GENETIC EVALUATION OF IMMUNOGENETIC VARIATIONS, MUTATIONAL LOAD AND EFFECTIVE POPULATION SIZE IN INDIGENOUS CHICKEN

ESTHER KULUNDU MBAKAYA

A thesis submitted to the Graduate School in partial fulfillment of the requirements for the Master of Science in Animal Breeding and Genetics of Egerton University

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has neither, wholly or in parts been presented nor concurrently been presented for the conferment of any degree in this or any other institution

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ESTHER KULUNDU MBAKAYA

KM111/13602/19

Recommendation

This thesis is the candidate's original work and has been prepared with our guidance and assistance. Therefore, it has been submitted with our approval as the official university supervisors.

Signature Date 10-11-2022

Dr. Kiplangat Ngeno, PhD

Department of Animal Science

Egerton University

Signature. Date 10-11-2022

Dr. Thomas Muasya, PhD (*Dr. rer. agr*)

Department of Animal Science

Egerton University

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DEDICATION

I dedicate this thesis to the memory of my late father Julius Shikuku Mbakaya and my loving mother Roselyne Ayako Mbakaya

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ABSTRACT

Modern poultry production houses large flock of birds at high stocking density thus increasing risk of diseases, and disease spreading. Control of diseases through improved management, vaccination and prophylaxis to a large extend increases costs and affects animal welfare negatively. One promising strategy to increase general disease resistance is by selectively breeding for immune traits using antibody titers against particular pathogen. The study aimed to examine the population structure of 150 Indigenous chickens (IC) at genome level. Secondly, it aimed to identify the impacts of deleterious mutations with relation to disease resistance and undertaking functional characterization of deleterious variants. Lastly, the study aimed to estimate Linkage Disequilibrium -based effective population size, rate and levels of inbreeding. Objective one addresses phenotypic and genetic clustering. Phenotypic clustering based on body weight and antibody titers revealed two significantly ($P < 0.001$) distinct groups of IC. Cluster with high mean in bodyweight and low mean in titers and vice versa. Genetic characterization at whole genome level grouped the IC into two groups providing a deeper understanding of the structure of IC population to supplement the use of phenotypes. At chromosome 16 level grouped IC as a single population. Part two of the study identified deleterious alleles and carry out gene ontology analysis. Using whole genome data, 182 deleterious genes were identified, of which, six were related to immune traits. The six genes included; *FANCA, RBBP5, CRB1, RUFY3, FBXO38,* and *PDE3A*. Results revealed numerous biological and KEGG processes that are involved in immune and disease resistance traits. Analysis of the ontology of the ENSGALG00000046739 candidate gene that was identified in chromosome 16 (MHC region) was linked with missense variants associated with the regulation of RNA polymerase II transcription. The third part of the study confirmed that effective population size of IC is decreasing whereas the inbreeding rate is increasing as the number of generations increase. The rate and levels of inbreeding seem to be in line with low effective population size thus leading to loss of diversity in IC. The findings in this thesis provide insight understanding on the immuno-genetic variations, genomic diversity. The knowledge generated in this study is useful for the development of a sustainable IC breeding programme for enhanced IC productivity and improve disease resistance. Consequently, reducing the need for vaccine and antibiotic treatments, thereby reducing drug residues in IC products.

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LIST OF ABBREVIATIONS

AB	Antibody
APC	Antigen presenting cells
CV	Cross Validation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent assay
FRS	Free-range system
IgA	Immunoglobulins A
IC	Indigenous chicken
IgG	Immunoglobulins G
IgM	Immunoglobulins M
IS	Intensive system
IgY	Immunoglobulins Y
LD	Linkage disequilibrium
MHC	Major Histocompatibility Complex
NAb	Natural antibody
ND	Newcastle Disease
N_e	Effective population size
NPDP	National Poultry Development Programme
PCA	Principal Component Analysis
SIS	Semi-intensive system
VEP	Variant Effect Predictor
BW	Body Weight
BWA	Burrows-Alignment tool
CV	Cross Validation
PCA	Principal Component Analysis
SNP	Single Nucleotide Polymorphism
F_{ST}	Fixation index

CHAPTER ONE

INTRODUCTION

1.1 Background information

Indigenous Chicken (IC) farming in developing countries has been gradually shifting from subsistence to commercial due to increased demand for IC products (Magothe *et al.,* 2012). Commercial farming of IC makes farmers house birds in large flocks at high stocking density, thereby increasing the risk and spread of diseases. Bwana et al. (2018) stated the effects of infectious diseases are so major on the productivity among IC famers since very few prophylactic measures and vaccinations are used. These infectious diseases lead to economic losses through reduced production, cost of disease treatment and control (Haunshi, *et al.,* 2015). Dar *et al.* (2018) argue that the effects of diseases in the poultry industry is directly hefty on the lives of the poor households in developing countries where up to 25% of their monthly earnings might be lost as a result of the diseases.

Disease control methods have contributed a great part in the treatment and prevention of diseases although they have become highly problematic and intolerable in several markets. Drugs like antibiotics, when used without following instructions leads to drug resistance and residues of the drug in the IC products (Nikolaidou *et al.,* 2008; Tajick & Shohreh, 2006). Vaccination is commonly used to prevent diseases, however, variations of pathogens' genetic makeup and frequent mutations render some vaccines ineffective (Müller *et al.,* 2012). Hence, it is significant to consider alternative disease prevention methods other than vaccination and treatment that can be helpful in the reduction of the effect of infectious disorders. According to San (2013), Berghof *et al.* (2015) and Khobondo *et al.* (2016), selective breeding based on genomic technologies may advance alternative and sustainable mitigation strategy against diseases. Improving genetic resistance to infectious diseases, which could potentially be genetically correlated to production traits are yet to be considered by the available technologies. Selection for growth and production traits has been associated with decreased immunity (Bayyari *et al*., 1997; Wondmeneh *et al*., 2015). Selection for increased body weight (BW) has been shown to be genetically associated with a reduction in disease resistance in chicken. Other studies have reported the opposite in turkey (Li *et al*., 2000). Phenotypic correlations between BW & antibody (AB) are very low. Genetic correlations between BW & AB in chicken (r_g) are medium to high (Mebratie *et al.,* 2019).

Artificial and natural selection in IC can increase inbreeding, deleterious genetic variants and reduce effective population size (Qanbari *et al.,* 2010; Okeno *et al.*, 2012; Khanyile *et al.,* 2015). Selection for gene variants that are favourable may increase homozygosity within domestic animals. If the selection coefficient against mutations is lower than the selection coefficient of the preferred allele that lies on the same haplotype, the allele frequency of the deleterious variants is expected to rise due to genetic hitch-hiking (Good & Desai, 2014). Therefore, slightly harmful mutations become over-represented in the regions of the genome under selection. The underlying genetic architecture of these deleterious effects in IC gene pools has not been documented.

Development of genomic selection and design breeding programs of IC can be attained by effective steps like exploring of the Linkage disequilibrium (LD) patterns. Effective population size (Ne) and LD influences the population structure (Wragg, *et al.,* 2012). This has been done and shown in domesticated animals such as cattle (Lee *et al.,* 2011; Porto-Neto *et al*., 2014), sheep (Liu *et al*., 2017) and chicken (Khanyile *et al.*, 2015a ;Pengelly *et al*., 2016). According to Karimi *et al*. (2016) and Porto-Neto *et al*. (2014), linkage disequilibrium is an important tool in assessing the inbreeding levels in relation to detection of long homozygous regions across the genome. In several studies, the LD method was implemented to estimate the Ne (Uimari & Tapio, 2011; Zalewski *et al*., 2016). However, no research has been conducted on the extent of LD and N_e in IC.

1.2 Statement of the problem

As intensification of IC farming increases, disease incidences are becoming a major challenge. Disease control measures like vaccines and antibiotics alleviates the disease problem, but leads to increased cost of production and reduced the quality of IC products due to drug residues. Use of antibiotics can also lead to environmental contamination as well antibiotic resistance. Breeding for disease resistance c is an alternative strategy of producing animals that are robust and resilient animals that can maintain production in the presence of diseases. However, breeding for disease resistance has not been done in IC because their genetic variation for disease resistance has not been quantified. Chicken genome carries some harmful mutations that can potentially alter fitness and health. However, the effects of these deleterious alleles in IC is not documented. Linkage disequilibrium based effective population (Ne) size explores the genetic basis of traits influencing productivity. Currently there have been no research aimed to

characterize the stages and extent of effective population size based on linkage disequilibrium, inbreeding rate, and levels of IC.

1.3 Objectives

1.3.1 Broad objective

To contribute to increased indigenous chicken productivity through enhanced disease resistance by assessing genetic and immunological diversity, and identification of deleterious alleles

1.3.2 Specific objectives

i. To assess immunogenetic variation of indigenous chicken genetic clusters
ii. To identify deleterious alleles & carry out their functional annotation in indigenous chicken
iii. To evaluate effective population size based linkage disequilibrium, inbreeding rate, and levels in indigenous chicken

1.4 Research questions

i. What are the differences in immunogenetic variation of IC genetic clusters?
ii. Which are the deleterious alleles and their functions in indigenous chicken?
iii. What are the estimates for effective population size based linkage disequilibrium, inbreeding rate, and levels in indigenous chicken?

1.5 Justification

Evaluation of genetic diversity of the immune system in chicken will provide the basis of understanding the differences in tolerance to diseases. Understanding genetic association between immune response and production traits in IC will lead to genetic improvement of the two traits. Understanding mutational load, LD-based Ne, inbreeding rate, and levels of IC will enable development of a breeding programme that maintains long-term genetic diversity and therefore fitness and viability of IC populations. Through breeding for disease resistance alongside performance traits will lead to reduction in the production costs, increased survival of the birds, avoid drug resistance and finally, there will be production of good quality chicken products thus increasing income of the farmers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Universally, chicken population is projected to be 25.9 billion with Africa consisting of about 1.3 billion of chickens and approximately 80% are said to indigenous chicken varieties kept in rural areas. About 100.8 million IC have been estimated to be kept in East Africa. (F.A.O. STAT, 2021) The Rwanda's poultry population is estimated to be about 5.3 million with IC consisting of nearly 70% (NISR, 2015). In Kenya, Ministry of Agriculture, Livestock, and Fisheries reported chicken population to be roughly 32 million with over 70% being Indigenous Chicken varieties. (MALF, 2015). Indigenous chickens play an important role by contributing to the food security of rural households across the developing world. According to Okello *et al.* (2010) and Magothe *et al.* (2012), Indigenous Chicken are vital source of both protein and income. The chicken business is therefore a vital source of employment, income, and food and has numerous cultural and social practices moreover having relations with other economic sectors (Kingori *et al.,* 2010).

Chickens (*Gallus gallus domesticus*) originated from the jungle birds (*Gallus gallus*) dwelling in Indo-China India, Philippines, Indonesia, and South China (Magothe *et al*., 2012). Human beings are perceived to have distributed chicken in the course of their movement. These birds were first presented through the Middle East into Egypt from South-western Asia. In many countries across the world, distinct indigenous chickens have been acknowledged and named (Vali, 2008). The names used define the mutual phenotypes in Africa include dwarf-sized, naked neck , frizzled feathered, bearded, feathered shanks, and barred feathered (Khobondo *et al.,* 2015; Kingori *et al.,* 2010; Ondwasy, *et al.,* 2006). Indigenous chicken vary in conformation, body size, performance and plumage colour (Mahoro *et al.,*2017; Nyaga *et al.,* 2007). Productivity is low due to factors like unimproved genotypes, poor nutrition, disease, and management. However, the birds are hardy and thrive under a harsh environment with minimal inputs, getting most of their food from scavenging and sporadically from the kitchen and other households (Moges *et al.,* 2010; Mwamachi *et al.,* 2000).

2.2 Purpose of indigenous chicken in the household economy

These chickens play an important role in household economies by converting feed resources existing in the household or homestead into rich nutritious protein products. Eggs and meats are the main animal protein source in the rural areas. Chicken eggs and meat provide a readily available, superior source of micronutrients, protein, and vitamins (Oloo *et al*., 2018). Eggs are an exceptional source of vitamin A, zinc and iron, all of which are crucial to wellbeing, health and growth (Chepkemoi *et al.*, 2017). Chicken and eggs play a crucial role in nutritious, balanced diet, which is especially key for children, nursing mothers, and many exposed groups. Other usages include dumping of kitchen remains, manure making, being biological clocks for telling the time of day especially in remote areas and a resource of monitoring sects insect (Magothe *et al.,* 2012b).

Chicken and eggs can be sold and the cash is used for home needs like clothes and buying medicines (Magothe *et al.,* 2012; Mahoro, *et al.*, 2017). In this way, they serve as a ready source of finance for small purchases , emergencies and source of capital accumulation (Ilatsia *et al.,*2017; Kingori *et al*., 2010) They also produce manure and contribute to pest control. Indigenous Chicken is also significant during special festivals, treatments, and traditional ceremonies such as cock fighting (Khobondo *et al.*, 2018).

2.3 Indigenous chicken production systems and performance

In the tropics, several production systems under which IC are kept have been acknowledged with various management regimes (Kingori *et al*., 2010). They comprise of free-range, semi-intensive and intensive production systems.

2.3.1 Free-range system (FRS)

Within this scheme, chicken are kept extensively for numerous motives including provision of meat and eggs for household nourishment, occasional basis of livelihood and various socio-cultural responsibilities. In this structure, birds leave their night housings in the morning and are allowed to move around as they source for any available food coffers within the homestead. Free-range food resources regularly comprise various seeds, insects, earthworms, and, grass (Moges *et al.,* 2010). Birds sometimes are restricted and supplemented with kitchen leftovers, maize, and any other availed food stuffs. Night shelters comprise stores, rudimentary coops, human habitats, and

kitchens. Due to limited inputs, production is also low but the value per unit of meat or egg is virtually negligible (Okitoi *et al.,* 2007).

