et al., 2011).

Of the four MHC haplotypes that had been tested the B130 was the only one that had a positive association with T cell proliferation. Figure 2.5 shows how proliferation in both the CD4 cells and CD8 cells in response to the NDV vaccine was quite good. On the opposite spectrum the B12 individuals had very low T cell proliferation in both cases. The individuals with the B13 haplotype had good CD4 proliferation while it was poor for CD8. On the other

hand the B201 haplotype showed good CD8 proliferation while poor for CD4. What must be taken into account is that although general trends could be observed, there is quite some variation within each haplotype. Norup et al. (2011) attributed this differences to the large period of time between NDV vaccination and the testing of the chickens.

In Tanzania, NDV specific antibodies using the haemagglutination inhibition test were detected (Allan and Gough, 1974). Of all the most frequent LEI0258 alleles from the two sampled populations only the 307bp and 205bp alleles showed significance to primary antibody response against the NDV vaccine that had been administered to the chickens (Lwelamira et al., 2008). Individuals with the 205bp allele had increased primary antibody responses while the 307bp allele was significantly associated with low primary antibody responses. The association in this study on the Tanzanian population partially correlates with the findings by Norup et al. (2011) where individuals with the 205bp allele had increased immune response to NDV in comparison with other MHC haplotypes (Norup et al., 2011). In the Tanzanian population the 205bp allele was among the most frequently detected alleles. This may be seen as evidence of the allele being beneficial to the chickens as in randomly mating populations, the process of natural selection increases the frequency of alleles that are beneficial to an individuals' survival in a specific environment (Jeffery et al., 2000; Verrelli et al., 2002). The same result in two vastly distant and different environments is also a good indicator that the 205bp allele is a good candidate for MAS for the resistance or decreased susceptibility against Newcastle disease.

2.8 Genetic Diversity of the Major Histocompatibility Complex using the LEI0258 marker

In addition to association studies it is also important to assess the genetic diversity in the chicken MHC in the various populations in different environments. The LEI0258 alleles that were reported by Fulton et al. (2006) have been found in other chicken populations around there world and there have been even more alleles that have been discovered (Banat et al., 2013; Chazara et al., 2013; Guangxin et al., 2014; Ncube et al., 2014; Ngeno et al., 2014). The number of LEI0258 varies from 22 to 79 which several reported private alleles. Genetic diversity is usually analysed using population genetics. This involves the modelling and examination of the changes of allele and genotype frequencies within populations over time, this typically involves using statistical software in order to compute the genetic information that is required to assess the diversity in a population. The criteria that has generally been used

in most MHC diversity literature include the observed mean number of alleles (Na), effective mean number of alleles (Ne), fixation index, within population inbreeding coefficient (F_{IS}), total inbreeding (F_{IT}), and among population genetic differentiation (F_{ST}). In addition to this observed heterozygosity (Ho), expected heterozygosity would be computed alongside with checking if the populations abided by guidelines to be in Hardy-Weinberg equilibrium.

Previously it has been reported that there were 26 alleles for the LEI0258 marker (Fulton et al., 2006). However with more studies in different locations there has been an increase in number of alleles being detected. In a study where the genetic diversity in Kenyan indigenous chickens from 8 regions was assessed, there were LEI0258 56 alleles which is more than double the number previously reported (Ngeno et al., 2014). A number of the chickens in the Fulton et al. (2006) study were commercial type chickens and the greater number of alleles in the Kenyan chickens may be due to the hypothesis that indigenous chickens are more exposed to harsh conditions (environment, diseases) than their commercial counterparts hence they have higher genetic variation in immune response genes in order to ward off a greater variety of diseases (Izadi et al., 2011) . Vietnamese chicken populations only had 19 alleles were detected (Schou et al., 2007) In Tanzania 22 and 23 alleles were found for Kuchi and Medium ecotypes respectively (Lwelamira et al., 2008), while in Cameroon 42 LEI0258 were found in the indigenous chicken population (Touko et al.,2015). These studies on Chinese chicken breeds yielded 22 alleles (Izadi et al., 2011) which is the same number as the alleles found in a study on South African village chickens (Ncube et al., 2014). Although the Chinese, Tanzanian and South African populations had the same number of LEI0258 alleles, South Africa did not share any alleles with the other two countries. On the other hand the Chinese chicken breeds had 11 of the same alleles that were found in the Tanzanian indigenous chicken populations. Although it seems as though the South African population had a lot of novel alleles however results from the study indicated a degree of inbreeding at the MHC locus as the allele and heterozygosity values were quite low (Ncube et al., 2014). The Kenyan ecotypes on the other hand had very high heterozygosity which was an indicator of the populations being outbred and authors attributed this to the populations' geographical isolation from each other and variation in ancestors (Ngeno et al., 2014).

There is also the bioinformatics approach that can be used to assess the genetic diversity in populations for the LEI0258 marker. After sequencing the LEI0258 markers in each chicken, the sequences can then be analysed using a wide array of bioinformatics programmes to find the differences and similarities at the sequence level. Chazara et al., (2013) used this approach

in very large study on the genetic diversity of the LEI0258 in 80 chicken populations that had been sampled from Africa, Asia and Europe. The Median-joining network (Figure 2.6) shows how the alleles that were detected were split into clusters A to L with L being the unique allele which was found in the Red Jungle Fowl. This reported Median-joining network took into account the allele size as well as the SNPs and indels present which is useful as two different alleles at the sequence level, may have the same allele size. Figure 2.6 also gives an idea of the relationships between alleles from around the world. Clusters D, E, F are composed of individuals that have no deletions in their MHC haplotypes while clusters G, H, I, J, K, is composed of individuals that 2 bp deletions in their sequences. Finally, clusters A, B, C, and L have 8 bp deletions. Interestingly, although the chickens are of different breeds and environments, 8 out of the 12 clusters are composed of chickens from all 3 continents which were sampled from. The observations made in this study agree with the suggestion that domestication events took place in Asia from the Red Jungle Fowl and that the LEI0258 diversity of domestic chickens has come from the ancestral Red Jungle Fowl (Liu et al., 2006; Chazara et al., 2013).

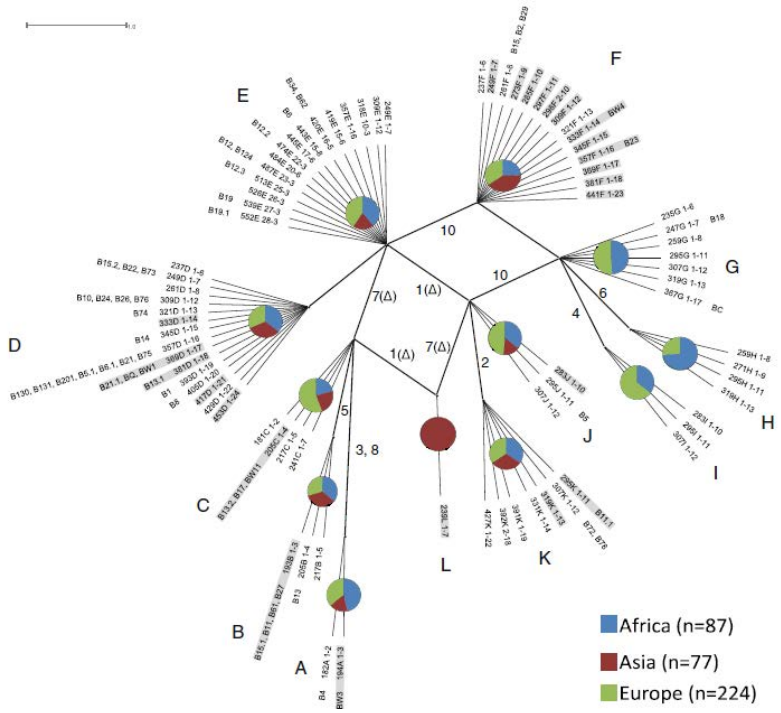

Figure 2.6: Median-joining network of the LEI0258 79 alleles that had been detected in 80 populations (Chazara et al., 2013).

2.9 Candidate genes for disease resistance in chickens

Poultry products (eggs and meat) are the main source of food poisoning (Hoelzer et al., 2011). One of the most common causes for Salmonellosis are *Salmonella enteritidis* (SE) is a gram negative rod-shaped bacteria which is part of the of the Salmonella family. This specific bacteria can become pathogenic and when infected poultry is ingested this can result in Salmonellosis which is a type of food poisoning (Velge et al., 2005). It is estimated that of the approximately 93.8 million salmonellosis cases that occur worldwide, 155 000 of those result in death (Majowicz et al., 2010). It is also reported that SE colonization from ingestion of contaminated poultry is the leading cause for food poisoning (Rabsch et al., 2001). The candidate gene approach has posed itself as possible alternative method to reduce SE infection rates as opposed to the tradition excessive use of antibiotics. This proposition has been repeated many including Vint et al. (1997), who reported that there is a real need for improving genetic resistance due to close-contact living conditions and large numbers of the birds and failure to successfully control SE populations in commercial operations (Vint, 1997). To achieve this, there are a number of undergoing studies where a number of candidate genes in which Single Nucleotide Polymorphisms (SNPs) can be found, are being exposed to SE infection to gage the differing degrees genetic resistance. Most of the candidate genes being studied are involved in the immunity and defence reactions that occur within the chicken.

2.9.1 Inducible Nitric oxide synthase (INOS)

Inducible nitric oxide synthase (INOS) is an enzyme has a host of immunological uses however on its main uses is to, with the aid NADH and tetrahydrobiopterin, convert L-arginine present in cells into nitric oxide. The production of nitric oxide which is cytotoxic to bacteria which could then reduce bacterial populations while also preventing bacterial translocation to other parts of the chicken using a various number of methods (Figure 2.7) (Berg, 1999). High INOS producing mutant mice had higher immune response than wild type mice (Wei et al., 1995). Studies on chicken macrophages have indicated that certain immunological stimuli activate the production of NO (Qureshi et al., 1996) It is also reported that after lab rats were challenged with *Eimeria separate*, INOS expression increased greatly (Shi et al., 2001). There is a paucity of studies on this candidate gene and SE however a study reveals that after 1 day old broiler chicks were exposed to SE, the expression of INOS had increased 298 fold by the 48 hour mark (Carvajal et al., 2008). The overexpression of this gene may be an indicator of role that INOS has in immune response to SE infections.

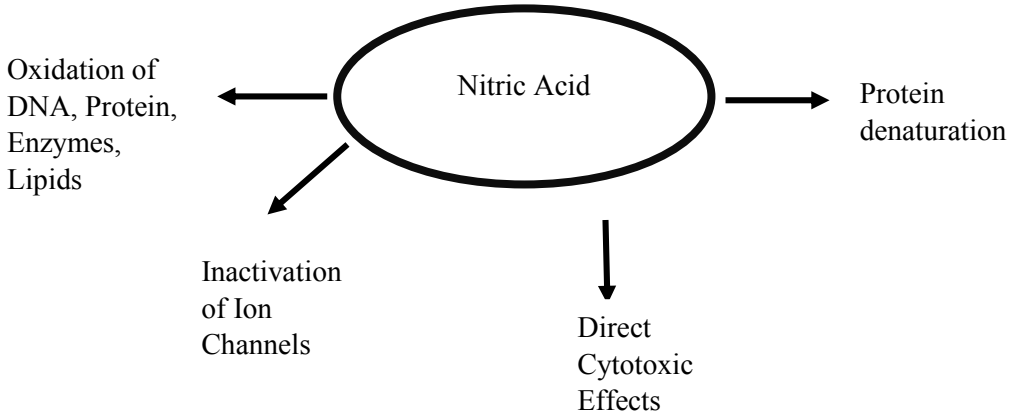

Figure 2.7: Image displaying different effects of Nitric Oxide on bacteria proliferation. Adapted from information from (Bredt et al., 1991; Marsden, 1995).

A recent study from Malaysia based on a C/T substitution in the intronic region of INOS in indigenous chickens findings demonstrated that individuals with the CC genotype for INOS had significantly high bacterial load in the cecum compared to the other two genotypes (Tamura et al., 2011). Tohidi et al. 2012 reported that the polymorphism in this gene was associated with SE load in the caecum significantly for this specific study (Tohidi et al., 2012). The INOS-*Alu I* polymorphism in a study on old Dutch breeds and broilers revealed that most of the chickens had the CC genotype followed by the CT genotype and only 3 individuals in the population had the TT genotype (Kramer et al., 2003). When the chickens were challenged with *S.enteritdis* there was an association between high caecum SE load and CC genotype in both groups of chickens ($p<0,001$). Tohidi et al., (2012) found that in a Malaysian population of indigenous chickens, for the INOS-*Alu* I locus the CT genotype was the most popular and it was in fact the TT genotype that was associated with low SE colonization numbers (Tohidi et al., 2012).

The expression of INOS has also been linked to specific MHC haplotypes (Hussain and Qureshi, 1998). This study was done on Cornell K-strain (B15), MQ-NCSU, GB1 (B13) and GB2 (B6) chickens. These chickens underwent LPS-stimulation and the INOS mRNA content in the macrophages from these chickens were analysed. After LPS exposure INOS mRNA expression was higher in the macrophages from the B15 and MQ-NSCU chickens whereas the

macrophages isolated from the B13 and B6 chickens had low INOS expression (Hussain and Qureshi, 1998). Another study on the B21, B13 and B19 haplotypes and the B21 chickens produced NO sooner and at higher levels than the B13 and B19 counterparts (Xing and Schat, 2000). Since there are interactions between these genes involved in immune response, it may be possible to breed for several diseases at once as some of these MHC haplotypes are associated with susceptibility to viruses.

2.9.2 Toll like receptors and myeloid differentiation protein 2

Currently there are 10 toll like receptors that have been identified in chickens (Temperley et al., 2008). The Toll like receptor 4 (TLR4) gene is located on microchromosome

E41W17 which was previously found to be a Salmonella susceptibility locus in the chicken genome (Hu et al., 1997). This gene codes for a 843 amino acid protein which is part of a group of highly conserved membrane associated receptors which recognize pathogen associated molecular patterns such as peptidoglycan, flagella and lipopolysaccharides (LPS) (Leveque et al., 2003). Hydrophobic as well as hydrophilic interactions are responsible for bringing together TLR4 and myeloid differential protein 2 (MD-2) in order to form a dimer (Figure 4.8) which is essential in structurally diverse LPS (Park et al., 2009). Lipopolysaccharides from gram negative bacteria such as *Salmonella* is reported to induce innate immune response in infected animals (Beutler and Rietschel, 2003). Once the bacteria enters the cell, LPS binding protein as well as the CD14 protein aid in the transferring of LPS to the TLR4-MD2 dimer (Bryant et al., 2010). It is this quality of TLR4 that makes it evident that this molecule has an important role in the immune system as it is able to detect infection by bacterial species and this is done by the receptors detecting pathogen associated molecular patterns (PAMPs) (He et al., 2006). Once PAMPs have been detected by TLR4, pro-inflammatory mediators such as nitric oxide and cytokines can be activated (Janeway Jr and Medzhitov, 2002). A G/C substitution in the intronic region of TLR4 has been selected as a point of interest in genetic resistance against SE. Some findings indicate that individuals with the CC variant of this polymorphism are more susceptible to SE infection and colonization as they had high *S. enteritidis* numbers in the chicken cecum (Tohidi et al., 2012).

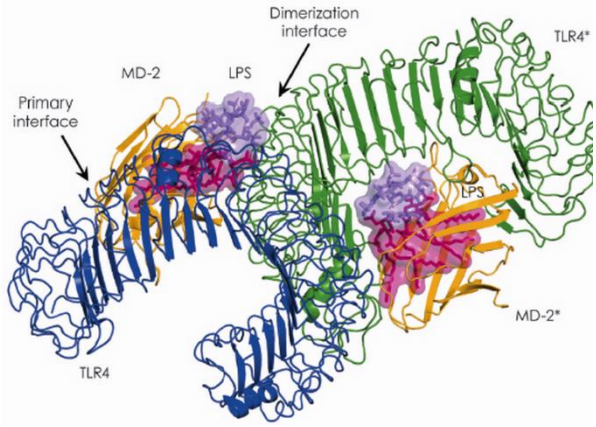

Figure 2.8: A ribbon structure representation of the top view of the complex formed by TLR4, MD2 and LPS (Park et al., 2009).

In yet another study, 14 TLR4 sequence variations were found across a range of Inbred White-Leghorn lines. Polymer chain reactions and sequencing revealed that 9 of these mutations were silent while the remaining 5 mutations resulted in amino acid variants with only three of these being a non-conservative mutation.

TLR4 is not the only toll like receptor that is expressed in chickens, there are several others and some have even been liked to disease resistance and susceptibility in chickens. To date there have been reports of type 1 and type 2 of TLR1 and TLR2 as well as TLR3, TLR4, TLR5, TLR7, TRL15 and TLR 21 expression in various types of tissue in chickens (Fukui et al., 2001). Of all the receptors, only TLR15 is unique as it is the only toll like receptor that is expressed in chickens and not in mammals (Higgs et al., 2006). The receptors can be separated into groups based on the type of pathogen they are able to detect. Toll like receptors 1, 2, 3, 4 and 5 have been reported to be able to recognize bacteria PAMPs and specifically toll like receptors 1, 2, 3, 4, 5, 15 and 21 related to *S. Enteritidis* infection (Abasht et al., 2008). Toll receptors such as TLR 3, 7 and 21 are known to recognize viruses (Hornung et al., 2005). These has been previously demonstrated in studies that investigate the relationship between toll like receptors and viral diseases such as Avian Influenza (AIV) and Infectious bursal disease (IBDV). One of these is a study where white Leg horn chickens were inoculated with AIV and IBDV and then the gene expression of toll like receptors 3, 7, 15 and 21 were monitored. After 7 days post inoculation, the expression of TLR 3 and 15 were up regulated and it was concluded that these toll like receptors play a role in the recognition of bacterial invasion in the early stages of infection and this is illustrated in (Figure 2.9) (Jie et al., 2013). The up regulation of TLR3 result is in agreement Rauf et al. (2011) who reported on increased toll like receptor 3

gene expression after chickens were infected with IBDV (Rauf et al., 2011). In another study the lung tissue of chickens that had been infected with Marek's disease the toll like receptors 3 and 7 gene expression was also up regulated in the spleens of infected chickens (Figure 2.9) (Abdul-Careem et al., 2009). It is illustrated in Figure 2.9a how at 72 hours post inoculation and onwards TLR 3 and 7 expression is up regulated and is at its highest at 168 hours.