2.3.2 Semi-intensive system (SIS)

In this system, between 5 and 50 chickens are kept majorly for sales and consumption. The commercial value of these birds is directly proportional to the input per stage which always ranges from low to medium. Chickens are allowed to wonder in free-range within the fenced runs or on the homestead with kitchen wastes, insects, grass, and any other available feed resource serving as their main feed (Mwamachi *et al.,* 2000). Birds are availed with different designs of shelter extending from basic shelters to properly built chicken house structures. Since the input stages are low, productivity is lower as compared to intensive System. This scheme is prevalent in areas with high human count density, remote and peri-urban areas. The commercial value attached to the enterprise remains the key determinant of the chicken health care. Sometimes veterinary or ethno-veterinary attention is provided though not effectively and mortality is 40-60% in undeveloped chicks (Moges *et al.,* 2010)

2.3.3 Intensive system (IS)

The production scheme entailing the confinement and isolation of birds with a constant balanced diet is known as the Intensive System (IS) or the restricted full-ration system (Moges *et al.* 2010). The most common components are slatted floors and deep litter. This system encourages vaccination against most endemic diseases. The bounded system shields the chicken from predators and thieves. However, the high input requirements deem it quite impossible to implement in the most developing countries (Harrison *et al.,* 2010; Msoffe *et al.,* 2010).

2.4 Performance of indigenous chicken

Performance of indigenous chicken varies in different production systems. The annual poultry meat production in Kenya is 20 tonnes while the annual egg produced is 1.3 billion eggs; this is according to Aviana Africa (2015). According to Victor *et al.* (2014), in both the extensive and semi-intensive production system, the first egg is laid between ages 190 and 240 days. Njenga (2005) reported that there are 10 to 18 eggs and 22 eggs per clutch in FRS while SIS systems respectively. The mean egg weight in all the production systems varies from 38 to 48 g.

Hatchability and fertility range within 60 to 93 %. Hatching weights are regularly low, ranging between 30 to 43g. Chick survival range between 13 and 84% in extensive and about 86% in IS (Olwande *et al.,* 2010).

2.5 Genetic improvement of indigenous chicken

The previous genetic improvement programs aimed at increasing these indigenous chicken productivity in developing countries majorly focused on use of imported temperate breeds (Rodríguez *et al*., 1989; Khobondo *et al.,* 2018). In Kenya, genetic improvement began in the 1960's with importation of exotic breeds like New Hampshire Red, Light Sussex, Black Australop and White Leghorns (Ngeno *et al.,* 2015). This approach involved interbreeding of unselected local chicken to a number of levels of exotic blood (Khobondo *et al*., 2018).

The agricultural ministry in Kenya in the 1970s, introduced a cockerel supply system, which involved importation and spreading of cockerels to be used as the males for breeding in rural areas. The genetic step up was introduced through a cross breeding system by the National Poultry Development Program (NPDP) (Khobondo *et al.,* 2018; Ngeno *et al.,* 2015). The initiative labelled 'cockerel exchange program' begun in 1976 originally in twelve districts and by 1980 another nine additional districts were brought onboard. More districts were latter introduced by 1993 into the program, by this time 26 out of 54 districts were taking part in the program. The local cock was exchanged with an exotic pure line and then all the local cocks were slayed. For the pullet switch, a farmer was needed to keep 10–15 pullets. Disease control through vaccination and poultry management trainings were undertaken as the main component of the program. The program led to step up performance in the crossbreds but degenerated in the subsequent generations. By 1994, the program had failed to meet stakeholders' expectations hence leading to its termination. Poor understanding and planning of the IC sub-sector with respect to production surrounding, needs of actors (farmers, marketers and consumers), lack of sustainable breeding objectives and lack of clear breeding programme to supply constant pure-line breeding stock are some of the factors related to its failure (Khobondo *et al.,* 2015).

2.6 Challenges of affecting indigenous production and disease mitigation strategies

2.6.1 Limitations to chicken production

Main challenges of IC production are Inappropriate construction, poor management, minimal medication, expensive and feeds of reduced quality, lack of genetically improved

breeding stocks, financial limitation, scarcities in channels for marketing, small flock size, poor performance of IC, illiteracy, limitations to extension and healthcare services access (Moges *et al.,* 2010) in the tropics, mostly in Sub-Sahara Africa. Inappropriate shelters restrict the practice of high sanitation especially dusting, (Okeno, *et al.,* 2012). This results to increased pest invasion and recurrent disease outbreaks, leading to high death count. A number of disorders have been recognized in FRS as major limitation both in terms of expenses of prevention, treatment and/or loss resulting from mortality or less productivity (Okeno, *et al.,* 2012). Newcastle Disease (ND) is the most fatal disease in poultry in tropical regions (Moges *et al.,* 2010; Okeno *et al.,* 2012; Olwande *et al.,* 2016).

2.6.2 Importance of diseases and impact of diseases in poultry production

Diseases come along if the body can no longer be protected by the immune system from the inflicted injuries resulting from the invading pathogens, this condition can be caused by aberrant , misdirected or lack of stable immune response (Zekarias & Rebel, 2014). Diseases in animals have direct effects in the livestock production, these include productivity reduction, mortality, mobility and other indirect impacts such as control measures and prevention. High population of birds at high stocking density are kept in modern poultry production and changes in production system increases the spread of pathogens because of frequent direct contact between chicken. The economical, societal, and animal level of diseases effects in poultry production is high. Increased frequency in administration of preventive antibiotic treatment in chicken has increased, raising concerns, since a number of bacterial pathogens developed resistance against most antibiotic types. Increased in antibiotic resistance, increases the vulnerability of the poultry industry to diseases resulting in reduction in income due to mortalities and the increase in the direct cost of treatment (Segal, 2011). Some of diseases such as salmonellosis are zoonotic can be transferred to human being and cause infection hence leading to death. The effects of diseases can be cut down by practice of intervention tactics to prevent, or control disease.

2.6.3 Disease intervention strategies

Segal (2011) argues that the effects of diseases and other disorders can be stepped down by applying disease intervention tactics that target either the pathogen or the animal itself. Quite a number of disease interventions are known and practiced this includes approaches such as;

vaccinations, management, antimicrobials, shelter, feeds, and microbiota are among the approaches. Biosecurity plan is a cluster of acts developed with the intentions of restricting the entry and spread of viral and infectious disorders into and from chicken farm. Biosecurity is embracing of a set of strict attitudes and behaviours by individuals, to lessen the risk in poultry production and marketing activities. Biosecurity measures prevent pathogens from invading the farm by distancing the potentially contaminate object and infected animals from the farm. Vaccination is one of disease intervention strategy used by poultry farmers (Lindahl *et al.,* 2019).

2.7 Immunological and genetic basis of disease resistance in chicken

In animals, innate immunity is the first line of defence against attacking disease causing pathogens. It includes reactions such as; inflammatory, phagocytosis, complement proteins, etc. that are constitutional nonspecific defence reactions. Adaptive immunity is another compartment of the immune system and is mostly involved in individual differences in resistance to infectious pathogens. The adaptive immunity operates through communications of the APC, T cells and B cells by direct cell-to-cell contact using MHC, TcR and immunoglobulins. The interactions of these cells brings about operations of the immune response (leukocytes) with the invasive disease causing organisms (antigen), infected cells and cells that are dedicated in scavenging and duly presenting antigens to lymphocytes.

The T cells , B cells and the antigen presenting cells (APC) are the major immune effector cell types (Carlander *et al.*, 2010). The contact between these cells occurs by way of surface molecules, and secreted proteins (antibodies) are important. Molecules of the major histocompatibility complex (MHC) are the main membrane proteins responsible for the communication of the immune cells, T cell receptors (TcR) and immunoglobulins (B cell receptors), and secreted proteins such as cytokines and antibodies. The named proteins act as immune mediators related in antigen binding and presentation, activating immune effector cells and linking the different events during the immune response. The above proteins scientifically known to be genetically diverse and naturally polymorphic. The resultant interactions between polymorphism of these proteins or their encoding genes and disease resistance or susceptibility has made them potential immune genetic markers for resistance or susceptibility (Zekarias & Rebel, 2014).

Disease resistant genes can be identified by a cumulative knowledge on chicken immunogenic. The quantitative trail locus (QTL) mapping of the combination of DNA variations, immune response by the host, and the transcriptome are prime examples (Dar *et al.,* 2018). Examples of the disease resistant genes that aid the host to slow down the damage caused by bacteria include: encoding antibodies, and microRNA (Dar *et al*., 2018). The MHC (Major Histocompatibility Complex) genes, the *NRAMP1* (Natural Resistance-Associated Macrophage Protein 1), *IFN* (Interferon), *Mx* (Myxovirus-resistance), *anti-ALV* (Avian leucosis virus), and the *Zyxin* gene have been associated with disease resistance amongst poultry (Dar *et al.,* 2018; He *et al.,* 2013; Lamont, 1998; Sophie *et al*., 2006*;*). Next generation sequencing, microarray analysis, RNA sequencing and high-density SNP genotyping are among the few technologies that assist with identifying disease resistant genes (Aubourg, 2001).

Selective breeding for general disease resistance is an approach of building up genetic resistance in a population. This scheme takes advantage of the natural ability of a population to elevate general disease resistance and has the major pros of boosting all individuals' resistance of the subsequent cohorts. In poultry production, selective breeding for elevated general disease resistance is a key disease intervention (Cheng *et al.,* 2013; Wijga *et al.,* 2009). Among the proactive strategies for controlling disease spread in poultry is the incubation and distribution of disease resistant flocks. This strategy complements other methods to manage disease spread through drugs and vaccinations.

2.8 Genetic diversity and population structure analysis in chicken

Population structure means the 'make up' or composition of a population. The arrangement of genetic variation is also the population structure and is determined by the combined impacts of evolutionary procedures consisting of genetic drift, recombination, mutation, and artificial and natural selection.

2.8.1 Phenotyphic characterisation

This involves aspects like how an animal appears physically or qualitative and qualitative traits of an animal which can measured (Nthimo *et al*., 2004). Various chicken types can identified by studying quantitative traits such as; height, body length, length of beak, wing length, and body weight) and qualitative features (color of feathers, skin, foot, and comb). Phenotypic

characterization is the first vital stage vital that the animal breeders use to get information utilization and conservation of indigenous chicken breeds. Phenotypic characterization is used by researchers in the identification phenotype variations existing within and between breeds, this could be very important for the improvement and selection programs for specific profitable traits. The characterization and comparison of various chicken breeds have been done by the use of phenotypic measurements (Mahammi *et al.,* 2016; Maharani, *et al.,* 2021; Mushi *et al*., 2020; Otecko *et al*., 2019).

2.8.2 Molecular characterisation:

This is process of investigating the genetic pattern of phenotypic traits, their mode of inheritance from one generation to another, relationships between breeds, levels of variability and within-breed genetic structure (Schmid *et al.,* 2015). Molecular characterization based on molecular markers is used in the estimation genetic variability at genome level and determining the biodiversity with high levels of accuracy. Molecular characterization plays significant part in the estimation the genetic diversity among livestock by comparing the genotypes at a number of polymorphic loci this is done by the aid of molecular markers (Gheyas *et al.,* 2015; Khoo, 2017). Examples of some molecular markers used include; microsatellites and single nucleotide polymorphism (SNP). Marker identification will help to enhance selection of quality genotypes for breeding to improve significant traits such as disease resistance and tolerance (Agarwal *et al.,* 2020). The molecular characterization and comparison of various chicken breeds have been done by the use of molecular markers. Microsatellites have been used to determine to cluster IC in Kenya (Ngeno *et al.,* 2015) and Rwanda (Habimana *et al.,* 2020). The studies found 2-3 genetic clusters in Kenya and 4 genetic clusters in Rwanda. In addition, single nucleotide polymorphism (SNP) makers have been used to liken the diversity and genetic structure of chicken populations. High density SNP chips have been successfully used in recent studies to molecularly characterize chicken (Molee *et al.,* 2016; Nie *et al.,* 2019; Qanbari *et al.,* 2010). These studies shows that with help of SNPs the chicken performance can be improved and also they aid in the selection, upgrading or crossbreeding programme of traits of economic importance.

2.8.3 Diversity in the chickens Major Histocompatibility Complex

Major histocompatibility complex (MHC), the B complex in the birds, is composed of heterodimeric transmembrane glycoproteins that are crucial in the presentation of antigens to T-lymphocytes (Sophie *et al*., 2006). The MHC molecules (class I, class II and class IV or B-F, B-L and B-G, respectively), belong to the immunoglobulin superfamily with extracellular regions composed of two N-terminal antigen binding domains and two constant domains that bind to the analogous receptor molecules on the T cell membrane. All cell types possess the class I molecule type. These molecules are characterized with endogenously synthesized peptides as well as self-derived and viral peptides. Class II MHC is expressed by antigen presenting cells (APC) with processed exogenous antigens such as bacterial antigens. Class I and II MHC molecules are restriction molecules in APC – T cell – B cell interactions. The T cells identify antigenic peptides and are activated by recognizing the MHC molecules expressed with the antigen on the surface of APC or infected cells. Major Histocompatibility Complex contributes majorly on the regulation of immune response and disease resistance. The humoral response to simple chemically defined antigens and to complex native antigen are examples of MHC control of immune response in chickens. The major role of this molecules is binding of antigens derived from pathogens and display them on the cell surface for acknowledgement by the suitable T-cells (Miller & Taylor, 2016). According to Chen et al (1997), the *MHC class II* gene is said to have a close association with disease resistance mechanisms and its location is on chromosome 16 (Li *et al.*, 1997). Some studies have reported *MHC* genes to have an important association with the disease resistance trait in various breeds of chicken (Boonyanuwat *et al.,* 2006; Molee *et al.,* 2016; Weigend & Lamont, 1999).

Esmailnejad *et al.* (2017) showed that LEI0258 alleles were associated with humoral and cell mediated immune response in broiler chickens. Several studies have used genotyping of the LEI0258 marker to assess the genetic diversity at MHC (Guangxin *et al.,* 2014; Han *et al*., 2013; Nikbakht *et al.,* 2013). In a study done on indigenous chicken breeds of India, Haunshi *et al*. (2020) concluded that the LEI0258 marker was highly polymorphic in these breeds, and the number of alleles identified in each breed was different therefore showing genetic diversity at MHC.

2.8.2 Functional annotation of variants

The process of gathering information and describing a gene's biological identity and molecular functions is called functional annotation. Through annotation, the biological information about a particular variant is identified based on the available information on nucleic acid and protein sequences (Aubourg, 2001). In the modern days, several integrated bioinformatics resources are accessible online for scientists to exploit genomic information. Ensemble, Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis *et al.,* 2003) and Variant Effector Predictor (VEP) (McLaren *et al.,* 2016) provides functional analysis tools for genes.

Ensemble is considered one of the most common genomic resources (Flicek *et al.,* 2014). A gene list can be presented to the database for annotation enrichment analysis. Gene ontology terms, protein-protein interactions biological pathways, and homology and disease associations are among the gene categories included. The functional analysis offers an organized annotation and consistent originating from a number of biological angles for all the genes in the lot. According to Dennis *et al.* (2003) and McLaren *et al.* (2016) these databases also offer the pathway viewer instrument to visualize genes in the batch on pathway maps convenient to decipher biological interaction networks.

2.9 Effective population size and linkage disequilibrium

2.9.1 Effective population size

The degree of the ideal magnitude of the population that experiences the same amount of allele frequency variation, loss of heterozygosity or inbreeding as the natural population under consideration is referred to as Effective population size (N_e). Effective population size is used to regulate the rate of inbreeding, loss of genetic variation, and rate of fixation of deleterious alleles of populations. Effective population size is a sign of the risk of populations to extinction. Critical Knowledge and understanding the stages of effective population size is significant in certifying sustainability of genetic diversity of a breed or population over time.

According to Lee *et al.* (2011), temporal and linkage disequilibrium-based are among the molecular approach methods that help in detecting the changes in effective population size related with allele's loss of heterozygosity and frequency variance in populations. The most commonly used is the linkage disequilibrium-based approach. According to Hurt and Hedrick (2004), It is

one of the most effective methods, since only single sampling is required as compared to the temporal methods.

2.9.2 Linkage disequilibrium

Linkage disequilibrium (LD) is the non-random association of alleles at two or more loci (Hurt & Hedrick 2004; Karimi *et al,*. 2020; Qanbari *et al.,* 2010; Tenesa *et al.,* 2007) . The most commonly used measures of LD are D´ and (r2). The D´ measure is a standardised LD value which is dependent on allele frequencies (Slatkin, 2008). D´ is calculated from D which is the difference between the frequency of gametes carrying allele A and B (pAB) at two Loci and the product of the frequencies of those alleles (pA and pB), DAB = pAB-pApB (Hedrick, 2004). LD measured using r^2 is defined as the squared correlation co-efficient between two loci (Hurt & Hedrick, 2004; Karimi *et al.,* 2020; Slatkin, 2008). It can be used for multiple pairs of loci and is less dependent on allele frequencies.

Linkage disequilibrium avails information on population demography and evolutionary history. According to Uimari & Tapio (2011), LD is also one of the most frequently used methods to estimate Ne. The LD, as a squared correlation coefficient of marker pair between two loci, is a function of distance between makers that is influenced by recombination rates. linkage disequilibrium of marker pairs at a close distance reflects more recent Ne while LD of marker pairs at two different loci far apart reflect ancient Ne of population. Knowledge of LD extension is crucial to determine the required marker density to achieve adequate accuracy in both genome-wide association studies (GWAS) and genomic selection.

New alleles are introduced into a population through gene flow. Gene flow or mixing of gametes from different population has an effect on LD. The mixing up of two populations carrying different allele at different frequencies will usually result in a new gene pool characterized by higher heterozygosity levels over time. This increased heterozygosity level will result in reduced levels of LD. The extent of reduction in LD will depend on the origin of the chicken subpopulation in such a way that greatly diverse subpopulations will result in high genetic variation and lower LD values than when genes are exchanged between closely related populations. Linkage disequilibrium has been used to reveal the demographic history including trends in effective population sizes of egg laying commercial chicken populations (Qanbari *et al.,* 2010) and extensively raised village chicken populations (Khanyile *et al.,* 2015). In other studies, in a number

of species, LD have been used to construct maps (Lee *et al.,* 2011), in fine mapping quantitative trait loci (QTL) and population parameters (Tenesa *et al.,* 2007), analysis of haplotype diversity and mating systems (Lee *et al.,* 2011; Uimari & Tapio, 2011). The availability of whole genome high density SNP data has even increased the use of LD based estimates of Ne and other population parameters particularly in humans and domestic livestock that lack pedigree data.

CHAPTER THREE

IMMUNOGENETICS AND GENOMIC VARIATIONS IN INDIGENOUS CHICKEN

Abstract

This study used data from 150 indigenous chicken from four agro-ecological zones in Rwanda to provide deep insight of the population structure and variation of the immunogenetics using several approaches based on phenotypic and SNP data. The population structure of indigenous chicken was analysed using Principal Component Analysis (PCA), ADMIXTURE analysis, and phylogenetic relationships for the whole genome and at chromosome 16. The study used 65,945 SNPs from the collected chicken. Phenotypic analysis was done for the Newcastle disease titer (ab) alongside bodyweight at 20 weeks with the highest having 1.6kg. The genome analysis was done using the genotyping-by-sequencing approach. The grouped the indigenous chicken into two genetic clusters, which was confirmed by ADMIXTURE analysis that revealed that the lowest cross-validation (CV) error (0.51) was at K = 2. The analysis of Population structure at chromosome 16 showed that the population had the lowest CV error (0.50) at K =1. Cluster one mean body weight and antibody titers were 1673.61 ±237.14g and 4912.5±55.35, respectively. Corresponding values for cluster 2 were 1311.34±121.9g and 8832.5±55.36, depicting an inverse relationship between bodyweight and antibody titers. The cluster means for body weight and antibody titers were significantly different ($P < 0.001$) for body weight and antibody titers. The indigenous chicken genetic clusters in Rwanda have variation in antibody titers which can be attributed to varied selection pressure. The observed genetic diversity of the indigenous chicken for disease resistance should be well-thought-out when scheming a selection programme to ensure that the ICs population is sustainable, flexible and simultaneous improvement of this trait. Based on this study's findings government should implement strategies that conserve and maintain the genomic diversity of Rwanda indigenous chicken.

3.2 Introduction

Indigenous Chicken farming has been gradually shifting from subsistence to commercial enterprise due to increased demand for its meat and eggs (Magothe *et al.*, 2019). The increase in IC demand makes farmers house birds in large flocks at high stocking density thus the increased risk of diseases, and disease spreading (Mujyambere *et al.,* 2022). Newcastle disease is one of the common diseases affecting IC farming (Kapczynski *et al.,* 2013; Walugembe *et al.*, 2019).

Identifying the genes that control disease resistance would make the selection of IC for improved production performance and enhanced disease resistance possible. Such useful spinoff would reduce cost of production due to decreased use of drugs, as well as better product quality due to lowered drug residues (Dar *et al*., 2018; Jie & Liu, 2011).

A number of efforts have been practiced worldwide to appreciate and improve resistance to disease in livestock through the application of immunogenetics. In pigs, improvement of resistance to disease was applied using gram–negative bacteria (Zhao & Chen, 2012) and in ruminants it was done using gastrointestinal nematodes (Sweeney & Good,2016). In bovine, immunogenetics was applied to improve resistance to mastitis (Sodeland *et al*., 2011). Information on both immunology and genetics of animal would well describe the disease phenotype (Bishop, 2014). Immune capability related to a particular disease can be used to indirectly select for resistance to disease because these traits can be assessed and measured in breathing animals (Luo *et al.,* 2013). Santos-Argumedo (2012) showed that antibody titers are immunological traits which can be inherited in poultry thus making it easy to determine loci or a particular gene associated to immune-related traits.

In chicken, selecting for growth and production traits has been related with decrease in immunity (Bayyari *et al*., 1997; Wondmeneh *et al*., 2015). Selecting chicken for increased weight is genetically related to a reduction in disease resistance. Genetic correlations between body weight and antibody titers in chicken (r_g) are medium to high (Mebratie *et al.,* 2019). Using microsatellite and sequence data, Habimana *et al*. (2022) showed genetic structuring in IC in Rwanda and reported body weight to be inversely related to Abs respectively. Microsatellites are more variable but suffer from ascertainment bias, homoplasy and amplification variation of primers (Tian *et al.,* 2008). High-density single nucleotide polymorphism has made it possible in the investigation of the population's genetic structure through use of large numbers of markers and identify regions in the genome where events related to the traits we are interested with (Groenen *et al*., 2008; Wollstein *et al.,* 2010). Single nucleotide polymorphism are more abundant and evenly distributed across the genome hence more informative. Therefore, the study aimed to examine the immunogenetics and genomic variations in indigenous chicken.

3.3 Materials and methods

3.3.1 Population and housing

A total 150 IC were sampled randomly from the northern, southern, central, and eastern agro-ecological zones of Rwanda. Birds were kept at the University of Rwanda under the same environmental conditions under deep litter system. Stocking density of 12birds/M^2 was adopted.

3.3.2 Collection of phenotypic data

Birds were vaccinated with two commercial Newcastle Disease virus live vaccines. At 2 days of age, AVI Newcastle Disease HB1vaccine was put in water and the second shot of the vaccine (AVI ND Lasota) were dropped on their eye when they were at 28 days old. On the 35th day, collection of blood was done without anticoagulant for separation of serum for detection of antibody titers against Newcastle disease vaccine. The detection of antibody responses to Newcastle disease was done using an indirect ELISA test. The Antibody levels were computed using the IDSoft TM data analysis programme. At the 20th week of age, body weight (BW) data of the IC were collected.

3.3.3 Extraction of genomic DNA and genotyping

Blood samples for DNA extraction were collected from wing vein using 2.5ml EDTA tubes. Genomic DNA from blood was extracted by a DNA extraction kit. The concentration of extracted genomic DNA and the qualities were evaluated using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific™ Nanodrop 2000) and gel electrophoresis (1% agarose) (Lu, *et al,* 2016). Raw reads were obtained using the Genotyping-by-sequencing (GBS) approach (Jain *et al.,* 2016).

3.3.4 Alignment of the reads and calling of the SNPs

Trimming of the raw reads was done using the sickle tool and then they were aligned to the Galgal4 chicken reference genome using Burrow-Alignment tool (BWA v0.7.17), afterward sorting of the reads was done. Removal of the duplicated reads was performed using SAMtools v1.3.1. The calling of SNPs was done using SAMtools v1.3.1.

3.3.5 Quality control of the SNPs

The SNPs obtained were thereafter subjected to the standard filtering procedures using Plink v1.07 software (Purcell *et al.,* 2007); minimum SNP quality of 20, 5% missing SNP genotypes, Hardy–Weinberg equilibrium ($P<10^{-6}$), call rate > 95%, heterozygosity > 0.4, and minor allele frequency > 0.05.

3.3.6 Extraction of SNPs in MHC region of chromosome 16

The study extracted SNPs from MHC region at chromosome 16 that covered from LOC425771 through CD1A1 (210.000 bp) The extraction was done using VCFtools v0.1.14 (Danecek *et al.,* 2011).

3.4 Statistical analysis

3.4.1 Phenotypic and immunogenetic clustering

Data collected on antibody titers against Newcastle disease and BW of ICs populations were entered into a database using Microsoft Office Excel 2016. The phenotypes (Abs and BWs) were used to place the chicken in unique clusters based on either AB, BW and AB and BW using PROC FASTCLUS in SAS software v 9.4 (2008). The within-cluster variation was described using descriptive statistics. The PROC GLM of SAS was used to determine whether the clusters were differing significantly for AB and BW based on the different clustering approaches. The following linear model was used:

$$y_{ij} = \mu + t_i + e_{ij}$$

where y_{ij} is the total IgY titer or body weight; μ is the overall mean; t_i is the effects of the fixed factor(sex and age) and e_{ij} is the residual term

3.4.2 Genotypic clustering of IC into genomic clusters

PCA analysis

The SNP genotypes of the IC were used to assess the genetic differentiation of the population. The study used PCA to minimize the dimensionality of the SNP data set with a large number of interrelated variables. This was achieved by converting the original variables into a new set of variables, the principal components (PCs), which were uncorrelated, and ordered so that the

first few retain most of the variation present in all of the original variables. The genomic clusters were determined by plotting PC1 and PC2 in Tassel 5.2.60 (Bradbury *et al*., 2007).

Admixture analysis

Admixture analysis was done to determine population relatedness and assigns populations to ancestral clusters. The population structure was done using the model-based clustering algorithm that was run in ADMIXTURE software (Alexander *et al*., 2011), from K = 2 to 4. The cross-validation method was used in estimating the number of clusters that are most likely to be found. The K value with the lowest cross-validation prediction error was then assumed as the most likely number of clusters (Evanno *et al*., 2005). The ipADMIXTURE R package was used to present the results in a graph (Amornbunchornvej *et al*., 2020).