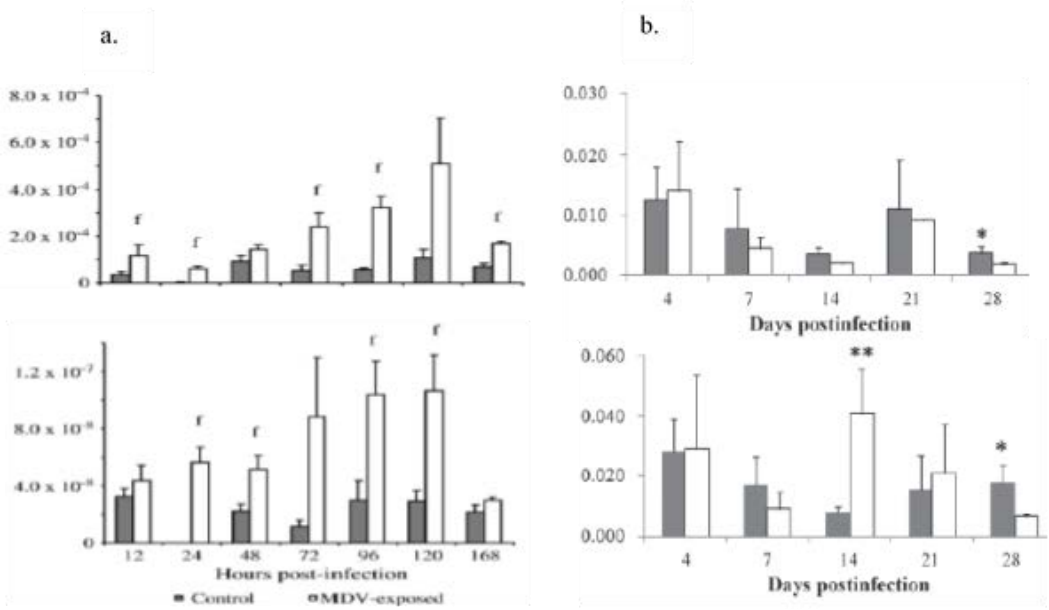

Figure 2.9: a. Expression of Toll like receptors 3 and 7 respectively in lungs of chickens infected with MDV (Abdul-Careem et al., 2009). b. Expression of toll like receptors 3 and 7 in the spleens of chickens infected with MDV (Jie et al., 2013).

As mentioned earlier TLR 15 is unique to the avian species and there isn't a great deal of information on this receptor. The mRNA of TLR15 can be found throughout various tissues of a healthy chicken however once infected with *Salmonella enterica* serovar *Typhimurium* the expression of TLR15 also became upregulated (Higgs et al., 2006).

2.9.3 Natural Resistance Associated Macrophage Protein/Solute Carrier family 1 (NRAMP1/SLC11A1)

The gene coding for natural resistance macrophage protein was first identified in mice. At this time, the gene was referred to as *Ity*/*Lsh*/*Bcg* due to its role in controlling the early stages of infection by different microorganisms and parasites. In 1974 scientists found that the

growth of the parasite *Leishmania donovani* was different amongst the lab mice strains and due to this the gene was dubbed *Lsh* (Bradley, 1974). In the same year it was discovered that mice with certain alleles at this gene responded differently to infection by *Salmonella typhimurium* therefore the gene was named the *Ity*. These two were followed by a study done in 1981 where it was discovered that the lab mice could be separated into groups based by their susceptibility to a *Mycobacterium bovi* infection, this allowed them to name the locus *Bcg* (Forget et al., 1981). It was later determined that all these susceptibility to infection findings were due to one locus which was then described as *Ity*/*Lsh*/*Bcg*. This locus was found and mapped onto chromosome 1 in the mouse genome (Mock et al., 1990) and coded for a 53kDa integral membrane protein which was proposed to function as a cation transporter (Vidal et al., 1993). The gene went on to be called NRAMP1 and now is known as SLC11A1, as the protein coded for by this gene belongs to the solute carrier family 11.

In chickens the SLC11A1 gene is located on chromosome 7 and plays in role in the mortality of chickens due to *S.typhimurium* infections amongst others (Hu et al., 1997). The protein coded for by this gene also transports cations such as Zn^{2+},Mn^{2+} and Fe^{2+} that are required cofactors in the cellular functions of various pathogens (Blackwell et al., 2000). SLC11A1 works with late stage endosome and lysosomes by modulating the amount of divalent cations aiding in the prevention of the pathogens replicating (Blackwell et al., 2000). The important role this gene plays in the response to infection is why it has been chose as a candidate gene worth of being investigated.

The effect of single nucleotide polymorphisms within SLC11A1 has been studied extensively in chicken populations. A number of these polymorphisms are silent mutations that have no effect on disease resistance. This was demonstrated by Hu et al. (1997) where 8 of the 11 SNPs found in the SLC11A1 region were silent mutations and have no effect in the performance or structure of the SLC11A1 gene and protein (Hu et al., 1997). Of the three non-silent polymorphisms, one at position 696 was a G →A to transition which resulted in an amino acid change from Arginine to Glutamine. This change was identified as non-conservative region within the TM5 region. Hu et al. (1997) found that this SNP at this position was specific to a line of chickens found to be susceptible to *S.typhimurium* infection. However it was found that when resistant lines were crossed with susceptible lines, SLC11A1 had no effect and it was concluded that genetic susceptibility to *S. typhimurium* infection is governed by more than just one gene. Although it was found that the relationship between *S. typhimurium* susceptibility was very limited, this was not the case for all *Salmonella* species. Findings by

Liu et al. for the G/A (Arg^{223}/Gln^{223}) polymorphism show the link between the polymorphism in SLC11A1 and response to infection by *Salmonella enteritidis* (Liu et al., 2003). This is supported by a previous study on mice where bacterial load of the spleen was associated with the SLC11A1 genotype (Lalmanach et al., 2001).

Another polymorphism in SLC11A1 identified through PCR-RF Ser^{379} polymorphism where there is a C/T change. This polymorphism or SNP is a synonymous one therefore the amino acid stays the same however, this does not always mean that the SNP does not have an effect on the phenotype. Depending on the nature in residue substitution, the SNP may be responsible for a change in the physical conformation of the SLC11A1 membrane protein hence altering the function of the SLC11A1 protein function resulting in substantial or subtle changes in phenotype (Hu et al., 1997). One candidate gene does not act alone in expressing certain phenotypes due to candidate gene interaction as well as linkage disequilibrium (Rothschild and Soller, 1997). In a study on different chicken lines of the Leghorn variety which had been challenged with SE, there were significant differences in individuals with different genotypes of this SNP. Blood was collected from these birds before they were euthanized and spleen and caecum bacterial loads were quantified. One fifth of the variation in the spleen bacterial load was due to the effect of Ser^{379} polymorphism whereas only 5,9% of the variation in antibody response was due to this polymorphism in SLC11A1 (Liu et al., 2003). Specifically it was the C allele that had a significant correlation ($P<0,02$) with higher antibody levels in one leghorn line however in another line the C allele was correlated with lower antibody levels (Liu et al., 2003). The spleen bacterial load measurements were lower in individuals with the C allele for SCL11A1 however no association was found between the SCL11A1 gene and cecum bacterial load. A similar study to the previously mentioned study was conducted however it was conducted on both commercial type chickens, namely broilers and Old Dutch indigenous breeds, SLC11A1- Sac I polymorphism used in the previous study was found to be associated with the SE bacterial load in the caecum's of both the chicken groups ($p<0.001$) (Kramer et al., 2003) as opposed to previous findings indicating no significance of this SNP to SE bacterial load in the caecum (Liu et al., 2003). Broilers that were homozygous for the C allele were found to have higher SE bacterial loads in the caecum and live while the TT counterparts had the lowest. However within the Old Dutch breed population, CC individuals had the lowest bacterial load in the spleen and in the broiler population the CT individuals had the lowest spleen bacterial load. In both instances, chickens that had the TT

genotype for this SNP, and this does indeed correspond with other findings on the Ser^{379} polymorphism in the SLC11A1 gene (Liu et al., 2003).

All of the candidate genes mentioned as well as the LEI0258 have been proven to have some degree of a relationship to disease resistance and susceptibility. Most of the research however, was performed on commercial breeds. It was hypothesized that indigenous chickens would be more genetically diverse (Izadi et al., 2011) therefore it is of interest to evaluate the frequency of the different alleles and overall genetic diversity in these genes in South African indigenous and commercial chickens. In Chapters 2 and 3 the genetic variation of the innate and adaptive immune response genes mentioned in this literature review, was assessed and compared to other studies.

2.10 References

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Chapter 3

Analysis of genetic variation of disease resistance and susceptibility genes in South African Chickens

Nonhlanhla Bridget Nduna

School of Life Sciences, University of KwaZulu Natal,

Private Bag X54001, Durban 4000, South Africa

Abstract

The genetic diversity in disease resistance/susceptibility genes was investigated in indigenous chicken populations as well as in one commercial population sampled in KwaZulu Natal, South Africa. The genes were the Myeloid Differentiation Protein 2 (MD-2), Toll like receptor 4 (TLR-4), Inducible Nitric Oxide Synthase (INOS) and Solute Carrier Family 11 member A1 gene (SLC11A1) which all play a role in the chicken's innate immune system response. Single nucleotide polymorphisms (SNPs) in these genes were detected in each chicken using the PCR-RFLP method. Restriction fragments from PCR-RFLP were then used to compute population genetics parameters. Of the four loci, the total sampled population was only found to be in Hardy-Weinberg equilibrium at the INOS-*Alu I* locus. Shannon information index values (I) ranged from 0.325 to 0.693 across the four different candidate gene polymorphism loci and the three population sample sites. The highest heterozygosity figures were observed in the TLR-4-*Sau 96 I* (0.78) and INOS-*Alu I* (0.51) loci and these figures corresponded with the negative fixation index (F) figures of -0.3885 and -0.028 respectively. On the other hand the MD-2-*Ase I* and SLC11A1-*Sac I* loci had excess homozygosity hence F values of 0.34 and 0.38 respectively, indicating a moderate degree of inbreeding at these two loci. The commercial chicken population also had low heterozygosity and high F of 0.167 and 0.556 respectively also indicating decreased genetic diversity. This study showed that the methods used were effective in genotyping for these important candidate genes and this allowed for genetic diversity to be measured. Such information will be useful in the development of marker assisted selection (MAS) systems for less disease prone indigenous chickens.

Key Words: candidate genes; single nucleotide polymorphism; PCR-RFLP; fixation index; genetic diversity; heterozygosity; inbreeding

3.1 Introduction

Since the 1960s, egg and poultry meat has seen a marked increment in production to cater for the growing world population as well as increased consumer demand for high animal protein diets (McMichael et al., 2007). Egg production has, in the last couple of decades, increased four times whereas poultry meat production is estimated to reach 300 million metric tons by the year 2020 (Speedy, 2003). With such projections, it is evident that chickens are a major source of protein worldwide. With the increase in protein requirements there is more pressure on commercial chicken farmers to produce sufficiently to cater for the demand. There are several *Salmonella* serotypes that are endemic in intensive poultry production systems. *Salmonella enteritis* is the most common to infect chickens as well as the eggs .This poses a large threat to the human health and animal welfare (Velge et al., 2005). *Salmonella enteritis* is pathogenic once ingested by humans leading to diseases such as gastroenteritis and human salmonellosis (Byrd et al., 1999). These diseases can be very severe and consequently lead to death in infants mainly from developing countries (Kosek et al., 2003). It is estimated that worldwide, 93.8 million cases of gastroenteritis were due to bacteria of the *Salmonella* species, 80.3 million were food borne and 155 000 deaths resulted from these infections in one year (Kosek et al., 2003; Majowicz et al., 2010). To combat these infections the commercial chicken industry relies heavily on the use of antibiotics and vaccines (Velge et al., 2005). There are instances where as soon as one chicken is discovered to have an infection, then the entire flock will receive antibiotics treatment and this is referred to as metaphylaxis where antibiotics are added to the water or food sources (McEwen and Fedorka-Cray, 2002). There is a perception amongst commercial farmers this seems to be an effective method for treating infected individuals as well as preventing secondary infections however, such practices are regarded as controversial by scientists because the extensive use of antibiotics may lead to the formation of antibiotic resistant bacteria which are more difficult to treat (O'Brien, 2002).

Animal breeding has historically always been based on artificial selection on the basis of phenotypic traits of important economic importance which takes several generations to achieve significant genetic progress. The advent of genetic molecular methods has ushered in complementary methods to achieve significant genetic progress which does not rely on the accumulation of massive pedigree information and performance records. It is now in common practice to use a genetic approach to breed for traits of interest (Muhammad et al., 2008) such as disease resistance. The two methods that have been investigated are the candidate gene approach as well as the genome wide linkage disequilibrium scan and these methods have been

used to identify the regions of the chicken genome that contribute to disease resistance (Kaiser and Lamont, 2002). These regions include genes such as sections of the Chicken Major Histocompatibility Complex (MHC), interferon-γ (IFN-γ), Inteleukin-2 (IL-2) and Toll like receptors (TLRs) (Zhou et al., 2001). The protein coded by the Toll like Receptor 4 gene is part of a large family of toll like receptors. Toll like receptor 4 is a membrane protein and is sensitive to pathogenic-associated molecular patterns (PAMPs) such as flagella as well as lipopolysaccharides (LPS), specifically those of *S. enteritidis* infection (Leveque et al., 2003). There are a number of association studies that have been conducted on the relationship between DNA variations in candidate genes and disease susceptibility. Specific polymorphisms found in the TLR-4 gene have been linked to spleen bacterial load (Malek et al., 2004a) as well as cecum bacterial load (Tohidi et al., 2012). Toll Like Receptor 4 generally works in conjunction with myeloid differentiation protein 2 as a dimer in order to detect structurally diverse forms of lipopolysaccharides from different bacteria (Park et al., 2009). Solute Carrier Family 11 member A1 (SLC11A1) as well as Inducible Nitric oxide Synthase (INOS) are two other important factors in immune response to *S. enteritidis* and has been proven to have a significant association to resistance of Salmonella (Hu et al., 1997; Liu et al., 2003; Tohidi et al., 2012).

The genes that will be analysed in this study which are Toll like Receptor 4 (TLR-4), Myeloid Differential protein 2 (MD-2), Inducible Nitric Oxide Synthase (INOS) as well as the solute carrier family 1 gene (SLC11A1). Knowledge of these genes gives the possibility of using Marker Assisted Selection in order to develop chickens that are capable of coping with infections due to desirable genotypes. This kind of breeding mechanism will mitigate the extensive use of antimicrobials and will therefore result in decreased development of drug resistance. In developing countries the indigenous chicken resources have until recently seldom been subjected to selective breeding for a trait as the commercial chickens. One of the contributing factors to this is that there is a paucity of information on the chicken genetic resources in South Africa. There has not been much genetic evaluation studies of the disease resistance genes in these indigenous chicken populations. Most of the South African genetic evaluation studies have been done using autosomal microsatellite markers, mitochondrial DNA as well as markers in the chicken MHC (Mtileni et al., 2011b; Mtileni et al., 2011c; Ncube et al., 2014). Indigenous chickens are the chicken of choice for small holder or sustenance farmers as they do not require as much upkeep (Kitalyi, 1998). These chickens are adapted to harsh climates and infections with no medications or special feed administered to them due to proposed high genetic variation in immune response genes (Izadi et al., 2011). Therefore

indigenous chickens are considered as important genetic resources that should be conserved against production threats and complete replacement by commercial hybrids (Mtileni et al., 2011a; Muchadeyi et al., 2007). Against this backgrounds, the current study is aimed to determine the population genetic diversity within genes that are linked to disease resistance in South African chickens.

3.2 Materials and Methods

3.2.1 Blood collection from indigenous chicken populations

110 chickens from different regions of KwaZulu Natal as depicted in Figure 3.1were randomly chosen to be a part of this study. Blood, as the source of the genomic DNA was extracted from the chickens into either 4mL tubes containing EDTA and anti-coagulant or immediately spotted directly onto Whatmann© FTA cards. Genomic DNA was extracted from the blood samples using Proteinase K and the conventional chlorophorm extraction method. The extracted DNA was eluted in TE buffer (Tris mM and EDTA mM at pH 7). DNA quality and concentration was assessed by using a NanoDrop™ spectrophotometer as well as agarose gel electrophoresis.

Figure 3.1: Map of KwaZulu Natal. Red stars indicating the areas where populations were sampled from.

3.2.2 SNP genotyping for disease resistance and susceptibility genes

The method used to genotype the chickens for the Single Nucleotide Polymorphisms present in these candidate genes was the PCR Restriction Fragment Length Polymorphism method (PCR-RFLP). The primers used for the amplification of portions of the TLR4, MD-2, SLC11A1 and INOS genes are reported in previous studies (Table 3.2). For all the PCR reactions the genomic DNA was initially denatured at 95°C for 5min and this was followed by 35 cycles of denaturation at 95°C for 1 minute, primers annealing conditions were as stated in Table 3.1, followed by extension at 72°C for 1 minute and finally the final extension at 72°C for 5 minutes.

Thermo Scientific Fast Digest enzymes with an incubation temperature of 37°C for a duration 15 minutes were used in order to digest the PCR products. Separation of the fragments was by electrophoresis though 2.5 % agarose gels. From this method we were able assign a genotype to each chicken for each gene.

3.2.3 Agarose gel electrophoresis

3.2.3.1 Preparation of reagents

3.2.3.1.1 Loading buffer

0.01 g of bromophenol blue was added to 1.5ml of 80% glycerol (1.5 ml) thereafter the volume was made up to 4 ml with dH2O. 3µl of GelRed^{TM} was then added to the loading buffer.

3.2.3.1.2 10X TAE

A 10 x TAE stock solution was made by dissolving 48.4g of Tris , 11.44 ml of glacial acetic acid (5.71 ml) and 20 ml 0.5 M EDTA (pH 8.0) in 1 litre of distilled water (dH2O.)

3.2.3.2 Method

After the genomic DNA was extracted from the blood samples it was visualized on 0.9% (w/v) agarose gels. The genomic DNA agarose gels were prepared by dissolving 0.9g of agarose in 100ml of 1 X TAE buffer while the PCR gels were 2.5% and were prepared accordingly. Once at the correct temperature the gels were poured into casting trays and allowed to polymerise for 30 minutes. The gels were run at 70V for approximately 1.5 hours. Thereafter the gels were photographed under ultraviolet light.

Table 3.1: Primer sets, annealing temperatures and restriction enzymes used for the identification of SNPs present in the four candidate genes.