Estimation of F_{ST} values

The unbiased genetic differentiation estimate, F_{ST} (Weir & Cockerham, 1984) was calculated using admixture software in Kenet vLAB (a virtual software) with the quality-controlled SNP dataset to estimate genetic differentiation between populations using the fixation index.

Phylogenetic analysis

The relationship among IC from the four agro-ecological zones was determined by constructing a matrix distance, from the matrix distance a phylogenetic tree was drawn in Tassel software v5.2.60 (Bradbury *et al*., 2007). The genetic relationship between the IC from the four zones was determined based on the neighbour-joining tree algorithm. The neighbour-joining tree cladogram generated by TASSEL was visualized in the archaeopteryx tree (Bradbury *et al.,* 2007).

3.4.3 Genetic diversity at MHC in chromosome 16

The study extracted 90 SNPs from MHC that covered from LOC425771 through CD1A1. Diversity was done following steps described above that is i) PCA ii) admixture analysis and iii) Phylogenetic relationship that is the neighbour-joining analysis. The SNP allele frequencies for chromosome 16, expected (He) and observed (Ho) heterozygosity and hardy Weinberg equilibrium (HWE) were computed using PLINK (v1.90b) software (Slifer, 2018).

3.5 Results

3.5.1 Phenotypic and immunogenetic clustering

Phenotypic clustering grouped the IC into two populations. Cluster one had mean body weight of 1673.61±237.14g and antibody titer of 4912.5±55.35. Cluster two had mean body weight

of 1311.34±121.9g and mean antibody titer of 8832.5±55.36. The two clusters differed significantly ($P < 0.001$) in body weight and antibody titers. The cluster one with high mean in bodyweight and low mean in titer and vice versa.

Table 3.1: Minor allele frequency of 20 variants extracted from the MHC region in chromosome 16

CHR	SNP	A1	A2	MAF	NCHROBS
16	100134902|F|0-45:G>A-45:G>A	G	A	0.4615	286
16	100149083|F|0-10:G>A-10:G>A	G	A	0.3169	284
16	100129173|F|0-17:C>A-17:C>A	A	C	0.003497	286
16	100034475|F|0-22:C>T-22:C>T	T	C	0.006993	286
16	100103111|F|0-39:T>C-39:T>C	C	T	0.003497	286
16	100094740|F|0-25:T>G-25:T>G	G	T	0.01748	286
16	100085316|F|0-26:T>A-26:T>A	T	A	0.465	286
16	100088251|F|0-14:A>G-14:A>G	A	G	0.01748	286
16	100163669|F|0-42:G>T-42:G>T	G	T	0.0979	286
16	100163035|F|0-13:G>A-13:G>A	A	G	0.0461	282
16	100125696|F|0-68:C>G-68:C>G	C	G	0.08741	286
16	100096683|F|0-36:G>T-36:G>T	T	G	0.1119	286
16	100021966|F|0-8:G>A-8:G>A	A	G	0.003497	286
16	100060608|F|0-32:A>G-32:A>G	G	A	0.09091	286
16	100077597|F|0-8:A>G-8:A>G	G	A	0.3741	286
16	100130892|F|0-21:T>C-21:T>C	C	T	0.3636	286
16	100147497|F|0-22:G>A-22:G>A	A	G	0.01049	286
16	100118266|F|0-26:G>C-26:G>C	G	C	0.5	266
16	100125885|F|0-14:G>A-14:G>A	A	G	0.01399	286
16	100033338|F|0-31:C>A-31:C>A	A	C	0.243	284

CHR is the chromosome, SNP is the SNP identifier, A1 is the allele 1 code (minor allele), A2 is the allele 2 code (major allele), MAF is the minor allele frequency, and NCHROBS is the non- missing allele count.

3.5.2 SNP Marker Characteristics

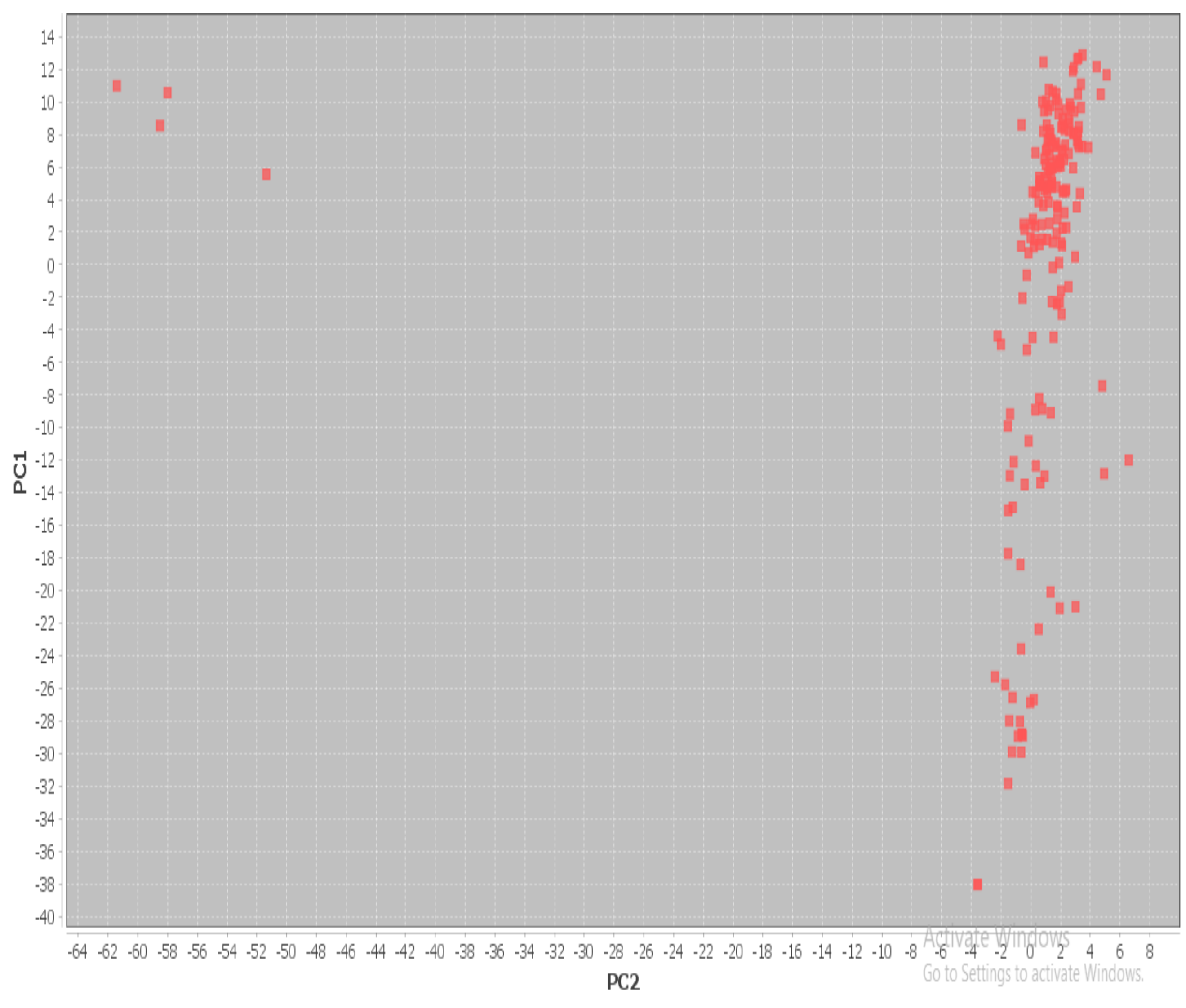
Minor allele frequency (MAF) had an average of 0.16 with SNP regions 100163035|F|0-13:G>A to 100021966|F|0-8:G>A-8:G>A having a MAF value below 0.05. An analysis of MAF distribution across the 20 variants in the MHC region revealed over 50% of markers were within the 0–10% MAF threshold.

3.5.3 The analysis of population structure

This was done using the principal component analysis (PCA), admixture and neighbour-joining tree analyses. This analysis was done at the whole-genome and chromosome 16.

3.5.3.1 Population structure analysis at whole genome level

At the whole genome level, PCA showed that principal component one (PC1) amounted to 38% and principal component two (PC2) amounted to 26% of the total variability (Figure 1 and 2).



Activate Windows
Go to Settings to activate Windows.

Figure 3.1: Plot of principal component analysis in tassel software showing the two genetic clusters of indigenous chicken from the four ecological zones

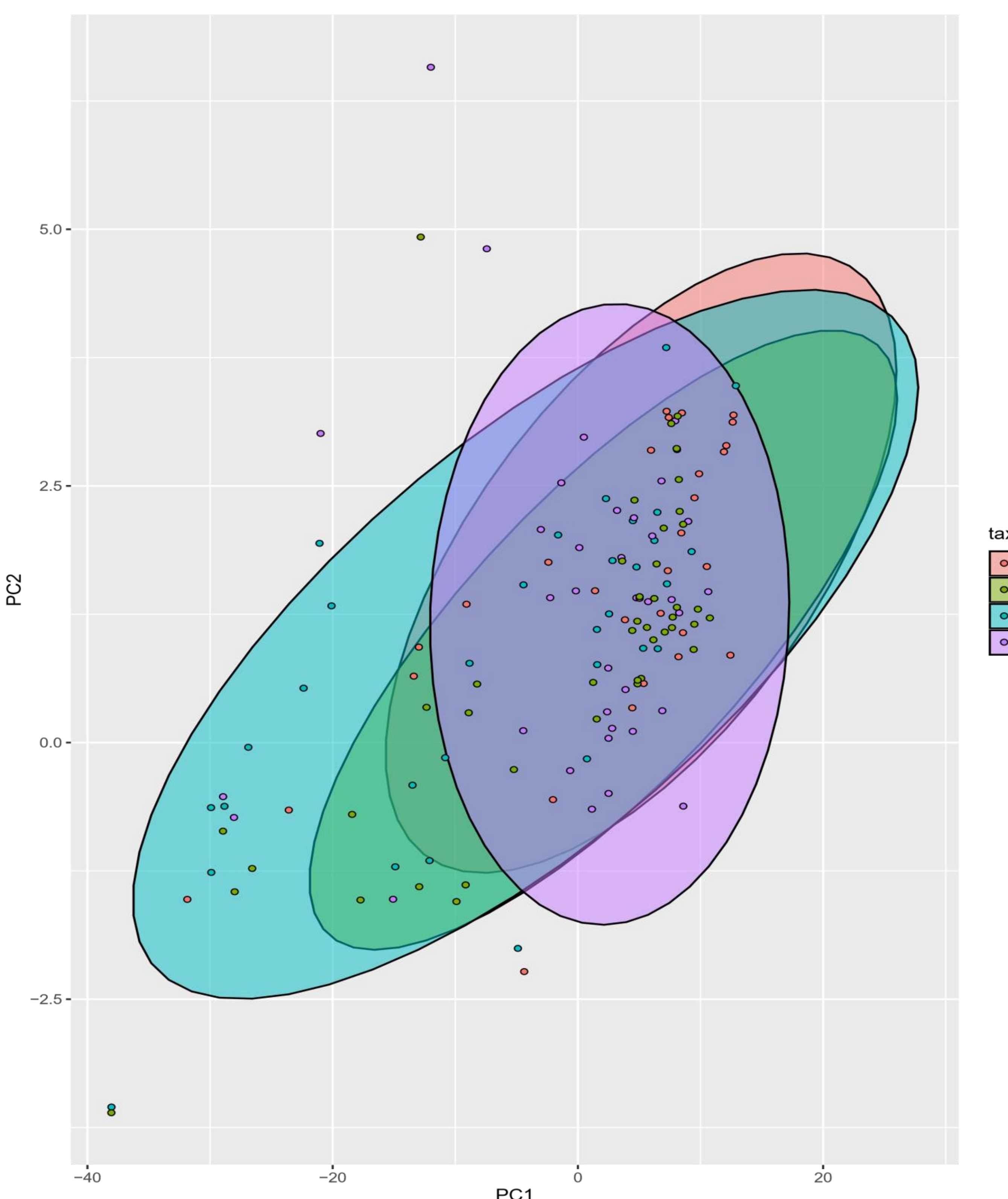


Figure 3.1: Principal component analysis of the whole genome showing two clusters

3.5.3.2 Admixture analysis

Admixture analysis performs maximum probability approximation of individual ancestries from multilocus SNP genotype data. An R package, ipADMIXTURE (Amornbunchornvej *et al*., 2020) was then used to plot the ancestry of the IC from the four zones. Admixture plots clustered the individuals according to the portion of origin they shared with other individuals. The Bayesian clustering analysis of ADMIXTURE for K values from K=2 to K=4 is shown in Figure 3 and 4. The lowest cross-validation error was found at K=2 (Figure 3). When K was 2, IC from the four agro-ecological zones displayed two distinct groups. When K was 3, IC from northern were pure. When the K value was 4, central and southern populations showed that the IC were pure breed.