Gene GenBank Accession no.	Primers	Annealing temperature	PCR Allele size (bp)	Reference
TLR4 AY064697	5'-CCTGGACTTGGACCTCAG-3' 5'-GGACTGAAAGCTGCACATC-3'	55/30s	257	(Malek et al, 2004)
MD-2 BI066409.1	5'-GTAACAACAAAGGCAGAA-3'5'-AGAAAAATCCACTGACTCC-3'	48.5/30s	252	(Malek et al, 2004)
INOS AF537190	5'-CCAATAAAAGTAGAAGCGA-3' 5'-CTCTTCCAGGACCTCCA-3'	50/1min	495	(Malek and Lamont, 2003)
SLC11A1	5'-GGCGTCATCCTGGGCTGCTAT-3'5'-AGACCGTTGGCGAAGTCATGC-3'	63/1min	801	(Lamont et al, 2003 Liu et al, 2003)

Table 3.2: Restriction fragment length polymorphism analysis of candidate genes

Gene	SNP Location	Restriction Enzyme	Incubation temperature (°C)	Incubation duration (minutes)	Reference
TLR-4 (G→C)	-3954bp of intron	*Sau96I* (1U/µl)	37	15	(Malek et al., 2004b)
MD-2 (G→A)	-102bp of exon 1 (G/A), Chr, 22	*Ase I* (1U/µl)	37	15	(Malek et al., 2004b)
INOS (C→T)	-173bp of intron	*Alu I* (1U/µl)	37	15	(Malek and Lamont, 2003)
SLC11A1 (C→T)	exons 8 to 11	*Sac I* (1U/µl)	37	15	(Lamont et al., 2002)

3.3 Statistical analyses

Each candidate gene genotype result from the PCR-RFLP fragments was uploaded on POPGENE version 1.32 (Yeh et al., 2000). POPGENE then computed expected and observed

heterozygosity and homozygosity (Levene, 1949) as well as the allele frequency, genotype frequency, Shannon Information index (I) (Lewontin, 1972), fixation index (F) and chi square (X^2). This was done for the total sampled for the total sampled indigenous chicken population, the commercial chicken population as well as for each indigenous chicken population based on the sample sites.

3.4 Results

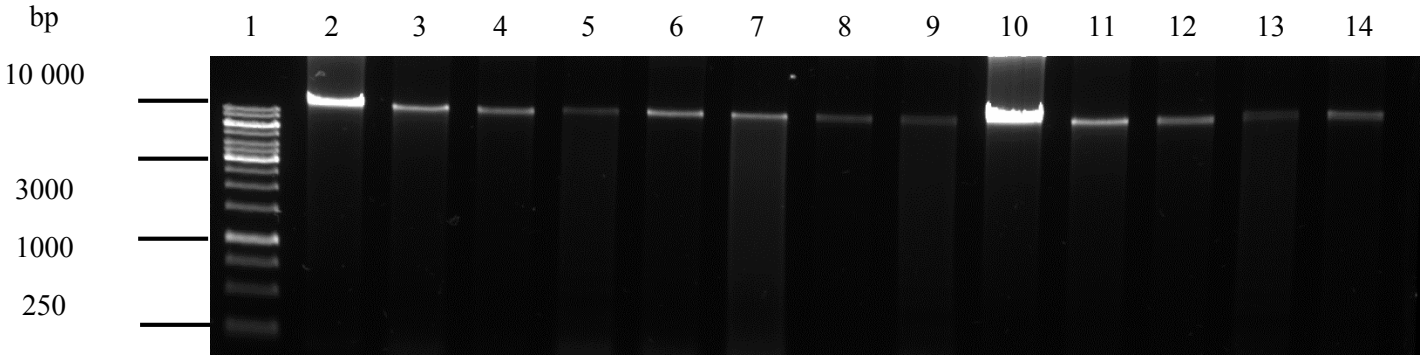

Figure 3.2: Image of a 0.9% Agarose gel run at 70V for 1 hour 15 minutes. Well numbers 2 to 8 contain Genomic DNA extracted using the Cholophorm method from *Gallus gallus domesticus* blood samples while in lane 1 is the Thermo Scientific 1kb Gene ladder.

NanoDrop readings confirmed that the extracted genomic DNA concentration varied from low concentrations of 7.7ng/µl to concentrations as high as 295.7ng/µl. The purity of the DNA was indicated by the A_{260}/A_{280} Ratio and majority of the samples were in the 1.8 region with the lowest being 1.11 to 2.03. DNA that is considered to be pure has a ratio between 1.8 and 1.99 (Wang et al., 1994).

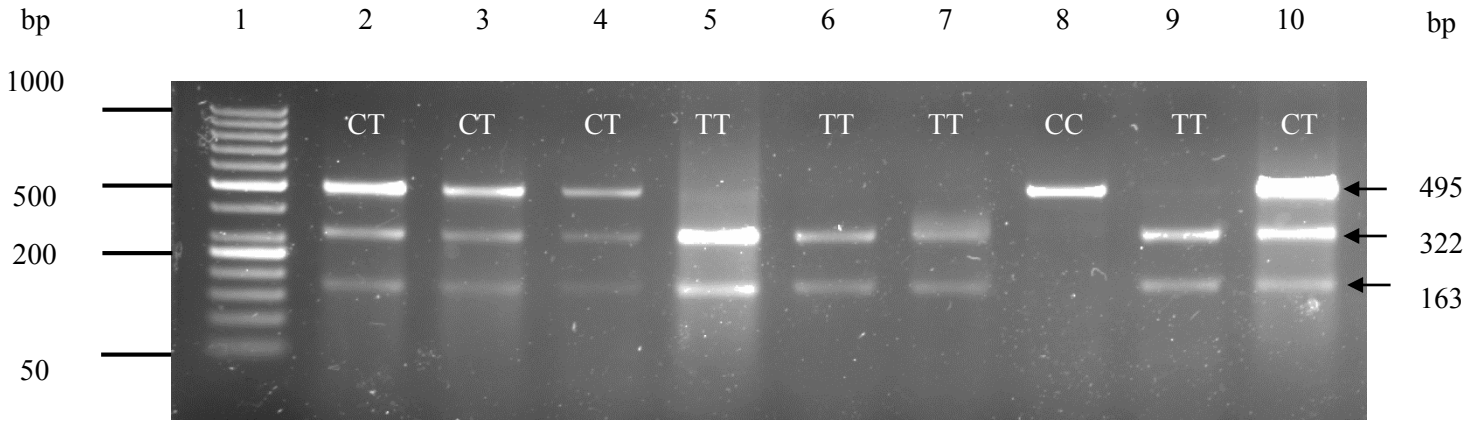

Figure 3.3: Image from a 2.5% Agarose gel showing PCR-RFLP fragments created from enzyme digestion of the iNOS gene fragment by *Alu I*.

The amplified portion of the INOS gene was a 495bp product and restriction enzyme digestion by *Alu I* of these PCR product resulted a band pattern of 322bp and 163bp for the T allele and the uncut 495bp band for the C allele. Heterozygotes had a combination of all three bands. Allelic frequency was 0.56 for the T allele and 0.61 for the C allele (Table 3.3).

Table 3.3 : Observed Gene and Genotype frequencies for the INOS-*Alu I* polymorphism across the different regions the chickens were sampled from.

Breeds	**Genotype Frequency**			**Allele Frequency**		**Probability**	χ^2	**I***
	CC	**CT**	**TT**	**C**	**T**			
Howick	0.12	0.42	0.46	0.33	0.67	0.79	0.07	0.64
Pietermaritzburg	0.33	0.53	0.13	0.60	0.40	0.67	0.19	0.67
Durban/KwaMashu	0.21	0.58	0.21	0.50	0.50	0.38	0.76	0.69

* I = Shannon's Information index (Lewontin, 1972)

None of the three populations had statistically significant chi square results (p>0, 05). For the INOS-*Alu I* locus only the Howick population had a significant chi square value. The population with the most heterozygotes was the Durban/KwaMashu population followed by the Howick and Pietermaritzburg respectively. The Howick population had the lowest observed heterozygote frequency as well as a significantly high observed frequency for the TT genotype. The mean value for the Shannon information index was found to be 0.67.

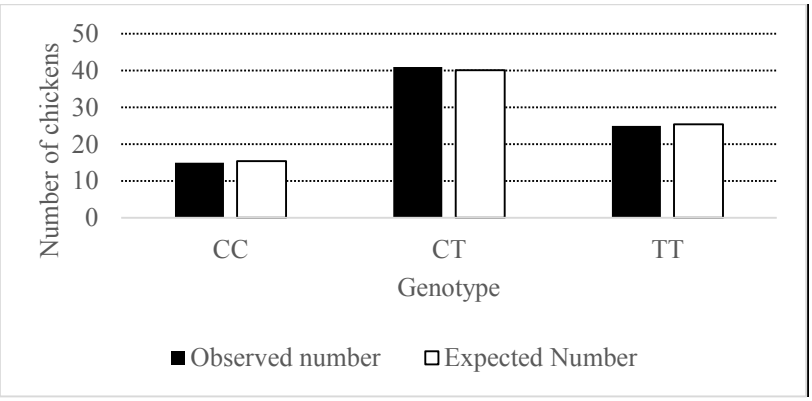

Figure 3.4: A visual representation of the observed number of indigenous chickens in present in each genotype group for the INOS-*Alu I* polymorphism as well as the expected number of indigenous chickens

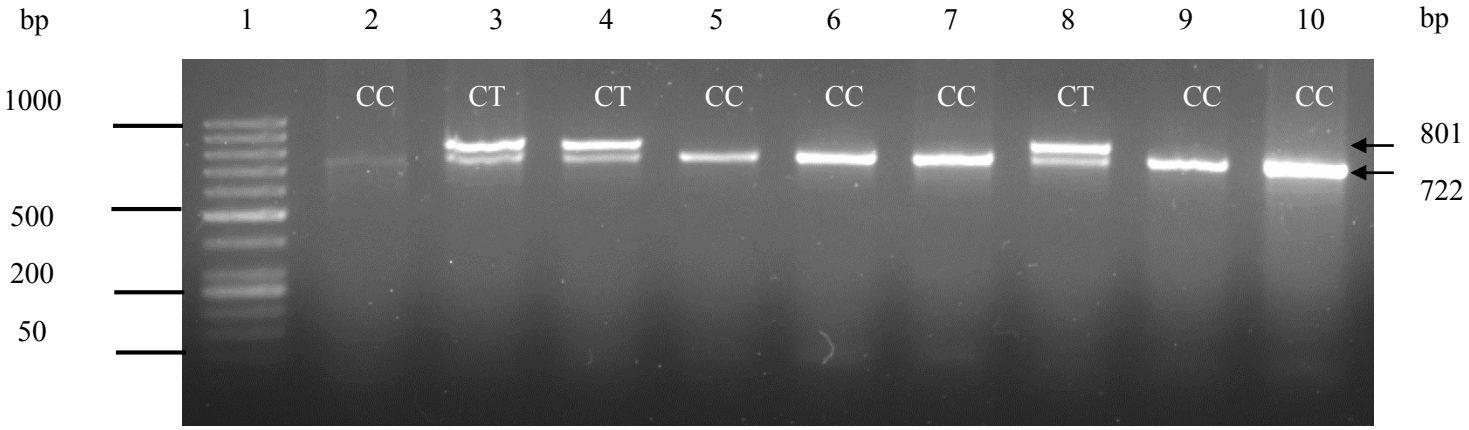

Figure 3.5 : Image of a 2.5% Agarose gel showing PCR-RFLP fragments created from enzyme digestion of the SLC11A1 gene fragment by *Sac I*.

The SLC11A1 PCR reaction resulted in an 801bp product which after digestion by *Sac I*, resulted in 722bp and 79bp fragments for the C allele, although the smaller fragment was too small to be visualized on the agarose gel, and the uncut 801bp represented the T allele. Allele frequencies at this loci were 0.39 for T and 0.61 for C (Table 3.4).

Table 3.4: Observed Gene and Genotype frequencies for the SLC11A1-*Sac 1* polymorphism across the different regions the chickens were sampled from.

Breeds	**Genotype Frequency**			**Allele Frequency**		**Probability**	χ^2	**I***
	CC	**CT**	**TT**	**C**	**T**			
Howick	0.56	0.29	0.15	0.71	0.29	0.09	2.89	0.61
Pietermaritzburg	0.47	0.27	0.27	0.60	0.40	0.09	2.96	0.67
Durban/KwaMashu	0.34	0.31	0.34	0.50	0.50	0.03**	4.50	0.69

* I = Shannon's Information index (Lewontin, 1972).

**p<0.05

The table above shows that the Durban/KwaMashu population is the only population with a significant chi square value (p<0.05) while the other two do not. This was also the population with the higher heterozygote frequency. However the most frequently observed

genotype for the SLC11A1-*Sac I* locus was the AA genotype across all three sampling sites. The mean value for the Shannon information index was found to be 0.66.

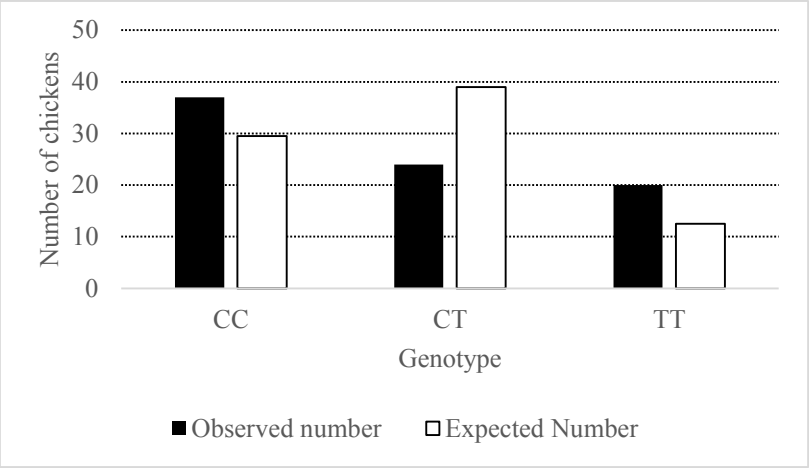

Figure 3.6: A visual representation of the observed number of indigenous chickens in present in each genotype group for the SLC11A1-*Sac I* polymorphism as well as the expected number of indigenous chickens

Figure 3.6 depicts how the CC genotype was detected in 37 of the 81 indigenous chickens while the number of CT and TT individuals was very similar.

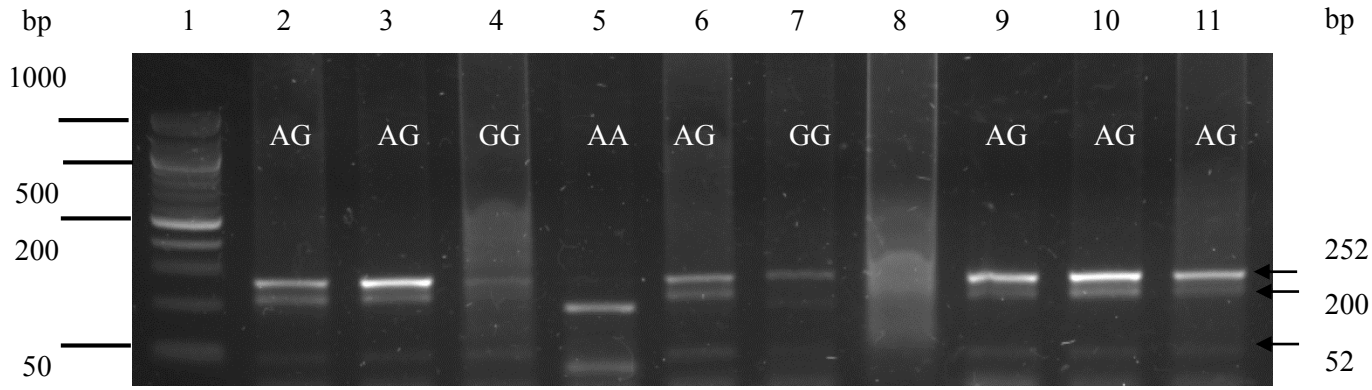

Figure 3.7: Image of a 2.5% Agarose gel showing PCR-RFLP fragments created from enzyme digestion of the MD-2 gene fragment by *Ase I*.

The MD-2 fragment that was amplified was 252bp long and the G/A polymorphism in this region resulted in a restriction site for *Ase I* which created 174bp and 78bp fragments representing the A allele and the undigested 252bp long fragment representing the G allele with 0. 27 and 0.73 respectively (Table 3.5).

Table 3.5: Observed Gene and Genotype frequencies for the MD-2 *Ase I* polymorphism across the different regions the chickens were sampled from.

Breeds	Genotype Frequency			Allele Frequency		Probability	χ^2	I*
	AA	AG	GG	A	G			
Howick	0.24	0.21	0.55	0.35	0.65	0,002**	9.37	0.65
Pietermaritzburg	-	0.20	0.80	0.10	0.90	0.67	0.19	0.33
Durban/KwaMashu	0.09	0.36	0.55	0.27	0.73	0.63	0.23	0.59

* I = Shannon's Information index (Lewontin, 1972)

**p<0.01

The chi square value for the Howick population was statistically significant however did not indicate that the population was in Hardy-Weinberg equilibrium. No AA individuals were found in the Pietermaritzburg population and very few in the Durban/KwaMashu population. The most frequent genotype across all three populations was the GG genotype. The mean value for the Shannon information index across all three populations was 0.52.

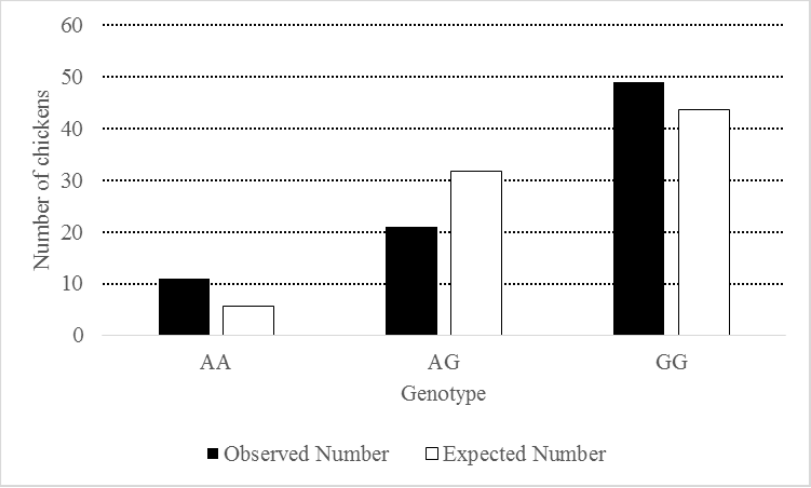

Figure 3.8: A visual representation of the observed number of indegenous chickens in present in each genotype group for the MD-2-*Ase I* polymorphism as well as the expected number of indegenous chickens.