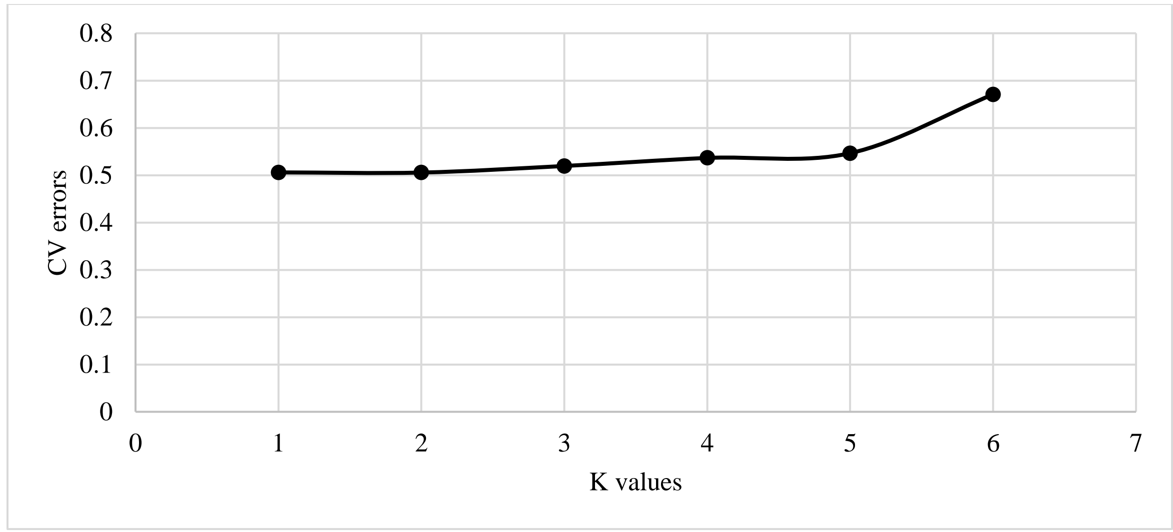


Figure 3.2: The cross-validation errors of the K values

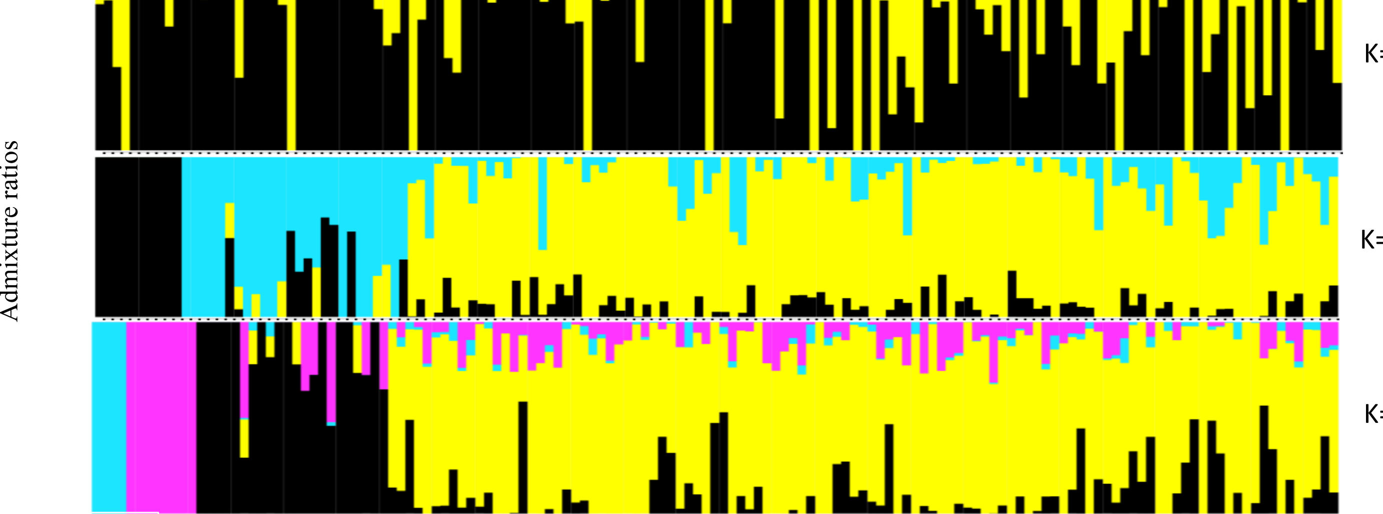


Figure 3.3: The ADMIXTURE analysis for IC from the four agro-ecological zones for K= 2 to K= 4. The colour of the vertical bar on the x-axis signifies the quantity of membership of each accession in each cluster. The geographic regions are indicated on the x-axis as follows N (Nothern), S (southern), C (central), and E (Eastern)

3.5.2.3 Analysis population differentiation based on Fixation index (F_{ST})

The magnitude of population differentiation between the different IC from the four agro-ecological zones was investigated using F_{ST} values which were calculated using genotype data (Table 3.1). The F_{ST} values ranged from 0.071 to 0.218, indicating genetic differentiation appeared between IC from the four regions. However, these F_{ST} values seem to increase between the zones. For instance, the F_{ST} value was 0.092 between northern and southern and increased to 0.186 between northern and central zones. The opposite was observed between southern vs central (0.176) and southern vs eastern (0.071).

Table 3.2:Fixation index (FST) of the population from the four agro-ecological zones

Agro-ecological zone	Northern	Southern	Central	Eastern
Southern	0.092			
Central	0.186	0.176		
Eastern	0.140	0.071	0.218	

3.5.4 Population structure analysis at MHC region in chromosome 16

Twenty variants were extracted from MHC region in chromosome 16. The variants were retained for downstream analysis that is, PCA, admixture analysis and neighbour joining analysis. The PCA and admixture analysis placed the IC as one population (Figures 3.5, 3.6 and 3.7). Admixture analysis revealed lowest cross-validation error when K= 1.

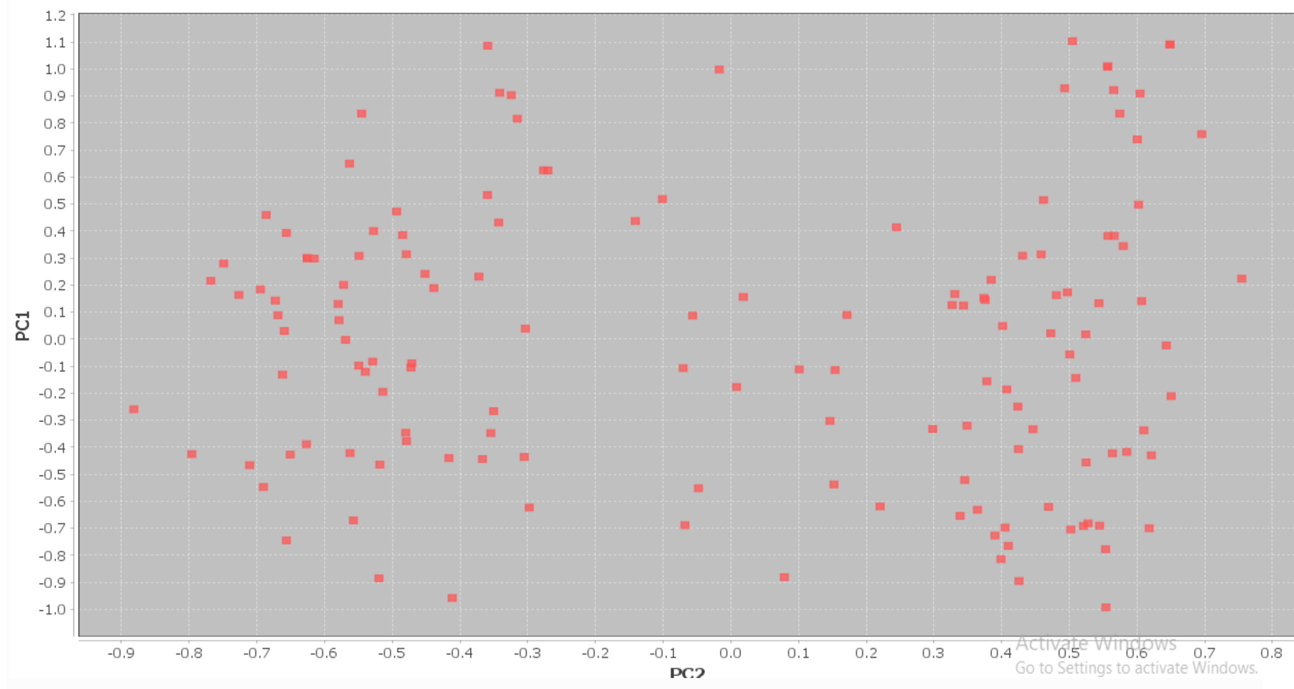


Figure 3.4: Plot of principal component analysis of indigenous chicken from the four agro-ecological zones

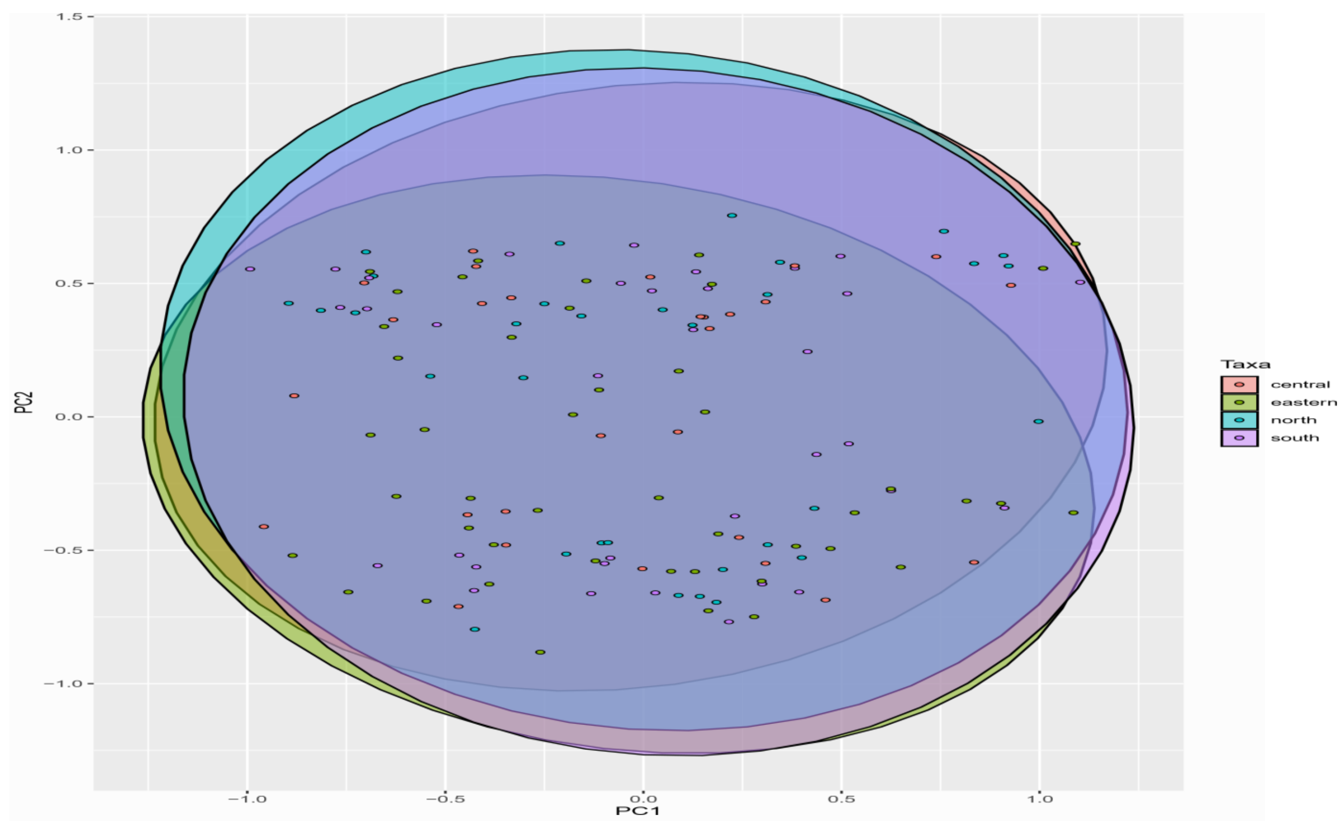


Figure 3.5: Principal component analysis of MHC region at chromosome 16 showing one cluster of indigenous chicken from the four agro-ecological zones

3.5.4.1 Phylogenetic relationship

The phylogenetic analysis of MHC region was used to deduce the relationships between IC collected from four agro-ecological zones. The phylogenetic tree, together with the details on each IC is shown in Figure 3.7. The neighbour-joining tree revealed all the IC from the four zones had one common origin thus grouping them as one population.

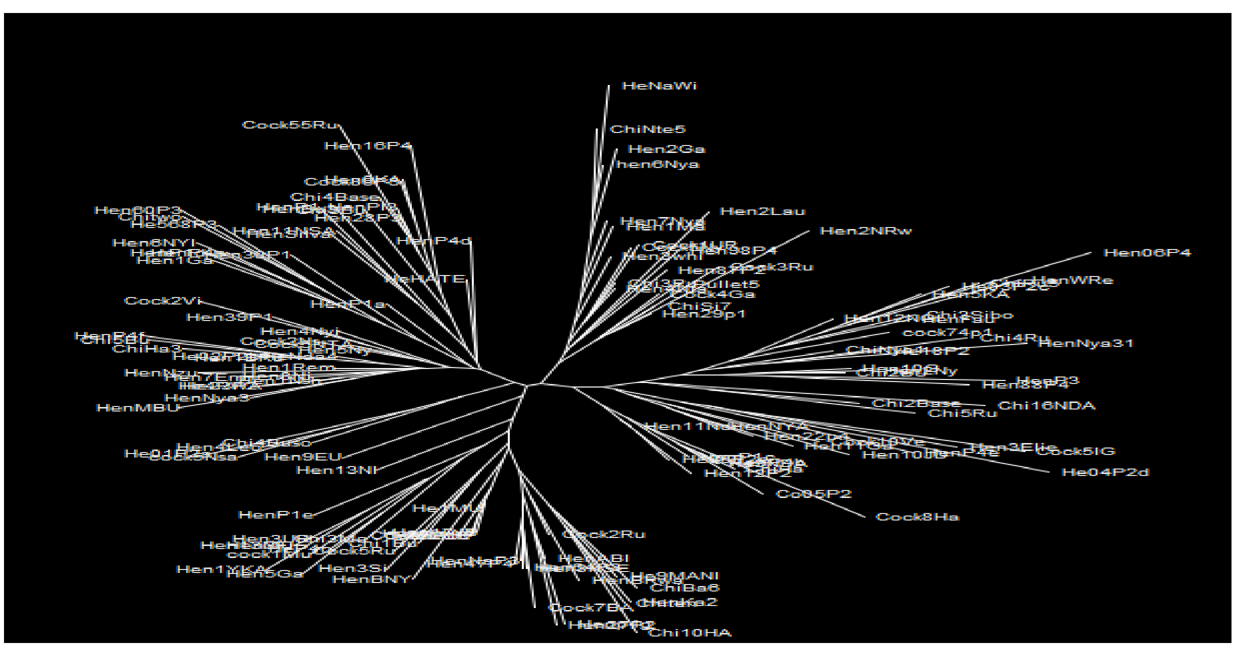


Figure 3.6: Neighbour-joining tree obtained from the distance matrix for the 142 indigenous chicken from the four agro-ecological zones

3.5.4.2 Admixture analysis

Results from Bayesian clustering analysis using ADMIXTURE software for the MHC region in chromosome 16 are presented in Figure 8. At K=1, cross-validation error was lowest (Figure 3.8), indicating IC to belong to one cluster.