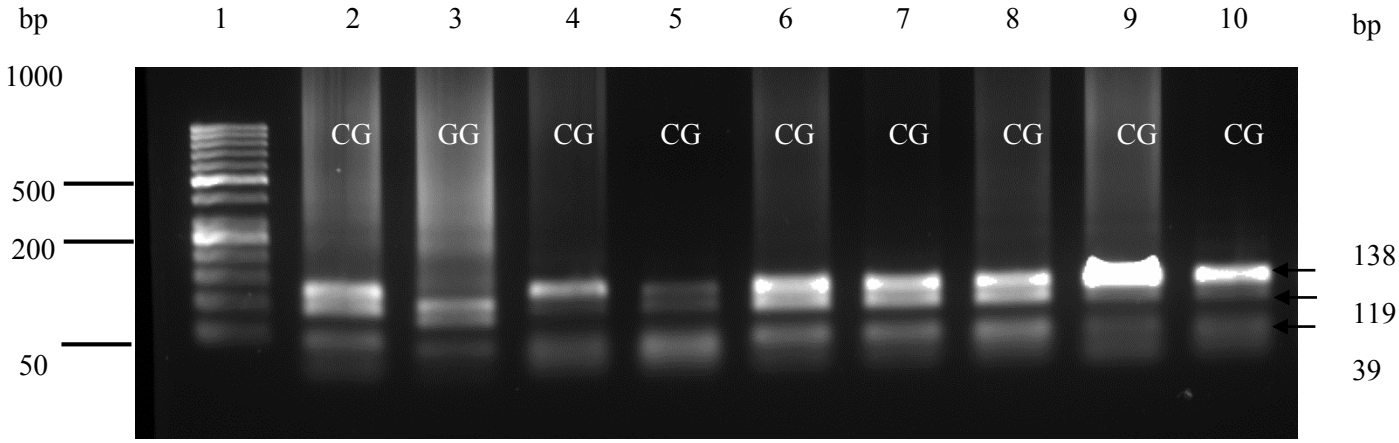

Figure 3.9: Image of a 2.5% Agarose gel showing PCR-RFLP fragments created from enzyme digestion of the TLR4 gene fragment by *Sau 96I*.

The C/G substitution in the 257bp PCR product of TLR-4 resulted in a digestion site for the *Sau 96I* enzyme which created 138bp and 119bp for the C allele and 119bp, 89 and 39bp for the G allele and there frequencies for these were 0.43 and 0.57 respectively (Table 3.6).

Table 3.6: Observed Gene and Genotype frequencies for the TLR-4 *Sau96 I* polymorphism across the different regions the chickens were sampled from.

Sample site	**Genotype Frequency**			**Allele Frequency**		**Probability**	**χ^2**	**I***
	CC	**CG**	**GG**	**C**	**G**			
Howick	0.12	0.79	0.09	0.52	0.49	0.001**	10.99	0.69
Pietermaritzburg	0.27	0.67	0.07	0.60	0.40	0.13	2.27	0.67
Durban/KwaMashu	0.03	0.58	0.39	0.32	0.68	0.06	3.53	0.63

*I = Shannon's Information index (Lewontin, 1972)

**p=0,001

Again chi square results were significant for the Howick population but did not indicate a population in Hardy-Weinberg equilibrium. Most of all three populations were composed of CG individuals. In the Howick and Pietermaritzburg populations very few GG individuals were

observed. The Durban/KwaMashu population had the lowest observed frequency for the CC genotype. The Shannon information index (I) mean was 0.66.

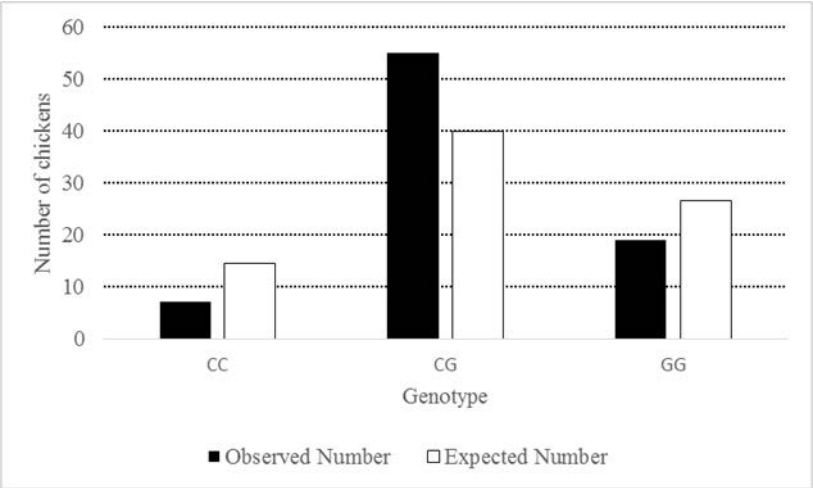

Figure 3.10: A visual representation of the observed number of indegenous chickens in present in each genotype group for the TLR-4-*Sau 96 I* polymorphism as well as the expected number of indegenous chickens

The bargraph above shows how the population was mostly heterozygous (CG) for the TLR-4-*Sau 96I* locus in the entire sampled population of indignous chickens.

Table 3.7: Genotype frequency, allele freuquency and χ^2 values for the entire sampled indiginous chicken population.

	Observed Genotype Frequency							**Allele Frequency**				**p-value**	**χ^2 value for HWE test**
Locus	**CC**	**CT**	**TT**	**AA**	**AG**	**GG**	**CG**	**A**	**T**	**G**	**C**		
TLR-4	0.09	-	-	-	-	0.23	0.68	-	-	0.57	0.43		11.85
MD-2	-	-	-	0.14	0.26	0.61	-	0.27	-	0.73	-		9.51
SLC11A1	0.46	0.30	0.25	-	-	-	-	-	0.40	-	0.61		12.10
INOS	0.19	0.51	0.31	-	-	-	-	-	0.56	-	0.44		0.84

Table 3.8: Observed, expected heterozygocity and homozogocity for the entire sampled indigenous chicken population.

Locus	Observed Heterozygocity	Observed Homozygocity	Expected Heterozygocity*	Expected Homozygocity*	Fixation Index (F)
TLR-4	0.78	0.22	0.78	0.22	-0.39
MD-2	0.26	0.74	0.39	0.61	0.33
SLC11A1	0.30	0.70	0.48	0.52	0.38
INOS	0.51	0.49	0.50	0.51	-0.03
Mean	0.46	0.54	0.54	0.46	0.08
Standard Deviation	0.21	0.21	0.15	0.15	0.31

* Expected homozygocity and expected heterozygocity were computed using Levene (1949)

** Shannon's Information Index (Lewontin, 1972)

Table 3.9: Gene and Genotype frequencies for the SLC11A1-*Sac I* polymorphism from the sampled commercial chickens.

	Genotype frequency			Allele frequency		p-value	χ^2	I*
Breed	**CC**	**CT**	**TT**	**C**	**T**			
Broilers	0.67	0.17	0.17	0.75	0.25	0.001	10.07	0.56

* I = Shannon's Information index (Lewontin, 1972)

Table 3.10: Observed, expected heterozygocity and homozogocity for the entire sampled indigenous chicken population.

Locus	Observed Heterozygosity	Observed Homozygosity	Expected Heterozygosity*	Expected Homozygosity*	Fixation Index (F)
SLC11A1	0.17	0.83	0.38	0.62	0.56

*Expected homozygosity and heterozygosity were computed using Levene (1949)

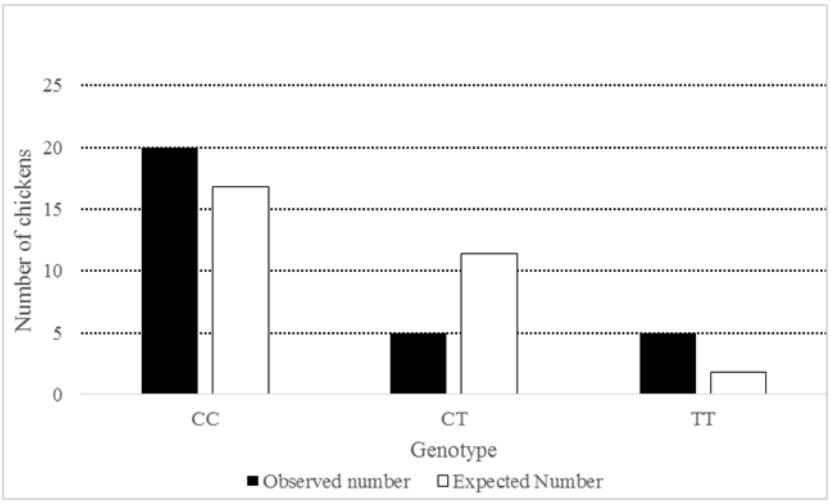

Figure 3.11: A visual representation of the observed number of commercial chickens in present in each genotype group for the SLC11A1-*Sac I* polymorphism as well as the expected number of commercial chickens.

3.5 Discussion

All the SNPs in this study have been previously characterized in other countries, different breeds and ecotypes (Liu et al., 2003; Malek et al., 2004b; Malek and Lamont, 2003) and we were able to detect all of these SNPs in South African chickens. All the genes that were chosen in this study play a role in the innate immunity against infection (Hussain and Qureshi, 1998; Kramer et al., 2003; Leveque et al., 2003; Li and Cherayil, 2003; Malek et al., 2004b; Tohidi et al., 2013; Tohidi et al., 2012). All but the INOS-*Alu I* SNP deviated from the Hardy Weinberg (HW) equilibrium. When a locus abides to the Hardy Weinberg equilibrium this shows that that at present, evolution is not occurring at this loci allowing the allele and genotype frequencies to remain constant. It is possible for certain loci in the same population to abide to HW equilibrium while others do not, as each locus has its own independent history in the population unless the loci are genetically linked. Therefore it is necessary to analyse more than one locus as what has been done in the current study.

Inducible nitric oxide synthase plays a major role in the innate immune response system as it is one of the messenger molecules with that has a various number of functions (Ramasamy et al., 2011). The main functions for INOS lies in the L-arginine pathway where the cytotoxic nitric oxide is produced to aid phagocytes in fighting pathogens (Schmidt and Walter, 1994). It is reflected in research that chickens of a different genetic background also differ in INOS

expression, activity and regulation hence its importance as a candidate gene for disease resistance (Hussain and Qureshi, 1998). The genotype and allele frequencies can be used as measurement of genetic variation in a population. It was determined that the total population was in Hardy Weinberg equilibrium at this locus ($p<0.05$). For the INOS-*Alu I* locus, a large portion of the chickens had the heterozygous CT genotype with a frequency of 0.51 (Table 3.7). Other studies on indigenous chickens in Malaysia as well as another study on indigenous Dutch breeds also reported that the CT genotype was the most prevalent in these chicken populations (Kramer et al., 2003; Tohidi et al., 2013). Another similarity with the Malaysian indigenous chicken population was that the second most prevalent genotype was TT with a frequency of 0.43 followed by very few individuals with the CC genotype (Figure 3.4) (Tohidi et al., 2013). The TT genotype has been reported to be correlated with low SE colonization numbers hence is said to have a positive effect on the immune system when combating infection (Kramer et al., 2003; Tohidi et al., 2012). The CC genotype had the lowest frequency in the population and is also genotype that has been found to have a negative effect on the immune system. In indigenous Dutch chicken breeds and Malaysian breeds it was found that individuals with this genotype had a higher SE load than the CT and TT counterparts (Kramer et al., 2003; Tohidi et al., 2012).

Natural resistance associated macrophage protein (NRAMP-1) or more recently known as Solute carrier family 11 member 1 (SLC11A1) is a membrane bound protein which works as a divalent metal cation transporter. This protein is reported to decrease intraphagosomal microbial replication by controlling the amount of cations within the phagosome (Hu et al., 1996). Observed heterozygosity for the SLC11A1 loci was 0.30 which is significantly lower than the expected value of 0.70. In the indigenous Malaysian chicken population study the most observed genotype for this polymorphism was the CT genotype with an observed frequency of 0.68 followed by CC with a frequency of 0.25 and a very small TT frequency of 0.07 so the drop in heterozygosity in the South African indigenous population is quite evident (Tohidi et al., 2013). Kramer et al.,2003 reported that there was a strong association ($p<0.01$) between this SNP and caecum SE load as well liver SE load ($p=0.05$) in both the old Dutch breeds as well as the broilers and no association between the SNP and spleen bacterial load (Kramer et al., 2003). From that study it was revealed that the broiler chickens with the CC genotype had the highest SE load in the liver and caecum while the broilers with the TT genotype had the lowest SE loads. Upon splitting the total population into separate populations based on the region the chickens were sampled from, the Howick and Pietermaritzburg populations were

found to not be in Hardy-Weinberg equilibrium. Although the Durban/KwaMashu population was the only one that showed significance in the Hardy-Weinberg equilibrium test (Table 3.4), the population was not in HW equilibrium as well. From this it can be inferred that in all 3 populations there was a departure in one or two of the HWE assumptions. Judging by the nature of these indigenous chicken populations, this may be an indication of natural selection for these disease resistance genes taking place which would cause this deviation from HWE in the 3 different populations (Raymond and Rousset, 1995).

For comparison, the SLC11A1 gene polymorphism was also detected in a population of commercial type chickens supplied by one of the large commercial chicken farms. The observed number of CC individuals shows that a large majority of the commercial chickens had the CC genotype for SLC11A1-*Sac I* (Figure 3.11). Due to the CC genotype being the most frequent followed by CT and TT both with a frequency of 0.17 (Table 3.9), the C allele had a rather large frequency of 0.75 (Table 3.9). Compared with the observed heterozygosity of 0.30 in the indigenous population, the observed heterozygosity in the commercial chickens was even lower at 0.17 (Table 3.9). This in conjunction with the high observed homozygosity of 0.83 is an indication an even higher deficiency of heterozygous individuals. High homozygosity in the commercial breed was to be expected as there are several reports on indigenous chickens being more genetically diverse than commercial chickens (Izadi et al., 2011; Okumura et al., 2006; Osman et al., 2006; Zhang et al., 2002; Zhou and Lamont, 1999). Commercial chickens are undergo stringent selection for economically important traits hence we can attribute the deviation from HWE to this selection process. The other factor that may be affecting this commercial population is that the mating is not random. Of the sampled commercial chickens, 67% of them had the CC genotype which is reported to be highly susceptible to *S. enteritidis*. At the present time genetic information is being used in the poultry industry in order to maximise on traits such as growth rate however a number of economically important traits are inversely related to health traits or genes (McGovern et al., 1999; Nardone and Valfrè, 1999). This may be the reason for a large majority of the commercial chickens to have the more susceptible genotype. Commercial chickens are also kept in as little contact as possible with bacteria and in conjunction with the stringent antimicrobial and vaccination programmes (Janmaat and Morton, 2010) that are followed in commercial farming, the industry may see no need for resistant chickens.

The gene that codes for toll like receptor 4 is found on micro chromosome E4/W17 in the chicken genome and codes for a 849 amino acid long protein (Leveque et al., 2003). Toll

like receptor 4 is part of a large family of proteins that work as pattern recognition receptors and these are able to detect pathogen associated molecular patterns such as lipopolysaccharides in order to activate the immune system (Köllisch et al., 2005). Studies that have found that after infraction by a pathogen, the expression of TLR4 had increased (Ramasamy et al., 2011). In the current study it was evident that more than half of the total population was heterozygous at the TLR4-*Sau 96I* locus (Figure 3.10) followed by GG individuals with a frequency of 0.24 and the least observed genotype was the CC genotype with a low frequency of 0.09 (Table 3.7). The observed allele frequencies were 0.57 and 0.43 for G and C respectively (Table 3.7). These results correspond with the allele and genotype frequencies reported in a Malaysian village chicken populations where (Tohidi et al., 2012). However the Malaysian study found that their population did not deviate from HWE whereas the South African population did. Deviation from HWE indicates changes in allelic frequencies in a population. These changes can be attributed to a number of factors such as genetic drift which is known to occur in populations that are not large as the ones used in this study (Corander et al., 2003) .The genotype for this SNP that was found to have the lowest observed frequency (CC) has also been reported to be more susceptible to caecum colonization by SE ($p = 0.03$) (Tohidi et al., 2012). These researchers also found that in the village chickens the CG genotype individuals had the lowest caecum ($p = 0.01$) and liver ($p = 0.29$) SE loads (Tohidi et al., 2012). Leveque et al., 2003 also reported on chickens that were heterozygous for TLR4 had higher survival rates (Leveque et al., 2003). As depicted in Figure 3.4, the total sampled population in the current study were mostly heterozygotes at this locus. This again echoes the same outlook on indigenous chickens having increased diversity in immunity genes in order to combat various infections found in natural environments.

Myeloid differentiation 2 (MD-2) is a highly conserved protein that also plays a role in the innate immune system. This protein forms a complex with TLR-4 and in conjunction they are able to activate a cascade of signalling events that result in the response of the innate immune system to LPS (Kim et al., 2007; Ohto et al., 2007; Shimazu et al., 1999). There is not a great deal of information on this polymorphism and chicken disease susceptibility however there is a study that found that the expression of TLR-4 and MD-2 increased after rabbits were challenged with *Escherichia coli* (Kajikawa et al., 2005). Another study also found an association between spleen ($p<0.04$) and caecum ($p<0.10$) SE bacterial load (Malek et al., 2004b). In the South African total sampled chicken population the GG genotype for MD-*Ase I* had an observed frequency of 0.61 followed by 0.26 and 0.14 for the AG ans AA genotypes

respectively. The AA genotype seems to generally be a a scarce genotype as other studies were not able to detect this genotype at all in village chicken populations as well as in jungle fowl populations (Tohidi et al 2012). A chi-square test revealed that the population is not in HWE for the MD-2-*Ase I* locus as the SLC11A1-*Sac I* locus.