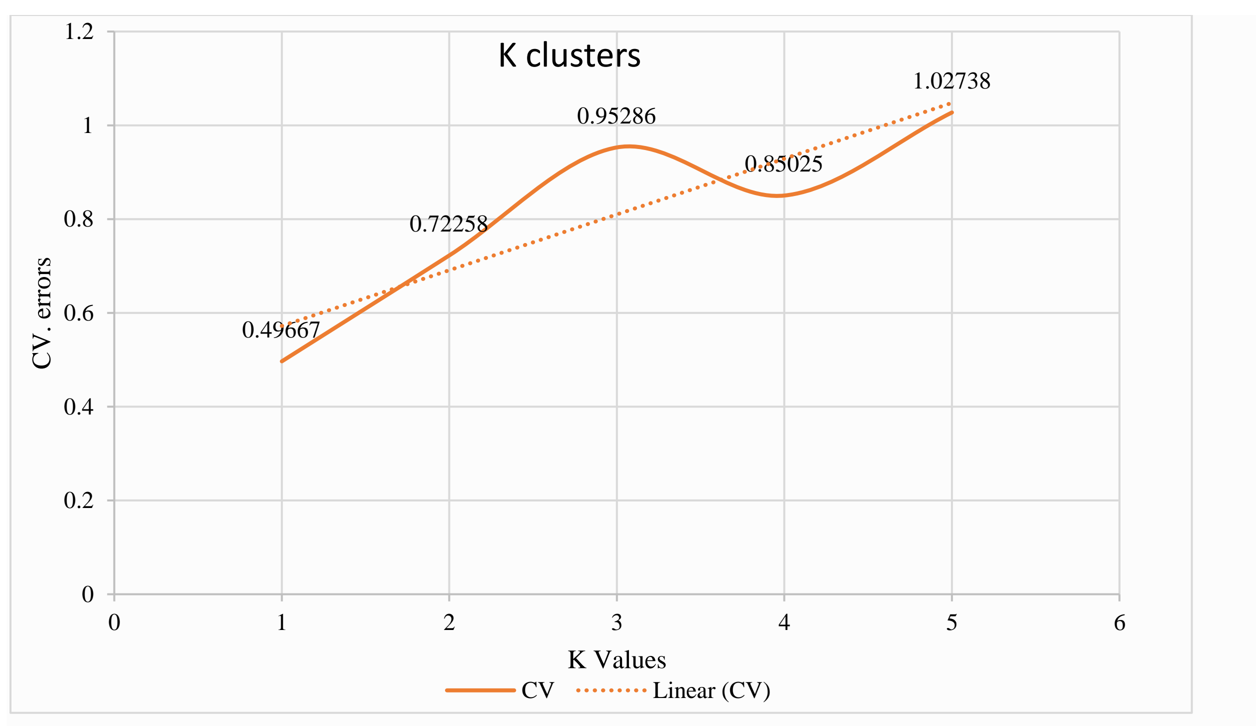


Figure 3.7: Cross-validation errors of the K clusters

3.6 Discussion

3.6.1 Phenotypic and immunogenetic clustering

Regardless of extensive information of the genomic foundations of phenotypes the G. gallus, few and non-exhaustive studies have measured genotype and phenotype variations in populations of this species. In this study, measurements of antibody titers and body weights were evaluated and compared among the IC populations from four agro-ecological zones. Phenotypic clustering using BW and antibody titers grouped IC into two clusters. The first cluster had a high mean body weight and low antibody titers as compared to cluster two which had a low mean body weight and high mean for antibody titers. This study focused on antibody titers associated with Newcastle disease because the disease is distributed worldwide and causes high mortality of up to 100% and severe clinical symptoms on the chicken populations (Kapczynski *et al.,* 2013).

Immune capacity associated with specific diseases may be useful indicators for indirect selection for general disease resistance because such traits can be evaluated and quantified in live animals (Luo *et al*., 2013). A study by Santos-Argumedo, (2012) shows that immunological traits such as antibody titers are heritable in poultry making possible to discover loci or genes related to immune or disease resistance traits. Antibody response plays an important role in host resistance

to Newcastle disease, and selection for antibody response can effectively improve disease resistance in chicken (Luo *et al*., 2013; Kapczynski *et al.,* 2013).

Selecting for growth and production traits has been linked with reduced immunity. Selection for increased body weight has been shown to be genetically associated with a reduction in disease resistance in chicken (Bayyari *et al*., 1997; Wondmeneh *et al*., 2015). This study's findings revealed that IC with high body weight have reduced antibody titers and vice versa when they have high antibody titers their body weight is reduced. It is unclear by what mechanism selection for increased body weight compromises immune traits. It may be down to simple energy allocation, in that resources spent on growth are no longer available for immune function and other energy-consuming processes (e.g. locomotion). However, increased body weight can also have detrimental effects, because it requires resources that cannot be spent on other vital traits. Similarly, although the benefit of an effective immune system is obvious, the wide spread susceptibility to disease suggests there may be downsides as well. It is also known that MHC haplotypes can affect growth (Lamont, 1998; Warner *et al.,* 1987), this can explain the results found in this study. In this study classification according to immunological traits based on Newcastle disease antibody titers revealed selection pressure of indigenous chicken in indigenous chicken in Rwanda. Hence development and distribution of disease-resistant chicken flocks represent a proactive approach for controlling diseases in chicken and complements current methods for disease control by drugs and vaccination (Dar *et al.,* 2018). Therefore, studying the negative effect production trait selection has on the immunity may give the breeder a tool not only to alleviate the effects but also to breed disease-resistant chicken.

3.6.2 Genetic diversity at whole genome

This study focused on genomic characterization of IC for the whole genome and the MHC region in chromosome 16. The study reports the diversity of the genome of Rwanda IC using 65,945 SNPs from 142 IC from four agro-ecological zones namely northern, eastern, southern and central. The analyses of the principal component and admixture revealed two ancestral gene pools across the Rwandan IC populations. A similar study done in Tanzania to investigate the population structure of IC using admixture, grouped the selected IC into two gene pools (Mushi *et al*., 2020). In agreement with the Principal Component Analysis results, admixture analysis grouped all the IC from the four agro-ecological zones into into two, contrary to a study done by Habimina *et al.*

(2020) which revealed four gene pools using microsatellite. When K=2 the IC were grouped into central and Eastern, with more proportion of the central ancestry cluster probably because of interbreeding among the IC (Mushi *et al.,* 2020). In overall, the IC population were admixed with most birds being from the central agro-ecological zone. Our results revealed that the F_{ST} values ranged from 0.071 to 0.218 thus indicating isolation between the IC populations, and this likely mean that the IC populations are presently breeding with one another. Findings for whole genome grouped IC into two clusters providing a deeper understanding of the structure of IC population to supplement the use of phenotypes for IC selection (Chiwanga *et al.,* 2020). Based on this study's findings, government should implement strategies that would conserve Rwanda IC genetic diversity and its characteristics

3.6.3 Genetic diversity at MHC region in chromosome 16

At MHC region in chromosome 16, the investigation of the population structure by Principal Component, admixture analysis and neighbour joining approaches grouped the Rwanda IC as one population. This means there is no horizontal gene flow probably due to small geographical size of the country. A study in Kenya using MHC-linked microsatellite markers grouped IC into three groups, composed of birds from dissimilar ecotypes while clustering based on non-MHC markers placed IC into two gene pools (Ngeno *et al*., 2015). The absence of geographical boundaries, the purchasing of the seeder flocks from one geographical location to another and the free-range system of chicken management in the tropics might be the reason of increase in the interbreeding among the IC resulting in one Rwanda IC population. Common cultural practices like intermarriages among the tribes might have attributed to recurrent flow of gene in the locations. . Studies have reported that MHC is related with disease resistance and immune traits (Fulton *et al*., 2016, 2017). These results indicate that at IC MHC region, there is significantly less genetic variation than the variation within the whole genome this is because of smaller chromosome length of chromosome 16 as shown in study done by Belew et al. (2018) on chicken of Ethiopia.

3.7 Conclusion

Phenotypic clustering found 2 gene pools of IC in Rwanda. This shows that phenotype can also be used in the identification of different IC population. Based on population structure analysis

using SNPs from the whole genome level and MHC region clustered IC two and one respectively. The observed genetic diversity of the indigenous chicken for disease resistance should be well-thought-out when scheming a selection programme that is sustainable, flexible and ensures there is simultaneous improvement of this trait. Based on this study's findings government should implement strategies that conserve and maintain the genomic diversity of Rwanda indigenous chicken.

CHAPTER FOUR

IDENTIFICATION AND FUNCTIONAL ANNOTATION OF THE DELETERIOUS VARIANTS WITH RELATION TO DISEASE RESISTANCE IN INDIGENOUS CHICKEN

Abstract

In animal breeding, identifying and purging the deleterious variants have been considered the best approach to reduce mutational load. Recently, advances in resequencing of DNA and sequence-based approaches have helped in the prediction of the functional consequence of certain mutation that allows the identification of putatively deleterious Single Nucleotide Polymorphisms on a genome. Using 65,945 SNPs from 150 indigenous chickens, we identified 182 deleterious alleles and their functions annotated. Out of 182 deleterious genes identified, six genes were related to immune traits. The six genes included; *FANCA, RBBP5, CRB1, RUFY3, FBXO38,* and *PDE3A*. Analysis of gene ontology revealed numerous biological and KEGG processes that are involved in immune and disease resistance traits. Functional annotation linked the *FANCA* gene to the regulation of the following; cell proliferation, T cell differentiation, inflammatory response, and CD40 signalling pathway. The *RBBP5* gene is linked to the cellular response to DNA damage stimulus and the *CRB1* gene is associated with the heterophilic adhesion of cell-cell through adhesion of the plasma membrane cell molecules and regulation of the synaptic vesicle exocytosis. The *RUFY3* gene is linked to positive regulation of cell migration. The *FBXO38* gene is linked to controlling T cell-mediated immune reaction to the cancer cell and finally and the *PDE3A* gene is linked to response to the drug. The analysis of the Gene ontology of the ENSGALG00000046739 candidate gene that was obtained in the MHC region in chromosome 16 was linked with missense variants associated with the regulation of RNA polymerase II transcription. In conclusion, this study reveals that the exploration of genes associated with immune and disease resistance traits offers direct applications for breeders to select chickens for increased disease resistance and allow breeding for disease resistance.

4.1 Introduction

New mutations occur in a population each generation; among these mutations, deleterious are the ones that mostly affect fitness (Keightley & Lynch, 2003). An increase in deleterious genetic variants can result in genetic drift and hitchhiking. In a population, impacts of an individual

deleterious variant is small, unlike collective variants that may affect the overall fitness of a population. A population that is having a small effective population size (Ne) can influence its fitness and may also lead to chances of high inbreeding depression, as a consequence of the build-up of deleterious variants that frequently arise due to genetic drift and mostly occur in domesticated populations (Lynch, 2010). Purifying selection is the most efficient way of getting rid of alleles that have deleterious effects on a population (Kimura & Crow 1963; Lynch, 2010), this will ensure they are at low frequencies. Some forces of evolution such as recombination rate and hitchhiking shape deleterious alleles. Hitchhiking is a change in the number of alleles caused by alleles being passed together with an allele undergoing selection.

The effectiveness of selection at eliminating deleterious alleles is determined by their fitness impacts (Henn *et al.,* 2015) thus this is summarized by Ne (Charlesworth, 2009). Most alleles that are considered deleterious are believed to be at least partially recessive (Simmons *et al.,* 1997; Agrawal *et al.,* 2012, 2011). Deleterious mutations that are weak can persist in populations, regardless of being selected against, therefore causing a reduction in the population's fitness thus referred to as mutation load (Hedrick *et al.,* 2016).

Artificial and natural selection in indigenous chickens can cause deleterious genetic variants (Khanyile *et al.,* 2015; Okeno *et al.,* 2012; Qanbari*et al.,* 2010). If the selection coefficient against mutations is lower than the selection coefficient of the preferred allele that lies on the same haplotype, the allele frequency of the deleterious variants is expected to rise due to genetic hitch-hiking (Good & Desai, 2014). Therefore, slightly harmful mutations become over-represented in regions of the genome under selection. Indigenous chicken genome may host some harmful mutations that can potentially alter fitness and health. However, the underlying genetic architecture of these deleterious variants in indigenous chicken gene pool is not clear. Identifying the deleterious variants and their impacts provides a chance to understand the historical selection forces, genes, and mutations that are responsible for the difference in phenotype in indigenous chickens (Agrawal *et al.,* 2012, 2011). This study aimed at identifying the deleterious alleles in indigenous chicken and carrying out their functional annotations.