The mean expected heterozygosity (He) for the total indigenous chicken population was 0.54 (SD = 0.21) which was higher than the average observed heterozygosity (Ho) of 0.46 (SD = 0.15) (Table 3.8). The highest observed heterozygosity in the population was 0.78 and was for the TLR4-Sau96I locus followed by 0.51 for the INOS-*Alu I* locus while the observed heterozygosity values for the MD-2-*Ase I* and SLC11A1-*Sac I* were both just under 0.30. The expected heterozygosity values for the MD-*Ase I* and TLR-4 values were much higher than the observed values at approximately 0.70 whereas they were relatively low for the other two loci (Table 3.8). High heterozygosity indicates high genetic variability for TLR-4 and SCL11A1-1 loci in the total population whereas on the opposite spectrum, for INOS-*Alu I* and MD-2 *Ase I* loci their low heterozygosity values indicate very little genetic variation. Heterozygosity can be taken as a measurement of genetic diversity and a low heterozygosity is generally a bad indicator as there is a correlation between heterozygosity and fitness. Therefore a low heterozygosity may be an indicator of inbreeding and low fitness (Keller and Waller, 2002). The loss of genetic variation can be attributed to factors such as a limited population size which applies to the current study (Allendorf and Leary, 1986). The excess homozygosity observed in the MD-2-*Ase I* and SLC11A1-*Sac I* loci is a reflection on why these two loci have deviated from HWE (Table 3.8)

The fixation index (F) measures the degree of the genetic differentiation within a population. When F results that were obtained from POPGENE showed that for the TLR-4 loci and the INOS-*Alu* I loci were -0.39 and -0.03 respectively (Table 3.8). The fixation index ranges from -1 to 1 therefore a negative fixation index suggests that the frequency of homozygotes was lower than expected under HWE as reflected in Table 3.8 and also that the population is outbred. Excess heterozygosity is an indicator that there is no inbreeding occurring in the population for the loci in question or the population is outbred. These fixation index figures correspond with the observed heterozygosity for these two loci. With the MD-2-*Ase I* and SLC11A1-*Sac I* loci, the F values were positive which indicates non-random mating of related individuals or inbreeding which would result in the excess homozygosity shown in Table 3.8. Previous studies on genetic diversity in chickens also showed this trend where chickens with a positive F value were found to have decreased heterozygosity (Vanhala et al.,

1998; Zanetti et al., 2007). It also to be mentioned that both loci with negative fixation indices were found to deviated from HWE. The lowest observed heterozygosity of was observed out of the indigenous chicken populations in the commercial chicken population (Table 3.10).

The Shannon information index (I) figures found in this study for the INOS SNP and SLC11A1 SNP loci ranged from 0.64 to 0.69 (Table 3.3) and 0.61 to 0.69 (Table 3.4) respectively. These I values are similar to those reported in 2013 on a Malaysian indigenous chicken population where the Shannon information index was 0.62 for INOS and 0.68 for SLC11A1 (Tohidi et al., 2013). The MD-2- *Ase I* locus had the lowest I value with a range beginning at 0.33 for the Pietermaritzburg population to 0.65 for the Howick (Table 3.5) population while the TLR-4-Sau96 I locus followed the general trend of the INOS-*Alu I* and SLC11A1-*Sac I* figures. The second lowest I value was for the SLC11A1-*Sac I* locus in the commercial chicken population which was sampled (Table 3.9). The Shannon information index gives an estimation of species richness and evenness in different locations. In a rich ecosystem generally it ranges from 1.5 to 3.5 therefore the higher the number the more the number of species increases or the more even the distribution becomes (McDonald and Dimmick, 2003) .It does happen however for the results to be below 1 as shown in a study in 2010 reported a range from 0.42 to 0.60 in a populations of indigenous chickens in Jordan (Al-Atiyat, 2010). Of the 4 sampled populations (3 indigenous and 1 commercial) the Shannon information index results the Pietermaritzburg population had the lowest genetic diversity for the MD-2-*Ase I* locus and the commercial population at the SLC11A1 locus as well. This was to be expected as both these populations showed deficit heterozygosity at both loci (Table 3.5, Table 3.10). Low genetic diversity in commercial chickens in comparison to the indigenous chickens as indigenous chickens are not constantly maintained and reproduction occurs randomly (Ahlers et al., 2009) whereas in commercial chicken farming systems all these factors are controlled.

3.6 Conclusion

The genetic diversity within indigenous chickens found in South Africa was assessed in this study using 4 single nucleotide polymorphisms as molecular markers. This was done by calculating population genetic parameters that assess diversity such as allele and genotype frequency, heterozygosity and homozygosity as well as the Shannon Information index. Results showed that across all the breeds the TLR-4-*Sau 96I* and INOS-*Alu I* loci, the genetic polymorphism was rather high as opposed to the SLC11A1-*Sac I* and MD-2-*Ase I* loci where

the observed genetic polymorphism was relatively low. The SLC11A1-*Sac I* polymorphism was also detected in a commercial chicken population for comparison and was found to have low genetic diversity as hypothesized. Also, majority of the commercial chicken population carried the unfavourable or susceptible genotype. Information on the variation of these candidate genes in these chickens is yet to be reported and information from studies such as this one can be used in marker assisted selection as well as conservation (MAS). There will however, be issues to consider as the heritability of immune response varying from low to average and can be affected by factors such as the environment.

3.7 References

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Chapter 4

Analysis of genetic variation in the LEI0258 microsatellite marker in South African indigenous chickens

Nonhlanhla Bridget Nduna

School of Life Sciences, University of KwaZulu Natal,

Private Bag X54001, Durban 4000, South Africa

Abstract

The Major Histocompatibility Complex (MHC) is a cluster of genes that play a large role in the chicken's adaptive immune system and several haplotypes have been found to affect resistance or susceptibility to disease. The LEI0258 variable number tandem repeat is positioned in the BF region of the MHC and was the molecular marker that was used to assess genetic variability in the MHC in 3 South African indigenous chicken populations. Heterozygosity, the Shannon information Index (I), Fixation Index (F), Number of alleles (Na) and allele frequencies were used to assess the genetic diversity. A total of 36 LEI0258 alleles ranging from 182 bp to 515 bp were detected in the chickens and 19 of these had been previously described in literature. There was a high number of private alleles which correlates with the evolutionary nature of LEI0258. Allele frequency ranged from 1.6 to 35%, the most frequent allele being the 321 bp allele which was not detected in the Pietermaritzburg population. Overall observed heterozygosity was substantially lower than expected indicating inbreeding. Lowered genetic diversity was also reflected in the positive Fixation indices with an average of 0.42 which also suggests inbreeding in the populations. An average Shannon Information Index value of 2.71 was determined inferring that the distribution of individuals across the species present, or relative abundance is high and desirable in relation to the conservation of a species. The Howick population had the highest number of alleles, observed heterozygosity, Shannon Information Index and lowest fixation Index and all these factors led to the conclusion that the Howick Indigenous chickens had the highest amount of genetic variation in the MHC. It was concluded that indigenous South African chickens are highly polymorphic at the LEI0258 locus. Multiple sequence alignment of sequences revealed similarities among as well as a number of conserved regions.

Keywords: Major Histocompatibility Complex; polymorphic; heterozygosity; relative abundance; fixation index

4.1 Introduction

Indigenous chickens (*Gallus gallus domesticus*) are the domesticated descendants of the Red Jungle fowl (*Gallus gallus*) and are a common farm animal in rural areas where subsistence farming is still a commonplace practice (Ahlers et al., 2009). It has been hypothesized that indigenous chickens have over time, acquired combinations of alleles of certain immune related genes which assist in the adaption to the harsh environments they are exposed to (Mwacharo et al., 2007). The Major Histocompatibility Complex (MHC) or the B-complex as it is referred to in chickens, is split into the BF, BG and BL which are composed of 19 genes located on microchromosome 16 (Kaufman et al., 1999). This cluster of genes plays an essential role in the chicken's adaptive immune defence mechanisms against different infections whether the source of infection be parricidal, bacterial or viral (Parmentier et al., 2004; Fulton et al., 2006). There are numerous studies depicting the effect of the MHC on disease resistance and susceptibility to diseases such as Avian influenza (Adams et al., 2009), Newcastle disease (Lwelamira et al., 2009) Avian coronavirus (Banat et al., 2013) and Marek's disease (Bacon et al., 2001) among a number of others. These diseases are the most common environmental factors that these animals routinely encounter and can be a danger to both the animals and humans who ingest them if the infections are of a zoological nature (Velge et al., 2005).

Fulton et al., (2006) characterized the LEI0258 marker in different commercial and indigenous chicken lines and reported 26 alleles that ranged from 182 bp to 552 bp. However due to the markers evolutionary nature, there are many more alleles that have been uncovered in more recent MHC genotype identification studies conducted all over the world (Gupta et al., 2011; Chazara et al., 2013; Guangxin et al., 2014; Ncube et al., 2014; Ngeno et al., 2014). The LEI0258 marker is found between the BG and BF regions of the chicken MHC (Fulton et al., 2006). LEI0258 is described as an atypical variable number tandem repeat (VNTR) which is composed of 12bp (TTCCTTCTTTCT) and 13bp (ATGTCTTCTTTCT) conserved sequences which are flanked on both sides by indels and SNPs (Fulton et al., 2006). Microsatellites such as these are widely used as markers to map genomes as well as determine genetic variation, diversity genetic structure as well as the phylogenetic backgrounds due to the fact that they are highly polymorphic and can be found throughout the entire genome in coding regions as well as in non-coding regions (Tautz, 1989; Cheng et al., 1995). Research into the use of microsatellites is ongoing however, some scientists believe that one of their roles could be to package eukaryotic chromosomes (Stallings et al., 1991).

In the current study the LEI0258 microsatellite marker, due to its highly polymorphic nature was used to assess the genetic variability in the chicken Major Histocompatibility Complex from chicken ecotypes emanating from different locations within the province of KwaZulu Natal, South Africa. Currently there is a paucity of information on the sequences for these microsatellites in chicken populations out of North America such as in South Africa. Therefore, the objective of the current study was to analyse the diversity of MHC in South African chickens by assessing the LEI0258 marker.

4.2 Material and Methods

4.2.1 Blood collection from indigenous chicken populations

A random sample of 81 indigenous chickens from different regions of KwaZulu Natal (Figure 4.1) were randomly chosen to be a part of this study during the period between July 2014 and May 2015. Blood, as the source of the genomic DNA was extracted from the chickens into either 4mL tubes containing EDTA and anti-coagulant or immediately spotted directly onto Whatmann© FTA cards. Genomic DNA was extracted from the blood samples using Proteinase K and conventional chlorophorm extraction. The extracted DNA was eluted in TE buffer (Tris mM and EDTA mM at pH 7). DNA quality and concentration was assessed by using a NanoDrop^{TM} spectrophotometer where DNA was measured in ng/µl as well as agarose gel electrophoresis.

Figure 4.1: A Map of KwaZulu Natal where the yellow stars indicate the areas where populations were sampled.

4.2.2 MHC microsatellite marker genotyping

A highly polymorphic microsatellite marker was required to assess genetic variability in at this locus hence the use of the LEI0258 marker.

Table 4.1: Primer sequence set, and the annealing for the LEI0258 marker.

Microsatellite marker	Primers	Annealing temp.	Allele size (bp)	Reference
LEI0258	5'-CACGCAGCAGAACTTGGTAAGG-3' 5'-AGCTGTGCTCAGTCCTCAGTGC-3'	57°C	182-539	(Fulton et al., 2006)

Thermo Fischer scientific master mixes were used for the amplification of these regions of the chicken MHC. The reactions were carried out in 50µl reaction volumes and the he cycling conditions for LEI0258 were as follows; initial denaturation at 94°C for 5 minutes followed by 35 cycles of 92°C for 45 seconds, annealing 57°C for 45 seconds, extension at 72°C for 45 seconds and a final extension at 72°C for 1.5 hours. The PCR samples representing each sampling site as well as each breed were sent to Inqaba Biotech for sequencing.

4.2.3 Agarose gel electrophoresis

4.2.3.1 Preparation of reagents

4.2.3.1.1 Loading buffer

0.01 g of Bromophenol blue was added to 1.5ml of 80% glycerol (1.5 ml) thereafter the volume was made up to 4 ml with dH2O. 3µl of GelRed™ was then added to the loading buffer.

4.2.3.1.2 10X TAE

A 10 x TAE stock solution was made by dissolving 48.4g of Tris , 11.44 ml of glacial acetic acid (5.71 ml) and 20 ml 0.5 M EDTA (pH 8.0) in 1 litre of dH2O.

4.2.3.2 Method

After the genomic DNA was extracted from the blood samples it was visualized on 0.9% (w/v) agarose gels. The genomic DNA agarose gels were prepared by dissolving 0.9g of agarose in 100ml of 1 X TAE buffer while the PCR gels were 2.5% and were prepared accordingly. Once at the correct temperature the gels were poured into casting trays and allowed to polymerise for 30 minutes. The gels were run at 70V for approximately 1.5 hours. Thereafter the gels were photographed under ultraviolet light.

4.3 Statistical analyses and bioinformatics

The GelAnalyzer software (Lazar and Lazar, 2010) was used in order to obtain accurate allele size readings for the LEI0258 marker. These readings were then used in GenAlex software version 6.5 (Peakall and Smouse, 2012), to calculate the effective number of alleles (Ne), Fixation index (F) , observed (Ho) and expected heterozygosity (He) as indicators of genetic diversity for the LEI0258 MHC marker in each breed as well as in the entire sampled population. Allele frequencies were determined and chi-square tests estimated in GenAlex as well in order to determine whether or not the population was in Hardy-Weinberg Equilibrium.

The sequence data was then edited using the Bioedit version 7.2.5 software package (Hall) which was followed by using Mega 6 (Tamura et al., 2011) to perform multiple sequence alignment using the sequences found in the South African as well as LEI0258 sequences extracted from GenBank, to see the similarities among MHC haplotypes as well as to find evolutionarily conserved regions of the marker. A pairwise genetic distance matrix as well as neighbour-joining dendrogram were constructed from the alignment with the aid of Mega 6 with 1000 bootstrap iterations.

4.4 Results

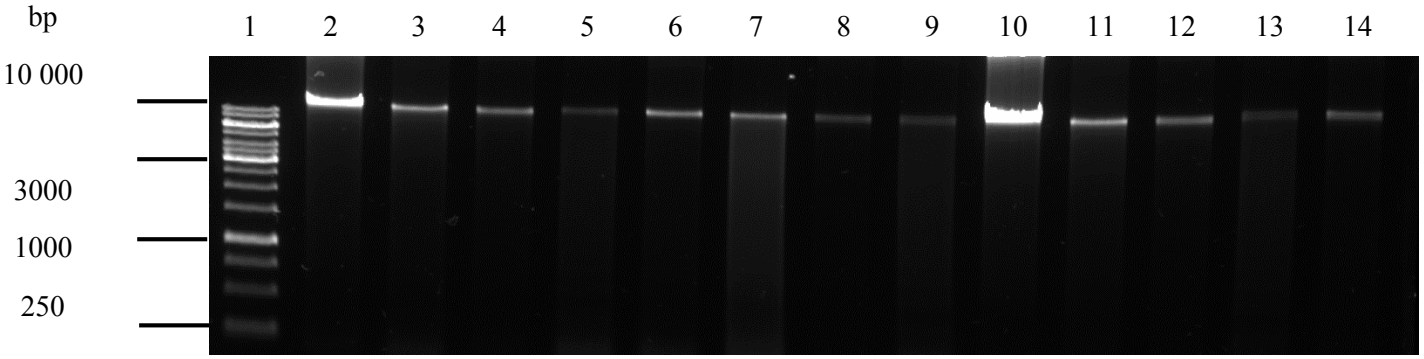


Figure 4.2: Image of a 0.9% Agarose gel run at 70V for 1 hour 15 minutes. Well numbers 2 to 8 contain Genomic DNA extracted using the Cholophorm method from *Gallus gallus domesticus* blood samples while in lane 1 is the Thermo Scientific 1kb Gene ladder.

Nanodrop readings confirmed that the extracted genomic DNA concentration varied from low concentrations of 7.7ng/µl to concentrations as high as 295.7ng/µl. The purity of the DNA was indicated by the A_{260}/A_{280} Ratio and majority of the samples were in the 1.8 region with the lowest being 1.11 to 2.03. DNA that is considered to be pure has a ratio between 1.8 and 1.99 (Wang et al., 1994).

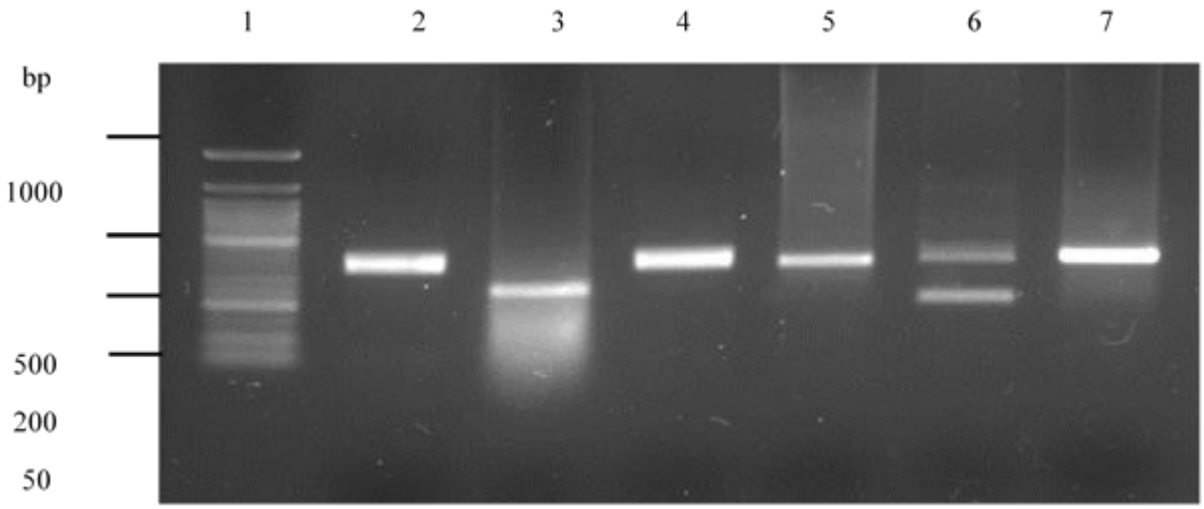


Figure 4.3: A 2.5% Agarose gel stained with GelRed, showing the band patterns for different LEI0258 haplotypes**.**

The band in lane 2 indicates a chicken that is a homozygote for the 393 bp allele while lane 3 indicates a chicken that is a homozygote for the 261 bp allele. Lanes 4, 5 and 7 all represent chickens which are homozygotes for the 414 bp allele while lane 6 represents a heterozygote chicken for the 432 bp and 247 bp alleles.

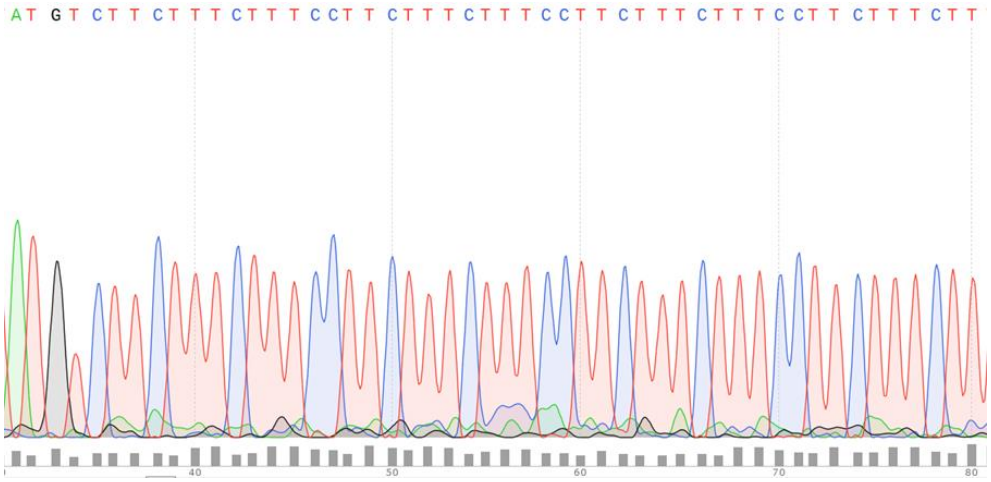


Figure 4.4: Chromatogram of the sequenced LEI0258 microsatellite.

LEI0258 is a VNTR and Figure 4.4 gives a visual representation of its repetitive nature.

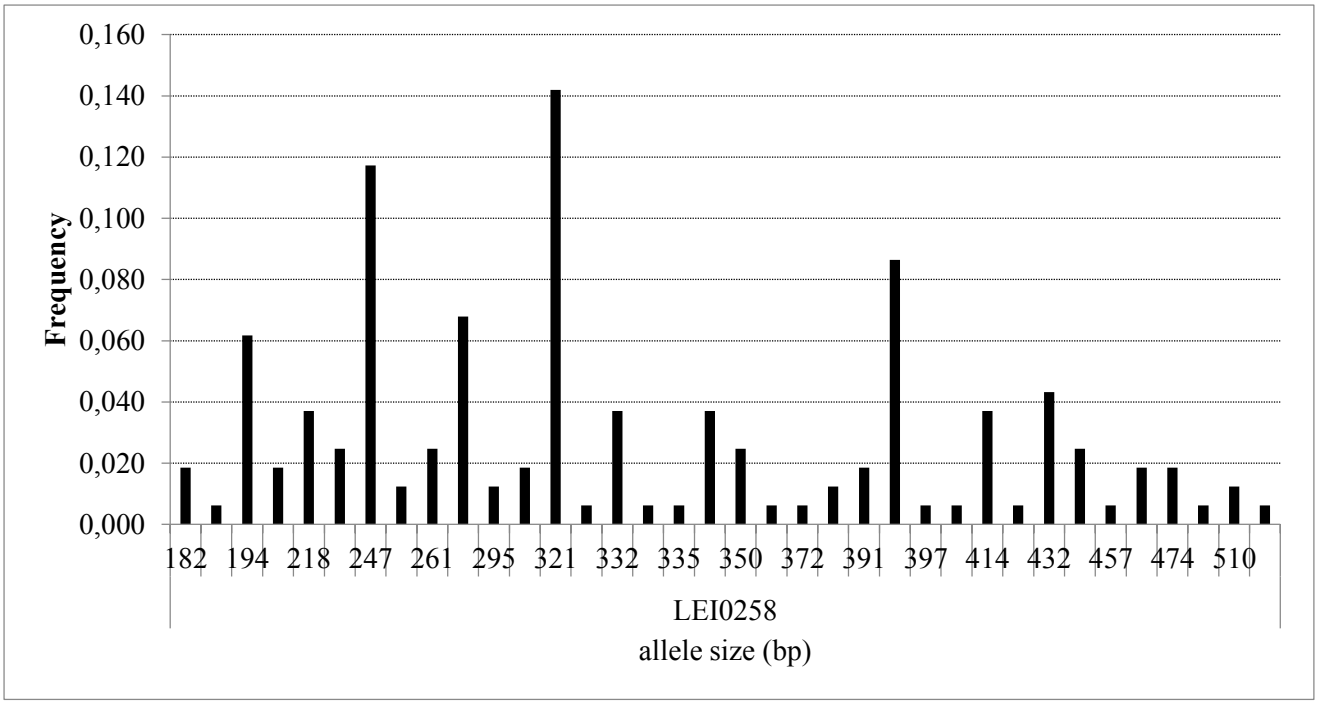


Figure 4.5: LEI0258 allele frequencies in the 3 populations collected from the different regions in KwaZulu Natal**.**

Figure 4.5 indicates how 321bp allele was the most popular allele overall followed by the 247bp and 393bp alleles. All of these alleles had been described previously in other literature (Fulton et al., 2006; Guangxin et al., 2014). Majority of the lower frequency alleles had not yet been reported in indigenous chickens.

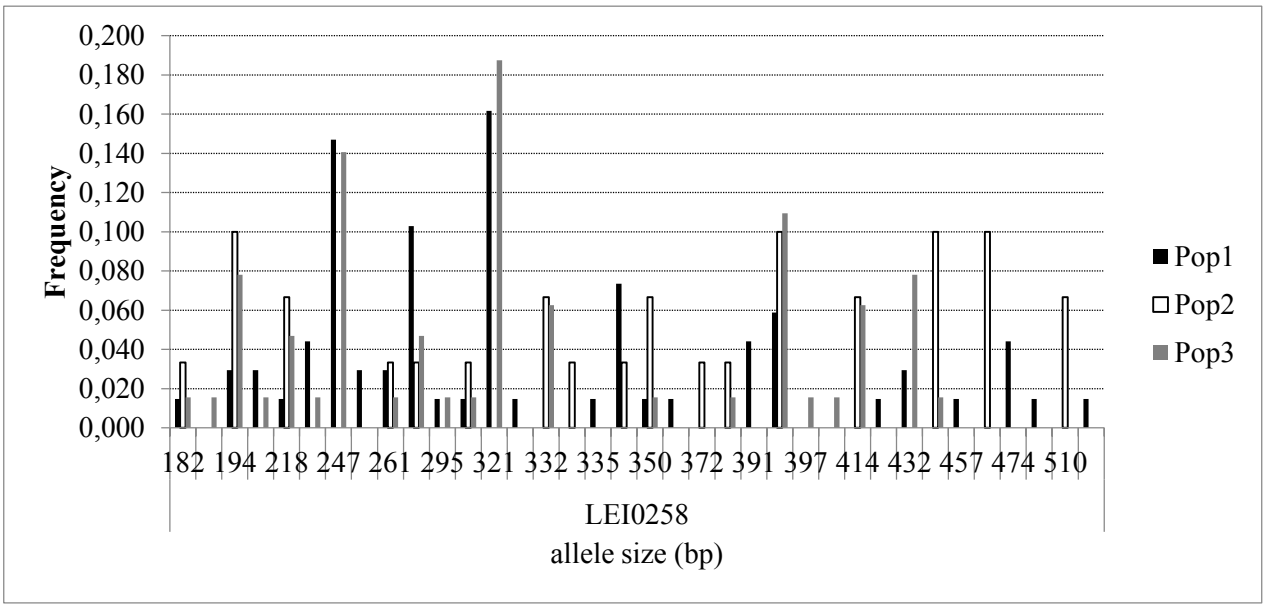


Figure 4.6: A comparison of LEI0258 allele frequencies across the 3 populations. Pop 1: Howick, Pop 2: Pietermaritzburg, Pop 3: KwaMashu/Durban.

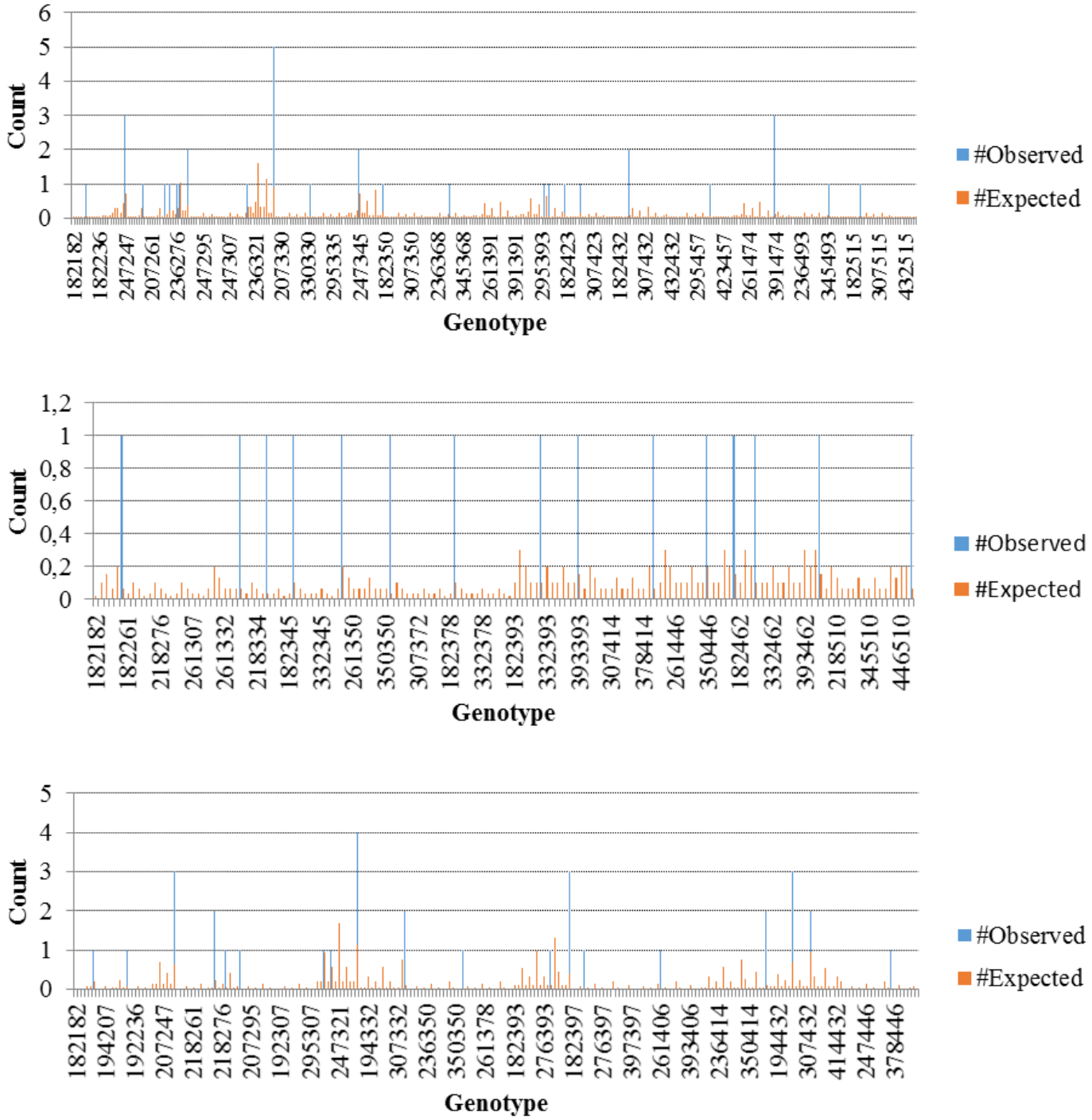


Figure 4.7: Observed versus expected genotype counts at the LEI0258 locus for the Howick, Pietermaritzburg and KwaMashu/Durban populations respectively.

The figure shows the large differences between the expected and observed allele counts in all 3 populations. This is an indication of all three populations deviating from Hardy Weinberg Equilibrium. Natural selection could be a factor that is contributing to this deviation from HWE however it must be considered that the marker being used to assess MHC genetic diversity in this study is a microsatellite. Microsatellites are known to have very high mutation

rates and have been described as evolutionary DNA markers (Schlötterer and Wiehe, 1999). For a population to abide by HWE rules, there must be no evolution occurring at the locus of question therefor, it is not expected for these populations to be in HWE.

Table 4.2: Number of different alleles (Na), and effective number of alleles (Ne), observed and expected heterozygosity (Ho and He), Shannon information index (I) and Fixation index (F) for the total number of indigenous chickens sampled.

Marker	Na	Ne	Ho	He	I	F
LEI0258	36.00	15.94	0.53	0.97	3.12	0.43

Table 4.3: Number of different alleles (Na), and effective number of alleles (Ne), observed and expected heterozygosity (Ho and He respectively), Shannon information index (I) and Fixation index (F) for the each of the indigenous chicken populations.

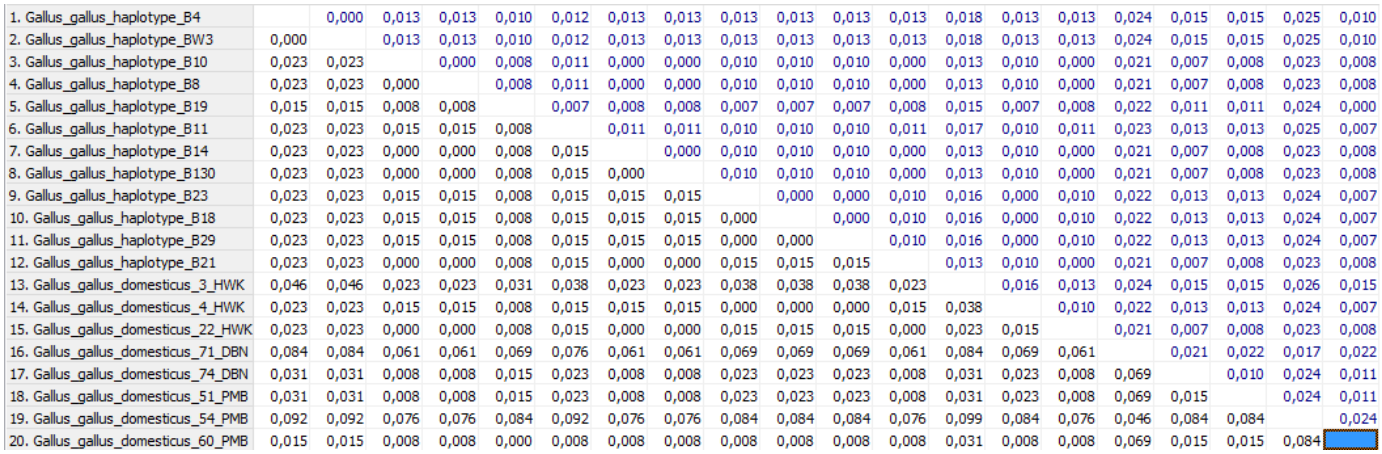
Marker	Chicken Breed	Na	Ne	Ho	He	I	F
LEI0258	**Howick**	25.00	12.17	0.58	0.92	2.84	0.37
	Pietermaritzburg	16.00	12.16	0.47	0,92	2.64	0.49
	Durban/KwaMashu	21.00	10.89	0.52	0,91	2.66	0.43
Mean		20.67	11.74	0.52	0,92	2.71	0.42
SD		3.68	0.60	0.45	0.01	0.89	0.05

Table 4.4: Allele frequencies (%) of LEI0258 in the chicken breed populations

	Population[1]			
Allele (bp)	**HWK**	**PMB**	**DBN/KWM**	**Pop[2]**
182	1.5	3.3	1.6	3
192	0	0	1.6	1
194	2.9	10	7.8	3
207	2.9	0	1.6	2
218	1.5	6.7	4.7	3
236	4.4	0	1.6	2
247	14.7	0	14.1	2
249	2.9	0	0	1
261	2.9	3.3	1.6	3
276	10.3	3.3	4.7	3
295	1.5	0	1.6	2
307	1.5	3.3	1.6	3
321	16,2	0	18.8	2
330	1.5	0	0	1
332	0	6.7	6.3	2
334	0	3.3	0	1
335	1.5	0	0	1
345	7.3	3.3	0	2
350	1.5	6.7	1.6	3
368	1.5	0	0	1
372	0	3.3	0	1
378	0	3.3	1.6	2
391	4.4	0	0	1
393	5.9	10	10.9	2
397	0	0	1.6	1
406	0	0	1.6	1
414	0	6.7	6.3	2
423	1.5	0	0	1
432	2.9	0	7.8	2
446	0	10	1.6	2
457	1.5	0	0	1
462	0	10	0	1
474	4.4	0	0	1
493	1.5	0	0	1
510	0	6.7	0	1
515	1.5	0	0	1
Na				

[1]DBN: Durban, PMB: Pietermaritzburg, DBN/KWM: Durban/KwaMashu

[2] Pop: number of populations sharing an allele



1. Gallus_gallus_haplotype_B4		0,000	0,013	0,013	0,010	0,012	0,013	0,013	0,013	0,013	0,013	0,013	0,018	0,013	0,013	0,024	0,015	0,015	0,025	0,010
2. Gallus_gallus_haplotype_BW3	0,000		0,013	0,013	0,010	0,012	0,013	0,013	0,013	0,013	0,013	0,013	0,018	0,013	0,013	0,024	0,015	0,015	0,025	0,010
3. Gallus_gallus_haplotype_B10	0,023	0,023		0,000	0,008	0,011	0,000	0,000	0,010	0,010	0,010	0,000	0,013	0,010	0,000	0,021	0,007	0,008	0,023	0,008
4. Gallus_gallus_haplotype_B8	0,023	0,023	0,000		0,008	0,011	0,000	0,000	0,010	0,010	0,010	0,000	0,013	0,010	0,000	0,021	0,007	0,008	0,023	0,008
5. Gallus_gallus_haplotype_B19	0,015	0,015	0,008	0,008		0,007	0,008	0,008	0,007	0,007	0,007	0,008	0,015	0,007	0,008	0,022	0,011	0,011	0,024	0,000
6. Gallus_gallus_haplotype_B11	0,023	0,023	0,015	0,015	0,008		0,011	0,011	0,010	0,010	0,010	0,011	0,017	0,010	0,011	0,023	0,013	0,013	0,025	0,007
7. Gallus_gallus_haplotype_B14	0,023	0,023	0,000	0,000	0,008	0,015		0,000	0,010	0,010	0,010	0,000	0,013	0,010	0,000	0,021	0,007	0,008	0,023	0,008
8. Gallus_gallus_haplotype_B130	0,023	0,023	0,000	0,000	0,008	0,015	0,000		0,010	0,010	0,010	0,000	0,013	0,010	0,000	0,021	0,007	0,008	0,023	0,008
9. Gallus_gallus_haplotype_B23	0,023	0,023	0,015	0,015	0,008	0,015	0,015	0,015		0,000	0,000	0,010	0,016	0,000	0,010	0,022	0,013	0,013	0,024	0,007
10. Gallus_gallus_haplotype_B18	0,023	0,023	0,015	0,015	0,008	0,015	0,015	0,015	0,000		0,000	0,010	0,016	0,000	0,010	0,022	0,013	0,013	0,024	0,007
11. Gallus_gallus_haplotype_B29	0,023	0,023	0,015	0,015	0,008	0,015	0,015	0,015	0,000	0,000		0,010	0,016	0,000	0,010	0,022	0,013	0,013	0,024	0,007
12. Gallus_gallus_haplotype_B21	0,023	0,023	0,000	0,000	0,008	0,015	0,000	0,000	0,015	0,015	0,015		0,013	0,010	0,000	0,021	0,007	0,008	0,023	0,008
13. Gallus_gallus_domesticus_3_HWK	0,046	0,046	0,023	0,023	0,031	0,038	0,023	0,023	0,038	0,038	0,038	0,023		0,016	0,013	0,024	0,015	0,015	0,026	0,015
14. Gallus_gallus_domesticus_4_HWK	0,023	0,023	0,015	0,015	0,008	0,015	0,015	0,015	0,000	0,000	0,000	0,015	0,038		0,010	0,022	0,013	0,013	0,024	0,007
15. Gallus_gallus_domesticus_22_HWK	0,023	0,023	0,000	0,000	0,008	0,015	0,000	0,000	0,015	0,015	0,015	0,000	0,023	0,015		0,021	0,007	0,008	0,023	0,008
16. Gallus_gallus_domesticus_71_DBN	0,084	0,084	0,061	0,061	0,069	0,076	0,061	0,061	0,069	0,069	0,069	0,061	0,084	0,069	0,061		0,021	0,022	0,017	0,022
17. Gallus_gallus_domesticus_74_DBN	0,031	0,031	0,008	0,008	0,015	0,023	0,008	0,008	0,023	0,023	0,023	0,008	0,031	0,023	0,008	0,069		0,010	0,024	0,011
18. Gallus_gallus_domesticus_51_PMB	0,031	0,031	0,008	0,008	0,015	0,023	0,008	0,008	0,023	0,023	0,023	0,008	0,031	0,023	0,008	0,069	0,015		0,024	0,011
19. Gallus_gallus_domesticus_54_PMB	0,092	0,092	0,076	0,076	0,084	0,092	0,076	0,076	0,084	0,084	0,084	0,076	0,099	0,084	0,076	0,046	0,084	0,084		0,024
20. Gallus_gallus_domesticus_60_PMB	0,015	0,015	0,008	0,008	0,000	0,008	0,008	0,008	0,008	0,008	0,008	0,008	0,031	0,008	0,008	0,069	0,015	0,015	0,084	

Figure 4.8: Estimates of Evolutionary Divergence between Sequences.