4.2 Materials and methods

4.3.1 Study population, chicken housing, and feeding

In total, 150 distinct indigenous chicken, were sampled from the northern, southern, central, and eastern agro-ecological regions of Rwanda. Sampled birds were kept at the University of Rwanda under a deep litter system. The stocking density was 12 birds per square-metre.

4.3.2 Extraction of genomic DNA and genotyping

Blood samples for DNA extraction were collected using EDTA tubes. Genomic DNA from blood was extracted by a DNA extraction kit. The concentration of extracted genomic DNA and the qualities were evaluated using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific™ Nanodrop 2000) and gel electrophoresis (1% agarose) (Lu, *et al,* 2016). Raw reads were obtained using the Genotyping-by-sequencing (GBS) approach (Jain *et al.,* 2016).

4.3.3 Alignment of the reads and calling of the SNPs

Trimming of the raw reads was done using the sickle tool and then they were aligned to the Galgal4 chicken reference genome using Burrows-Alignment tool (BWA v0.7.17), afterward, sorting of the reads was done. Removal of the duplicated reads was performed using SAMtools v1.3.1. The calling of SNPs was done using SAMtools v1.3.1.

4.3.4 Quality control

The SNPs obtained were subjected to the standard filtering procedures using Plink v1.07 software (Purcell *et al.,* 2007); minimum SNP quality of 20, 5% missing SNP genotypes, Hardy–Weinberg equilibrium ($P < 10^{-6}$), call rate > 95%, heterozygosity > 0.4, and minor allele frequency > 0.05

4.3.5 Extraction of SNPs from MHC region in chromosome 16

The study extracted SNPs (20 SNPs) from MHC region (position 1 to 652338) in chromosome 16 that covered regions LOC425771 through CD1A1 (210.000 bp) (Fulton *et al*., 2016). The extraction was done using VCFtools v0.1.14 (with parameters vcftools –vcf –chr 16 –from –bp 1 –to 652338 –recode –out). (Danecek *et al.,* 2011)

4.3.6 Identification of deleterious variants

Identification of deleterious variants were done using Variant Effect Predictor (Ensembl 87 chicken, *Gallus gallus*) (https://www.ensembl.org/info/docs/tools/vep/online/index.html). Missense mutations were considered a potential cause of loss of function and variants labelled as non-synonymous variants were retained for downstream analyses. Variants effect prediction for the deleterious impacts of missense mutation was done using Sorting Intolerant from Tolerant (SIFT) (predictor and score) SIFT of <0.01(Sim *et al.,* 2012). The SIFT option predicts the functional impacts of SNPs and categorizes them as intolerant (those that have impacts on the function of the protein) or tolerant (functionally neutral) grounded on the properties of the amino acids (Kumar *et al.,* 2009). When the SIFT score is bigger or equivalent to 0.05 that variant is thought to be (TOL) meaning the variant is tolerant while those with a score below 0.05 are considered as (INTOL) meaning these variants are intolerant and thus hypothetically deleterious (Choi & Chan, 2015; Sim *et al.,* 2012). The ratio of the mutational load (Derks *et al.,* 2018) was calculated as: $L = \frac{D}{S}$, where D is the deleterious missense mutation; S is the synonymous variants.

4.3.7 Functional annotation of genomic variants and Gene Ontology (GO)

The variants that were missense/non-synonymous were extracted and genes related to them were analyzed for GO. Overrepresentation of Gene Ontology terms enrichment for the biological process and other functions was checked by BinGO v3.0.3 a package found in Cytoscape v.3.4.0 (Maere &Kuiper, 2005) using chicken, (*Gallus gallus) genome*. To test the significance, the study used Benjamini and Hochberg correction procedures at 0.05.

4.4 Results

4.4.1 Identification of deleterious variants

Annotation of 65,945 SNPs against the ENSEMBL-VEP gene annotation database, revealed 61.52% of SNPs to be a mixture of intronic, exonic, UTRs and splicing genes, meaning they are within genes while the rest, 61.52% (100) of the SNPs are found as intergenic and upstream/downstream (outside genes) (table 4.1). The SNPs that were in the exonic regions were further categorized into; synonymous (0.87%, these are variants that do not change the sequence of the amino acid in a protein), and non-synonymous amounted to 2.06% (1481, these are variants that alter the sequence of the amino acid in proteins) and 0.17% (126) were stop gain/loss variants

(these are variants that results to the gain or loss of the stop codon). Using SIFT scores, 0.07% (n = 50) of the non-synonymous variants were predicted as INTOL ('intolerant' variants that have a major impact on protein) and 0.49% (356) as 'TOL (tolerant) (figure 4.2). This study also reported 0.36% splicing variants. The 3′ and 5′ UTR regions had variants that accounted for 1.08% and those within upstream or downstream accounted for 22.27%.

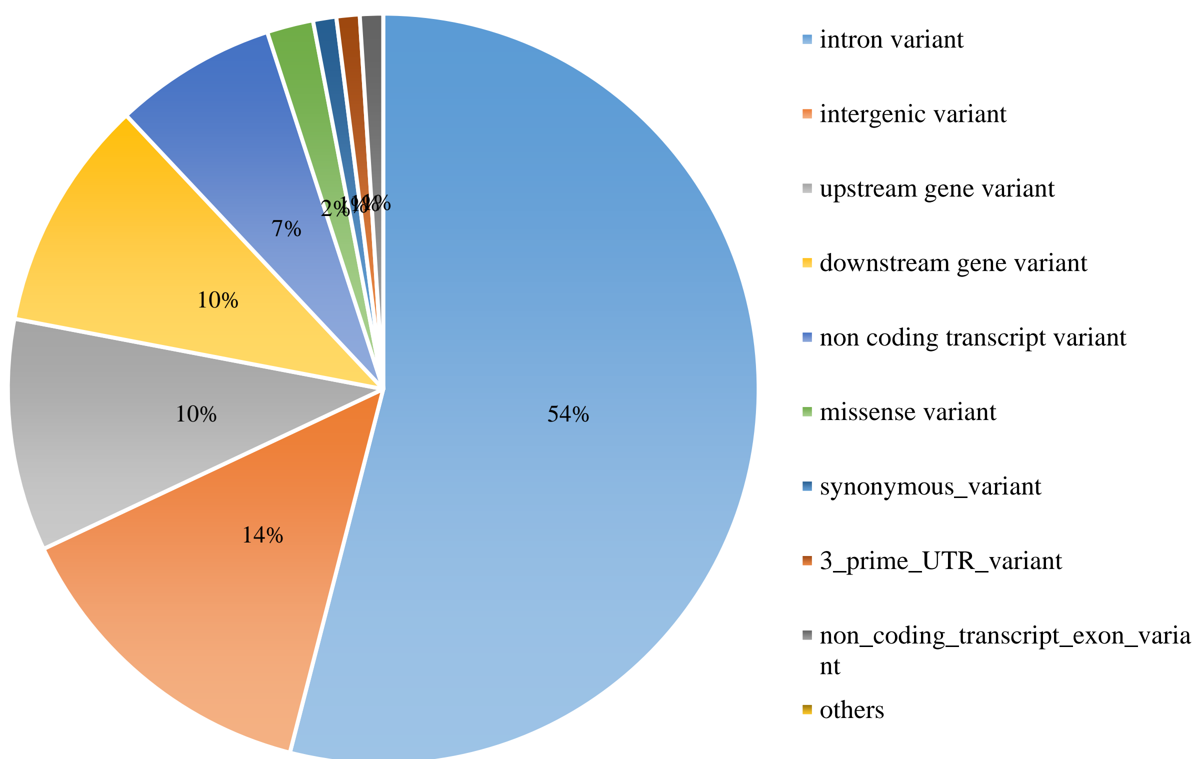


Figure 4.1: VEP-based SIFT analysis for all the consequences of SNPs (%) in the whole genome

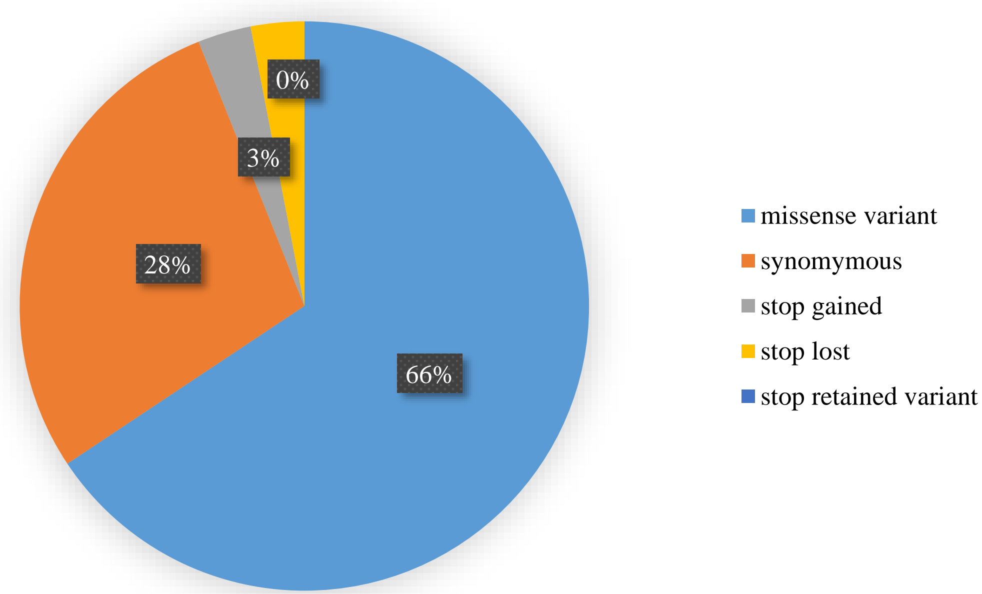


Figure 4.2: SIFT analysis for the Amino acid-based on VEP

Table 4.1: Summary of the functional annotation of SNPs in indigenous chicken

	Annotation category	Number
Exonic	2.23	1607
Nonsynonymous	2.06	1481
Nonsynonymous deleterious<0.05	0.52	375
Nonsynonymous deleterious>0.01	0.17	125
Nonsyonymous tolerated	0.49	356
Stop-gain/loss	0.17	126
Intergenic	14.66	10559
Intronic	57.85	41667
Synonymous	0.87	626
Splicing	0.36	261
Upstream/downstream	22.27	16041
UTR3'UTR5'	1.08	778

Mutational load was calculated by taking non-synonymous deleterious variants (<0.01) over the synonymous variants which is 375/626 (Table 4.1), which is equal to 0.59. The fitness of

the population was calculated by deviating 0.59 from 1, hence the fitness is 0.41.

Annotation of 20 SNPs obtained from MHC region of chromosome 16 against the ENSEMBL gene annotation database revealed 5% of SNPs as intronic, exonic, UTRs and splicing, meaning they are located within the genes and the rest are intergenic and upstream or downstream, meaning they are located outside the genes (Figure 4.3.)

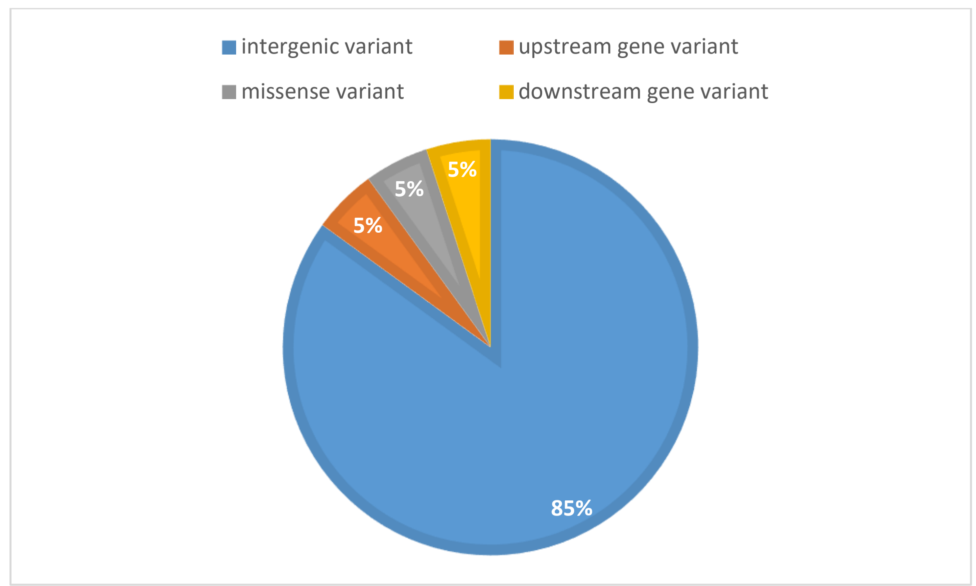


Figure 4.3: VEP-based SIFT analysis for all the consequences of SNPs (%) at the MHC region in chromosome 16

4.4.2 Functional annotation and enrichment analyses

Annotation of non-synonymous deleterious SNPs with SIFT score of < 0.01 from 142 indigenous chicken revealed 182 genes. Out of the 182 genes, only six genes (Table 4.2) were related to immune and disease resistance traits. Characterization of the functions for the six genes has shown that they are attributed to regulation of cell population proliferation, differentiation of regulatory T cell, inflammatory response, CD40 signal pathway, and cellular reaction to DNA injury stimulus, Heterophilic adhesion of cell-cell through membrane cells of the plasma adhesion

Table 3.2: GO term names and GO definition of the six genes related to immune traits for deleterious variants in the 142 IC based on SIFT score prediction of <0.01

Gene name	GO term name	GO definition
FANCA	Modulation of regulatory T cell differentiation	Processes which moderate the occurrence of how regulatory T cells differentiate
	Control of proliferation of cell population	Any processes that moderate the incidence and extent of proliferation of cells.
	Moderation of response to inflammation	Processes that moderate the incidence of an inflammatory response
	Regulates CD40 signal pathway	The process that controls the occurrence, and degree of signalling through the CD40 signalling path.
RBBP5	Regulates the response to damages to the DNA caused by a particular stimulus	The processes that cause a variation in the activity of a cell thus this stimulus may indicate damage to DNA from environmental impacts or faults that occur at the time of metabolism.
CRB1	Heterophilic adhesion of cell-cell via molecules that adhere to cells of plasma membrane	The attachment of a molecule adhering to a non-identical molecule that is adhering to a neighbouring cell.
RUFY3	Positive control migration of cells	Processes that stimulate the incidence of migration of cells
FBXO38	Control or moderation of T cell-mediated immune response to tumour cell	Processes that initiate the occurrence of immune response that is mediated by T cell to the tumour cell.
PDE3A	Reaction to drug	Processes that lead to alteration in the activity of a cell or an organism (in terms of its motion, what it secretes, the enzymes it produces, expression of several genes etc.) as a result of stimuli such as drugs.

molecules, control of synaptic vesicle exocytosis, positive modulation of migration of cell, and T cell-mediated immune reaction to the tumour cell and reaction to drug (Table 4.2). Functional annotation of the genomic variants located at the MHC region revealed most of the variants to be intergenic (86%) (Figure 3). Variants considered as missense contributed about 1%. Analysis of GO of the ENSGALG00000046739 as the major gene linked with missense variants is related to control of RNA polymerase II transcription.