Figure 4.8 depicts the number of base differences per site from between sequences. Standard error estimates are shown in the last column. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 131 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.

Table 4.5: The MHC haplotype sequences that were extracted from GenBank and compared with sequences from South African chickens.

***Gallus gallus* haplotype microsatellite LEI0258 sequence**	**GenBank Accession number**
B4	DQ239540
BW3	DQ239561
B10	DQ239494
B8	DQ239556
B19	DQ239516
B11	DQ239495
B14	DQ239508
B130	DQ239506
B23	DQ239532
B18	DQ239515
B29	DQ239539
B21	DQ239529.1

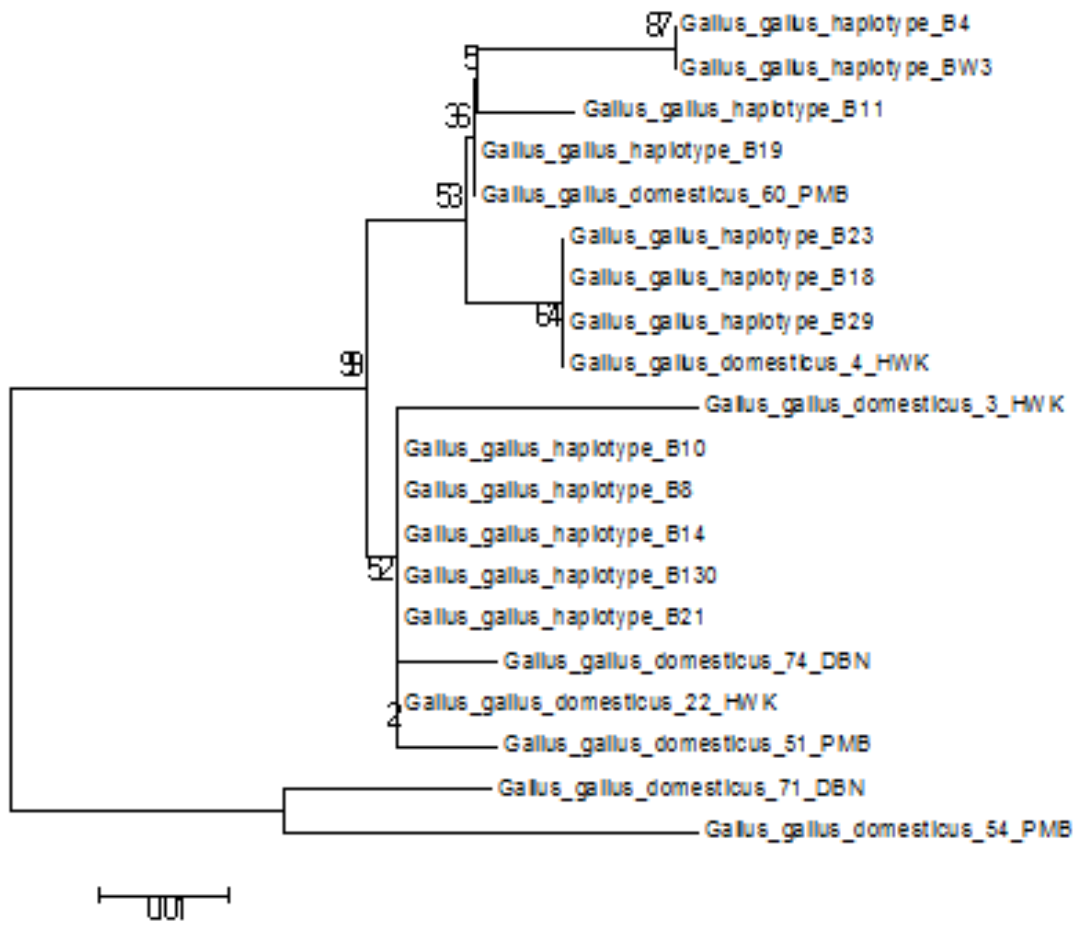


Figure 4.9: Analysis of 12 GenBank sourced and 8 South African LEI0258 sequences. Neighbor-joining dendrogram calculated using a maximum-likelihood computation with 1000 bootstrap iterations.

There is very strong evidence (99%) that the MHC LEI0258 haplotypes are related and similar except for a chicken from the KwaMashu/Durban population (ID = 54) and a fowl from the Pietermaritzburg population. It is interesting that the Pietermaritzburg sequence shared 96% identity with the B130 sequence. From Figure 4.9 it is evident that the sequence from the Pietermaritzburg chicken has undergone a number of nucleotide substitutions or changes and may be the reason it has been excluded from the other sequences. There was also moderately high evidence of the B23, B18 and B29 haplotype sequences being related to each other as well as to a LEI0258 sequence from a chicken from the Howick population (ID = 4). Nucleotide Blast results corroborate this relationship with the Howick sequence and B18 sequencing having 93% identity.

4.5 Discussion

Across all 3 populations that were sampled, a total number of 36 alleles (Na) were detected while the number of effective alleles (Ne) was 15.94 (Table 4.2). And these alleles ranged in size from 182bp to 515bp (Table 4.4). The range in this study correlated with the LEI0258 size ranges previously reported in other literature (Chazara et al., 2013; Ngeno et al., 2014). The frequencies for each allele were quite varied as they spanned from a frequency of 0.62% for the 372bp allele to 14.20% for the 321bp allele (Figure 4.5). In addition to this, seven of the Na alleles were shared in all 3 populations. The 247bp allele also had a higher frequency of 11.72% as well as the 393bp allele with a frequency of 8.64% (Figure 4.5). 19 of the 36 total alleles that were detected had been previously reported in different parts of Canada, Asia and Africa (Fulton et al., 2006; Guangxin et al., 2014; Ncube et al., 2014) .

The Howick indigenous chicken population, although the same size as the Durban/KwaMashu population, had the highest number of alleles which was 25 of the total 36 reported alleles (Table 4.3). There were 21 LEI0258 alleles detected in the Durban/KwaMashu population, followed lastly by the Pietermaritzburg population which only had 16 alleles (Table 4.3).

Table 4.4 shows that the alleles 182, 194, 218, 261, 276, 307 and 350 were common among all 3 sampled populations. Studies have uncovered a correlation between the 261 bp LEI0258 allele which was found in all 3 of the South African indigenous chicken populations. Fulton et al., 2006 assigned this allele the B2 haplotype and in another study chickens with this haplotype exhibited decreased signs of illness after being infected with Infectious Bronchitis Virus disease (Banat et al., 2013). Other work demonstrated that the B2 haplotype in fact expressed dominance over other haplotypes (Joiner et al., 2007) hence making the conservation of this allele in the local populations quite important. There were also 12 more alleles that were found present in at least two of the sampled populations (Table 4.4). Out of the 36 alleles detected, 20 of them were found to be private alleles. The Howick population had majority of the private alleles while the Pietermaritzburg and Durban/KwaMashu populations only had 5 and 4 private alleles respectively. The Howick population had the highest heterozygosity of 0.58 (Table 4.3) indicating that this population had more heterozygotes for the LEI0258 marker than the other two. The high heterozygosity may be linked to the excess number of private alleles (11) found in this population. Private alleles are alleles that are not shared among the sampled populations and due to their scarcity, can be considered rare alleles. Therefore in the

goal of conservation of indigenous chickens, these alleles would be a priority as they could become extinct quite easily.

The Pietermaritzburg and Durban/KwaMashu observed heterozygosity figures were 0.47 and 0.52 respectively (Table 4.3). All the observed heterozygosity values were under 0.60 across all 3 populations and considerable lower than the expected heterozygosity (He) figures which were all above 0.90. In Kenyan indigenous chicken ecotypes the observed heterozygosity values for LEI0258 ranged from 0.82 to 0.98 while another study on the marker in indigenous Egyptian ecotypes had He values which ranged from 0.67 to 0.88. The high values indicated high genetic variation at this locus reported in the Kenyan population may be a result of geographical isolation (Izadi et al., 2011; Ngeno et al., 2014). Although the chickens in this study were sampled from different areas, there would not be considered geographically isolated as these areas were all with KwaZulu Natal and this is what may have contributed to a lower heterozygosity in the populations. Overall, as the observed heterozygosity were mostly over or close to 0.5, we cannot say that there was no genetic variation in these populations. However it is possible to deduce from the He values that the South African chickens are moderately inbred at this MHC locus. Ncube et al. (2014) reported similar findings in South African village chickens populations. It would also be beneficial from a disease prevention stand-point to cultivate chickens that are more heterozygous. Research into the chicken MHC reports on how an increased LEI0258 heterozygosity frequency can also in turn increase the diversity of antigens being presented to T cells which should increase tolerance to infections (Chazara et al., 2013). The vast difference between Ho and He is an indicator of each of the populations not being in Hardy-Weinberg equilibrium (HWE). There a number of factors that can cause this deviation from HWE but due to the natural conditions such as harsh climate and viral/bacterial infections that indigenous chickens are exposed to, the occurrence of natural selection at the MHC locus to combat these challenges could be the main cause for deviation (Zhang et al., 2002; Osman et al., 2002; Ncube et al.,2014) . Natural selection is a mechanism of evolution where by the alleles that are advantageous for the survival of the organism are selected for in the population and to abide by HWE assumptions, a population in HWE should not be undergoing evolution at the locus (Hartl et al., 1997).

The fixation index (F) values ranged from 0.37 to 0.49 with the Pietermaritzburg population being the highest (Table 4.3). Since the fixation index value can fall between -1 to 1, these are relatively high F values as each population had a value over 0.37. These values lean towards the possibility of a degree of inbreeding in each of these populations (Simon and

Buchenauer, 1993). The Shannon information index (I) was also calculated to give an idea of the genetic population structure as it measures the species richness and evenness in populations in different regions. The I value for the three populations were within the same region with a mean I value of 2.71 (Table 4.3). In what is considered to be a "rich eco-system", the I value generally falls between 1.5 and 3.5 and the higher the I value the more the number of species increases or the more even the distribution becomes (McDonald and Dimmick, 2003). Therefore all 3 populations reported in this study can be considered as genetically evenly distributed and this can be said for the total sampled population as the overall I value is 3.12 (Table 4.2).

4.6 Conclusion

The Howick indigenous chicken population had the highest heterozygosity, the highest number of alleles, the highest Shannon information index value as well as the lowest fixation index value (Table 4.3). This data alludes to the conclusion of the Howick population having the most genetic variation in the MHC when compared to the other two populations. Between the Pietermaritzburg and KwaMashu/Durban populations it is difficult to say which has the least variation as the results for these two populations are very similar. As previously mentioned the overall heterozygosity value for the total sampled South African indigenous chickens indicated a degree of inbreeding in the population and this may be due to natural selection. It can be concluded that the populations of South African indigenous chickens in this study were polymorphic at the LEI0258 locus however diversity across the sampled populations is moderate. Information such as this is important in preventing the erosion of these genetic resources.

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Chapter 5: General Discussion, Conclusions and Recommendations

5.1 General Discussion and Conclusions

Alternative methods to using antibiotics and methods that can be used in conjunction with vaccination are currently being investigated. Using the genes that play important roles in the innate and adaptive immune system to select for more resistant livestock is a practice that is becoming popular Several studies have reported on the correlation between the chicken adaptive immune and disease resistance/susceptibility. These studies found that certain MHC haplotypes resulted in lower or higher response to viral, bacterial or parasitic diseases such as Marek's disease (Bacon et al., 2001), Infectious Bronchitis (Banat et al., 2013), *Salmonella* (Liu et al., 2002) and Avian Influenza Virus (Boonyanuwat et al., 2006). The LEI0258 marker is a highly polymorphic microsatellite marker found in the MHC and was the molecular marker used to assess the genetic variability in the MHC in non-commercial chickens as little is known of the variation at this locus in South African Indigenous chickens. The findings reported in this study correlated with those that were reported by Ncube et al., (2014) which also concluded that genetic variation at the LEI028 locus was lower than expected in South African chickens (Ncube et al., 2014). This deficiency in genetic variation at this locus has been reported in other African chicken studies and has been attributed to natural selection occurring in these indigenous chicken populations that are exposed to disease vectors and this likely could have happened in the South African chicken populations in this study (Pinard-Van Der Laan, 2002; Izadi et al., 2011).

The 261 bp allele was detected in all 3 populations. This allele has been classified as the B2 haplotype (Fulton et al., 2006) and has an association with decreased signs of illness caused by IBV and has also been reported to express dominance over other haplotypes (Joiner et al., 2007; Banat et al., 2013). The Pietermaritzburg population had the highest frequency for this allele at 3.3% while the Durban/KwaMashu population had the lowest frequency of 1.6%. The 307bp allele was also detected in all three sampled populations and once again the highest frequency of this allele was in the Pietermaritzburg population. A Tanzanian study has previously found a correlation between the 307bp allele and lowered primary antibody response to Newcastle Disease ($p<0.05$) (Lwelamira et al., 2008). From this study 11 LEI0258 alleles that remain undescribed in other literature were detected and this means that there is no information on the association between the unique alleles detected in South Africa the various diseases there are.

Using Marker assisted selection with the aid of candidate genes is a practice that has been used a lot in order to increase productivity such as in the dairy industry were certain genes have been found to increase milk yield as well as genes that when selected for, make cattle genetically more resistant to tick infestation (Grisart et al., 2002; Muhammad et al., 2008). By investigating the innate immune system genes polymorphisms present in local populations, the goal is to also select for genetically disease resistant chickens (Malek and Lamont, 2003). Using the single nucleotide polymorphisms in INOS, TLR-4, MD-2 and SLC11A1 candidate genes, the genetic variation in these innate immune system genes was examined. High to moderate variation was observed in the TLR-4 and INOS loci while the SLC11A1 and MD-2 loci indicated that there was inbreeding, across the total sampled indigenous chickens. It had been hypothesized that the indigenous chickens would have more genetic variation than the commercial population and the results in this study corroborated with that hypothesis. The observed heterozygosity, fixation index at the SLC11A1 locus indicated much higher levels of inbreeding compared to the indigenous counterparts.

Results from investigating the genetic diversity in innate and adaptive immune response genes as what has been done in this study, can be used as an initial guide in the development of conservation programmes and even in the development of breeding stocks for additional disease resistance studies. Disease resistance gene association studies have been conducted in many environments however none have been reported on in South African indigenous. Disease resistance is a poly-factorial trait with the environment being one of these factors. The environment has an influence on the heritability of a trait (Gibson and Bishop, 2005) therefore, it is essential for more of these association studies to be designed and carried out in South Africa based on MHC and innate immune system genes genetic evaluation information.

5.2 Recommendations

Although the PCR-RFLP method is affordable and practical for a study such as the current one, using high throughput SNP genotyping technologies such as the (60k) Illumina SNP BeadChip, This technique is becoming popular in association studies as well as a breeding tool in what is being referred to as genomic breeding (Groenen et al., 2011). Large SNP information would also help in the evaluation of the genetic population structures from the different areas.

For future studies increasing the number of areas sampled within South Africa and even the surrounding countries will build an even more comprehensive study in which the data will

give a better representation of the variation in the chicken MHC. From this, association studies must be implemented in order to see the effect of different MHC haplotypes on diseases in South Africa and the neighbouring countries. Although studies like this this such as the Tanzanian study have been conducted, it is necessary for different regions to conduct their own studies as phenotypes, such as disease resistance, may also be affected by the environment as well as the genetic component.

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Appendix

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Table S1: The innate immune system candidate genes' genotypes for the total sampled indigenous chickens.

Chicken ID	Locus			
	SLC11A1-*Sac I*	**INOS-*Alu I***	**MD-2-*Ase I***	**TLR4-*Sau 96I***
1	CT	CT	GG	CG
2	TT	CT	GG	CG
3	TT	TT	AG	CG
4	TT	TT	GG	CC
5	CC	TT	AG	CC
6	TT	CT	GG	CG
7	CT	TT	AA	CG
8	CC	TT	GG	CG
9	CC	CT	GG	CG
10	CC	TT	AA	CG
11	CC	CT	GG	CG
12	CC	CT	GG	CG
13	CC	TT	GG	CC
14	CT	TT	AG	CG
15	CC	CT	AA	CG
16	CC	TT	AA	CG
17	CT	CT	AA	CG
18	CC	CC	GG	GG
19	CC	TT	AA	CG
20	CC	TT	GG	CG
21	CC	CT	AA	GG
22	CC	TT	AG	CG
23	CC	TT	AG	CG
24	CC	CC	AA	CG
25	CT	CC	AG	CG
26	CC	CT	AG	CG
27	CT	CT	GG	CG
28	CC	CC	GG	CG
29	TT	TT	GG	CC
30	CT	CT	GG	GG
31	CT	CT	GG	CG
32	CT	CT	GG	CG
33	CT	TT	GG	CG
34	CC	CC	GG	CG
35	TT	CC	AG	CG
36	CT	CT	GG	CG
37	CT	CT	GG	CC

38	CC	CT	AG	CG
39	CT	CC	GG	GG
40	CC	TT	GG	CC
41	CC	CT	GG	CG
42	CC	CT	GG	CG
43	TT	CT	AG	CG
44	CC	CC	GG	CC
45	CC	CT	GG	CC
46	CC	CT	GG	CG
47	CT	TT	GG	CG
48	TT	CC	GG	CG
49	TT	CT	AG	CG
50	TT	CT	AG	GG
51	CC	TT	GG	CG
52	CC	CC	GG	CG
53	CT	TT	AG	CG
54	CT	TT	GG	GG
55	CC	CT	AG	GG
56	CC	CC	GG	CC
57	CC	TT	AG	CG
58	CC	CT	GG	CG
59	CT	CT	GG	GG
60	CC	CC	GG	GG
61	CC	CC	GG	CG
62	CC	CT	GG	GG
63	TT	TT	GG	CG
64	CT	TT	GG	CG
65	TT	CT	AG	GG
66	TT	CT	GG	GG
67	CT	CC	GG	CG
68	TT	CT	GG	GG
69	TT	CT	AG	GG
70	CT	CT	GG	CG
71	CT	CT	AG	GG
72	TT	CC	GG	CG
73	TT	CT	GG	CG
74	TT	CT	AG	CG
75	CT	CT	AG	CG
76	CT	CT	AA	CG
77	TT	CT	AG	CG
78	TT	CT	AG	CG
79	CT	TT	AA	CG
80	CC	CC	AA	GG

81	CC	CT	GG	GG

Table S2: Sample Size, Information Index, Observed Heterozygosity, Expected and Unbiased Expected Heterozygosity, and Fixation Index for SLCA11A1-*Sac I* locus.

Pop	**Locus**	**N**	**I**	**Ho**	**He**	**uHe**	**F**
HWK	SLC11A1	33	0.606	0.294	0.415	0.421	0.292
PMB	SLC11A1	15	0.673	0.267	0.480	0.497	0.444
DBN/KWA	SLC11A1	33	0.693	0.313	0.500	0.508	0.375

Table S3: Sample Size, Information Index, Observed Heterozygosity, Expected and Unbiased Expected Heterozygosity, and Fixation Index for INOS-*Alu I* locus.

Pop	**Locus**	**N**	**I**	**Ho**	**He**	**uHe**	**F**
HWK	INOS	33	0.637	0.424	0.444	0.451	0.045
PMB	INOS	15	0.673	0.533	0.480	0.497	-0.111
DBN/KWA	INOS	33	0.693	0.576	0.500	0.508	-0.152

Table S4: Sample Size, Information Index, Observed Heterozygosity, Expected and Unbiased Expected Heterozygosity, and Fixation Index for TLR4-*Sau 96 I* locus.

Pop	**Locus**	**N**	**I**	**Ho**	**He**	**uHe**	**F**
HWK	TLR4	33	0.693	0.788	0.500	0.507	-0.577
PMB	TLR4	15	0.673	0.667	0.480	0.497	-0.389
DBN/KWA	TLR4	33	0.625	0.576	0.434	0.441	-0.327

Table S5: Sample Size, Information Index, Observed Heterozygosity, Expected and Unbiased Expected Heterozygosity, and Fixation Index for MD2-*Ase I* locus.

Pop	**Locus**	**N**	**I**	**Ho**	**He**	**uHe**	**F**
HWK	MD2	33	0.647	0.212	0.454	0.461	0.533
PMB	MD2	15	0.325	0.200	0.180	0.186	-0.111
DBN/KWA	MD2	33	0.586	0.364	0.397	0.403	0.083

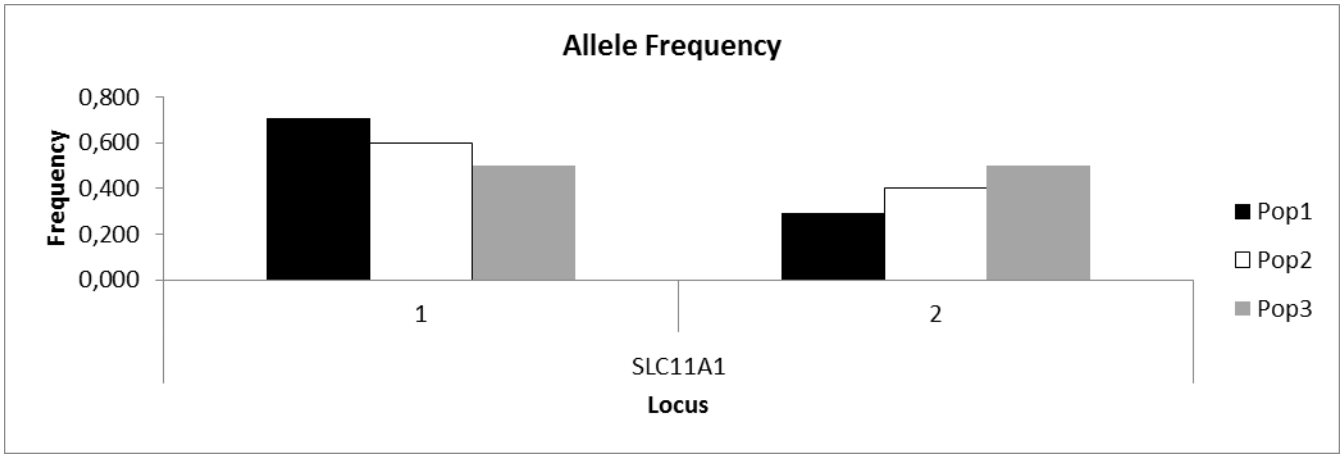

Figure S1: Allele Frequencies by Population for SLCA11A1-*Sac I* locus. Pop 1; Howick, Pop 2; Pietermaritzburg, Pop 3; Durban/KwaMashu.

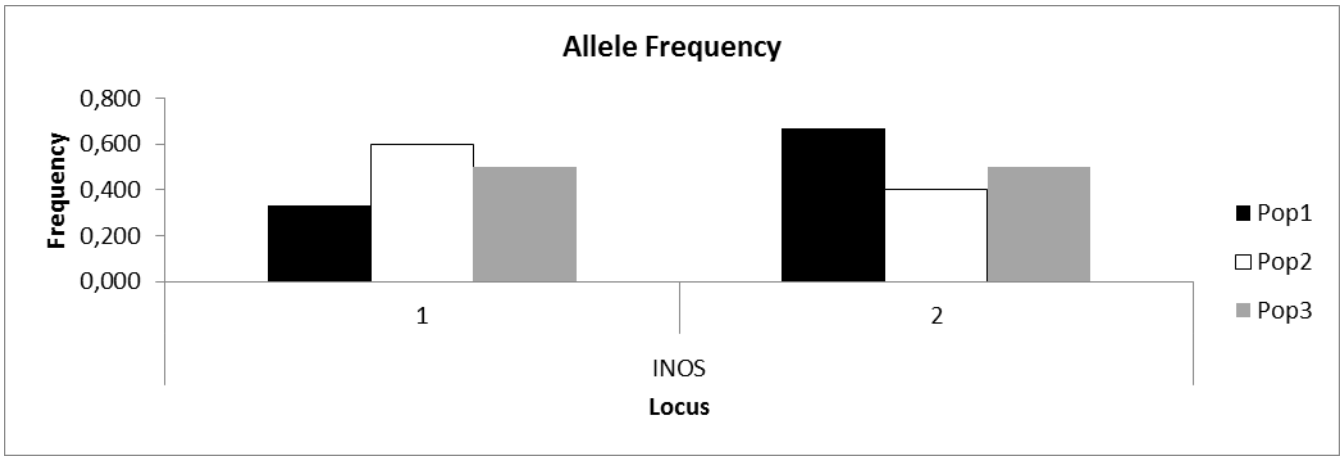

Figure S2: Allele Frequencies by Population for INOS-*Alu I* locus. Pop 1; Howick, Pop 2; Pietermaritzburg, Pop 3; Durban/KwaMashu.

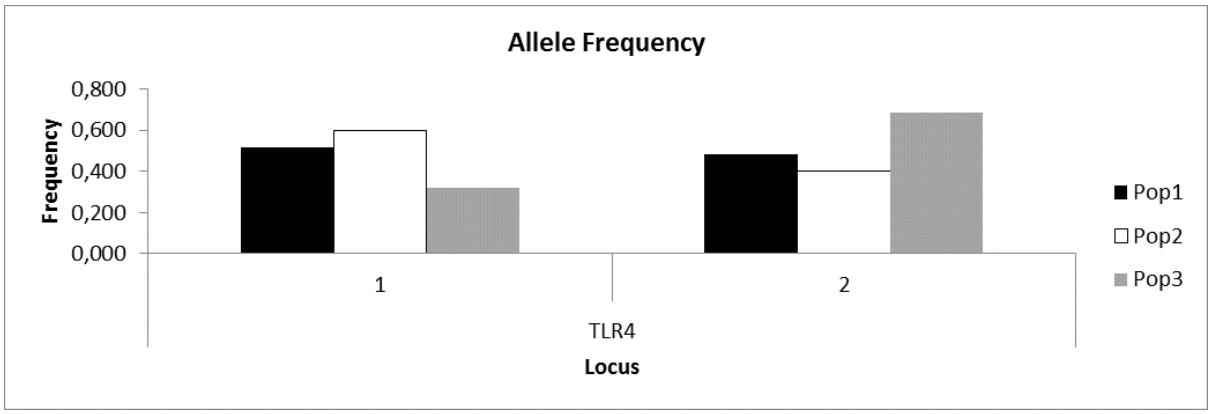

Figure S3: Allele Frequencies by Population for TLR4-*Sau 96 I* locus. . Pop 1; Howick, Pop 2; Pietermaritzburg, Pop 3; Durban/KwaMashu.

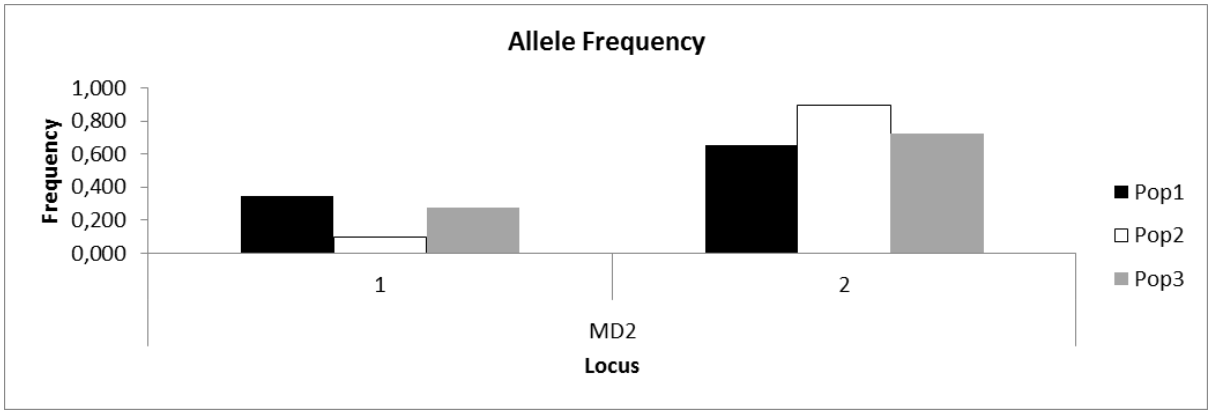

Figure S4: Allele Frequencies by Population for MD2-*Ase I*. Pop 1; Howick, Pop 2; Pietermaritzburg, Pop 3; Durban/KwaMashu.

Table S6: SLC11A1-Sac 1 genotypes detected in commercial chicken population.

Chicken ID	**Locus SLCA11A1-Sac I**
C1	TT
C2	TT
C3	CC
C4	CT
C5	CC
C6	CT
C7	CC
C8	CC
C9	CC
C10	CC
C11	CC
C12	CC
C13	CC
C14	CC
C15	CC
C16	CC
C17	CC
C18	CC
C19	CC
C20	CC
C21	CC
C22	TT
C23	CC
C24	CT
C25	CC
C26	CC
C27	TT
C28	CC
C29	CT
C30	TT

Table S7: LEI0258 alleles detected in indigenous chicken populations.

Sample no.	Pop	LEI0258 allele size (bp)	
1	HWK	273	273
2	HWK	249	249
3	HWK	247	247
4	HWK	273	273
5	HWK	247	247
6	HWK	345	247
7	HWK	457	345
8	HWK	287	236
9	HWK	345	247
10	HWK	287	171
11	HWK	322	194
12	HWK	273	247
13	HWK	262	262
14	HWK	322	322
15	HWK	335	182
16	HWK	423	247
17	HWK	346	346
18	HWK	322	322
19	HWK	322	322
20	HWK	322	322
21	HWK	322	322
22	HWK	207	207
23	HWK	393	330
24	HWK	474	391
25	HWK	474	391
26	HWK	515	225
27	HWK	493	351
28	HWK	368	290
29	HWK	393	305
30	HWK	474	391
31	HWK	432	239
32	HWK	247	247
33	HWK	432	239
34	PMB	393	393
35	PMB	462	266
36	PMB	462	462
37	PMB	446	350
38	PMB	510	510
39	PMB	393	305
40	PMB	446	446
41	PMB	393	393

42	PMB	372	184
43	PMB	354	174
44	PMB	345	165
45	PMB	334	274
46	PMB	332	332
47	PMB	215	215
48	PMB	414	414
49	DBN/KWA	378	199
50	DBN/KWA	393	393
51	DBN/KWA	332	332
52	DBN/KWA	312	220
53	DBN/KWA	397	208
54	DBN/KWA	378	192
55	DBN/KWA	393	393
56	DBN/KWA	247	247
57	DBN/KWA	414	414
58	DBN/KWA	432	247
59	DBN/KWA	332	332
60	DBN/KWA	247	247
61	DBN/KWA	393	393
62	DBN/KWA	432	247
63	DBN/KWA	322	322
64	DBN/KWA	322	322
65	DBN/KWA	322	322
66	DBN/KWA	322	322
67	DBN/KWA	170	170
68	DBN/KWA	287	171
69	DBN/KWA	414	414
70	DBN/KWA	446	350
71	DBN/KWA	287	171
72	DBN/KWA	287	236
73	DBN/KWA	223	223
74	DBN/KWA	295	183
75	DBN/KWA	393	305
76	DBN/KWA	322	194
77	DBN/KWA	406	262
78	DBN/KWA	247	247
79	DBN/KWA	432	247
80	DBN/KWA	432	329
81	DBN/KWA	432	329

Table S8: Allele frequencies for LEI0258 locus.

Locus LEI0258	Allele	HWK	PMB	DBN/KWA
	182	0.015	0.033	0.015
	192	0.000	0.000	0.015
	194	0.030	0.067	0.091
	207	0.030	0.000	0.015
	218	0.015	0.067	0.045
	236	0.045	0.000	0.015
	247	0.152	0.000	0.136
	249	0.030	0.000	0.000
	261	0.030	0.033	0.015
	276	0.106	0.033	0.045
	295	0.015	0.000	0.015
	307	0.015	0.033	0.015
	321	0.167	0.000	0.182
	330	0.015	0.000	0.000
	332	0.000	0.067	0.061
	334	0.000	0.033	0.000
	335	0.015	0.000	0.000
	345	0.076	0.033	0.000
	350	0.015	0.067	0.015
	368	0.015	0.000	0.000
	372	0.000	0.033	0.000
	378	0.000	0.000	0.030
	391	0.045	0.000	0.000
	393	0.030	0.167	0.106
	397	0.000	0.000	0.015
	406	0.000	0.000	0.015
	414	0.000	0.067	0.061
	423	0.015	0.000	0.000
	432	0.030	0.000	0.076
	446	0.000	0.100	0.015
	457	0.015	0.000	0.000
	462	0.000	0.100	0.000
	474	0.045	0.000	0.000
	493	0.015	0.000	0.000
	510	0.000	0.067	0.000
	515	0.015	0.000	0.000

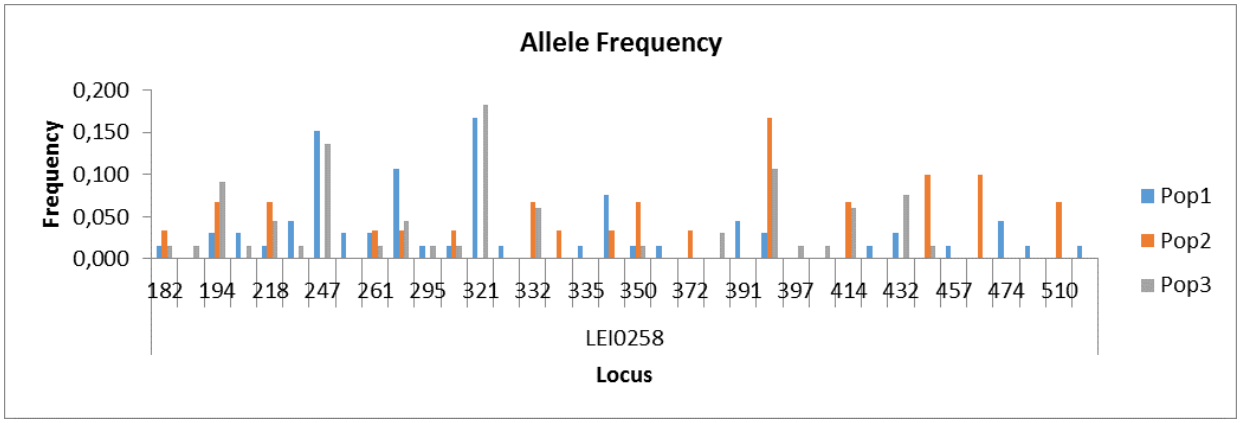

Figure S5: Allele Frequencies by Population for LEI0258 locus. Pop 1; Howick, Pop 2; Pietermaritzburg, Pop 3; Durban/KwaMashu.