4.5 Discussion

The study used SNPs to identify non-synonymous deleterious alleles in indigenous chicken populations of Rwanda. This study also presents a vital understanding of the role of the MHC region in chromosome 16, which is associated with immune traits. The study further reports mutational load to be 0.59 which was higher than 0.018 reported in commercial chicken and 0.014 that was observed in African village chicken (Bosse *et al.,* 2018). A study done by Makino et al. (2018) reported a higher mutational load of 1.19 in domesticated dogs and 0.83 in rabbits which were higher than what we observed. In relation to the results mentioned above on chicken, it can be concluded that the mutational load of Rwanda's indigenous chicken is high, this may be attributed to their production environment which is under scavenging system thus this contribute to deleterious variants as a result of selection pressure. Several studies in animal have shown deleterious mutations mostly accumulates in domestic lineages (Kadri*, et al.,* 2014; Kono *et al.*,2016; Marsden *et al.,* 2016; Schubert *et al.,* 2014). The large number of variants that are non-synonymous in a population are either deleterious variants that are neutral or weak (Eyre-Walker & Keightley, 2007). The proportion of mutations that are advantageous, effectively neutral and deleterious varies between species, and differs between coding and non-coding DNA. Despite these differences between species and genomic regions, some general principles have emerged indicating the advantageous mutations are rare, and those that are strongly selected are exponentially distributed (Renaut & Rieseberg, 2015). According to Makino *et al.* (2018), variants that are non-synonymous are between 15 and 50% and are thought to be damaging, hence the 60% observed in the indigenous chicken are categorized to be damaging.

4.5.1 Functional annotation of variants in indigenous chicken populations

The information on the functions of the non-synonymous variants can help to forecast phenotypes or genetic importance. Annotation of the 65,945 SNPs against the ENSEMBL gene annotation database revealed that 61.52% of the SNPs are intronic, exonic, UTRs and splicing, meaning they are located within the genes and the rest are intergenic and upstream or downstream meaning they are located outside the genes (Sabarinathan *et al.,* 2013). Wong *et al*. (2014) did a study that revealed that 37% of the genomic variants were located inside the genes and 1.2% located in regions that code for a protein. Belew (2018) reported that the Ethiopian indigenous chicken had 46.36% of SNPs are intronic, exonic, UTRs and splicing meaning they are located within the genes and the rest are intergenic and upstream or downstream while 1.6% of the SNPs were in regions that code for a protein (exonic). Genomic regions said to be preserved through distantly associated species are presumed to be undergoing selection purification, thus the variants located in such regions might be detrimental (Gheyas *et al.,* 2015).

Functionally annotation of 72,032 variants from the 142 indigenous chicken were checked for their functions and biological pathways . Functional annotation of deleterious SNPs (non-synonymous variants) with a SIFT score of < 0.01 revealed 182 genes. From the 182 genes, only six genes were associated with immune and disease resistance traits which include; *FANCA, RBBP5, CRB1, RUFY3, FBXO38* and *PDE3A*.

The *FANCA* gene is responsible for the regulation of the following; cell population proliferation, T-cell differentiation, inflammatory response and CD40 signalling pathway. A study done on humans by Ben *et al.* (2021), reported that in the human population where the *FANCA* gene has undergone mutation, the individuals had the following characteristics: short size, microcephaly pigmentation of the skin and abnormalities of the skeletons. In the human population, studies have examined the correlation between different mutations on *FANCA* and human phenotype. Castella *et al.* (2011) did a study on the Spanish human population and revealed that there was no association between the mutations of the *FANCA* gene and haematological disease incidences and malformations of the somatic cells. The *RBBP5* gene*,* on the other hand, is responsible for the cellular reaction to DNA injury stimulus, which encompasses processes that causes a variation in the function of a cell resulting in the introduction of a particular stimulus signifying damages to its DNA from mistakes throughout metabolism process. Diseases associated with *RBBP5* include Retinoblastoma. Retinoblastoma (RB) is a primary neuroectodermal tumour

that arises from immature retinoblasts, resulting from a mutation that occurs in chromosome 13. This tumour is a result of inactivation of the alleles of the Retinoblastoma (Rb) gene causing faulty development of pRB protein. The *pRB* is a gene is that suppresses the tumour and helps in the progression of the cycles of the cell and replication of the DNA. Damage to *pRB* functionality in the retinal progenitor cells causes impairment of the cell cycle and uncontrolled proliferation of the cell (Sachdeva & O'Brien, 2012). A study on dogs showed that dogs had the same characteristics as humans (Syed *et al.,* 1997). In chicken, a similar gene as *RBBP5* known as Retinoblastoma 1 (*RB1*) was studied. Results on Retinoblastoma 1 (RB1) revealed that it is a harmful regulator of proliferation of 254 preadipocytes, and causes inhibition of the cell cycle of preadipocytes by G1 arrest (Wang *et al.,* 2018). The *CRB1* is a gene that is responsible for heterophilic adhesion of cell-cell through the adhesion of molecules to plasma membrane cells. Studies on human showed that *CRB1* mutations thicken the retina (Jacobson *et al.,* 2003). Rashbass and Skaer (2000) hypothesized that *CRB1* has a role in the localization of the proteins that are responsible for phototransduction to the photoreceptor's membranes. Therefore, non-functional *CRB1* may obstruct phototransduction, hence leading to progressive photoreceptors dystrophy. Hence, from several studies, we can conclude that *RBBP5* and *CRB1* genes have almost similar functions they are associated with retinal phenotypes. The similarity of the experimental findings to the human condition offers robust evidence that the loss-of-function mutation in *CRB1* and *RBBP5* results in disease in chicken

The *RUFY3* gene, which was also found to be non-synonymous in the indigenous chicken, is involved in processes that stimulate or escalate the occurrence, rate or extent of migration of cells (Vadlamudi & Kumar, 2003). Several studies have reported the *RUFY3* gene to be involved in the increase of migration of the tumour cells of the gastric (Kumar & Vadlamudi, 2002; Wang *et al.,* 2015). Yoshida *et al.* (2010) found out that *RUFY3* is linked to huge vesicle structures and co-immunoprecipitation with *Rab5*. The *FBXO38* is a protein coding gene attributed to the control of immune response mediated by the T cell that is associated with the cancer cell. Diseases associated with *FBXO38* include neuronopathy and distal hereditary motor (Rossor *et al.,* 2012). Phosphodiesterase 3A (*PDE3A)* is a protein-coding gene, which is associated with response to drugs and the study found this gene with non-synonymous deleterious variants in indigenous chicken. Through genetic adaptation, indigenous chickens may have developed effective strategies

to cope with diseases; hence those chickens without this gene might have a problem with the response to drugs and thus increase disease incidences in the population.

Functional annotation at MHC region in indigenous chicken populations revealed one gene (ENSGALG00000046739) associated with missense variants. Analyses of gene ontology (GO) enrichment of the ENSGALG00000046739 gene was linked with regulation of RNA polymerase II transcription. Since this gene was categorized as missense variants but not deleterious and it does not cause deleterious mutations in the indigenous chicken population.

4.6 Conclusion

Exploiting sequence data allowed us to reveal six deleterious genes (*FANCA, RBBP5, CRB1, RUFY3, FBXO38,* and *PDE3A)* that are linked to immune traits of indigenous chickens. The results of this study provided the foundation for genome-assisted methods for animal improvement efforts. From our results, despite the weak artificial selection on IC, mutational load can be high. In most scenarios deleterious mutations are said to be harmful but in some cases they can be considered beneficial, in our current study we found out mutations like *CRB1* mutations etc. are of importance especially in relation to disease resistance of an animal. Therefore, the results of this study should be used when designing a breeding programme to improve the fitness of indigenous chicken population and also ensure there is simultaneous improvement of disease resistance and immune traits. Additionally, since 182 alleles were identified to be deleterious further, research is necessary to evaluate the other remaining genes influencing other traits other than immune traits

CHAPTER FIVE

EFFECTIVE POPULATION SIZE, AND LEVELS OF INBREEDING IN INDIGENOUS CHICKEN

Abstract

This study was achieved through the estimation of effective population size (Ne), inbreeding rates and levels using SNP data of 150 indigenous chickens from four agro-ecological zones of Rwanda, namely; Northern, Southern, Central, and Eastern. Blood samples were collected, DNA extracted and genotyped with the genotyping-by-sequencing (GBS) approach. After quality control procedures in raw data, 65,945 SNPs were retained for analysis. Effective population size-based linkage disequilibrium, inbreeding rate, and levels in indigenous chicken were estimated using different mathematical formulas. Our results showed and that the rate of inbreeding of averaged 0.080%. The rate of inbreeding for the Northern, Southern, Central, and Eastern IC population were 0.087%, 0.080%, 0.095%, and 0.0575%, respectively. The levels of inbreeding decreased as the number of generations increased. At the 400th generation, inbreeding levels reached the fixation point in all the birds. At the 13th generation, the central agro ecological zone had the highest rate of inbreeding of0.31%. The current average levels of inbreeding for IC from Northern, southern, central, and Eastern agro-ecological zones were 0.33%, 0.32%, 0.34%, and 0.34%, respectively. The results revealed an increase in Ne as the number of generations kept increased. Estimated Ne for Northern and central regions overlapped from 297 to 267 and from 660 to 758 generations. Even though the rates and the levels of inbreeding are lower than the recommended rate of 1%, all the agro-ecological zones had an upward trend, implying declining effective population size in the recent generation. To arrest the increasing inbreeding and downward Ne trends, a breeding programme should include management of genetic diversity of IC to enable long-term viability of these populations.

5.2 Introduction

The genetic diversity of the indigenous chicken (IC) population is thought to be threatened by crossbreeding with exotic or imported breeds and uncontrolled mating systems (Halima *et al.,* 2007). Developing countries over the past years have been using genetic programmes that use imported chicken breeds to improve IC productivity (Dana *et al.,* 2011; Reardon *et al.,* 1998). The consequences of using genetic programmes to improve IC productivity include high inbreeding

rates and levels and low effective population size (Beleke *et al.,* 2010; Bush, 2006). A study done by Hedrick, (2005) showed that inbreeding rates results due low effective population size especially in a small population. Inbreeding tends to result in more homozygous animals (Charlesworth & Willis, 2009). Effects of inbreeding manifest as a reduction in production (loss of vigour), fertility, and viability through low rates of survival (Flock *et al.,* 1991; Tongsiri *et al.,* 2019).

Understanding indigenous chicken's genetics and its genomic makeup is an important step in coming up with conservation and genetic improvement programmes (Abebe & Johansson, 2015). The availability of sequenced data is beneficial in getting to know the history of the evolution of indigenous chicken populations and in estimation of factors like LD, Ne, rate and level of inbreeding, in the lack of pedigree data and their records (Khanyile *et al.,* 2015). Estimation of Ne is a useful way (Khanyile *et al.,* 2015; Wright, 1938) to evaluate and understand the loss or gain of genetic diversity across time. (Addis & Aschalew, 2014; Hedrick, 2005; Tenesa et al., 2007). Conversely, Ne is a vital concept in evolutionary biology and the preservation of genetic resources (Frankham, 1995; Hayes *et al*., 2003).

Estimation of Ne, rate, and levels of inbreeding provide important information about strategies that could be used in selection, conservation, and monitoring of genetic variability in IC (Addis & Aschalew, 2014; Khanyile *et al.,* 2015). Studies on the rate and levels of inbreeding have not been done, especially on the IC of Rwanda. Therefore, this study aimed to estimate LD-based Ne, inbreeding rate, and levels of IC from Rwanda.

5.3 Materials and methods

5.3.1 Study population

Studied population comprised of 150 indigenous chickens collected from the northern, southern, central, and eastern agro-ecological regions of Rwanda (Figure 1). The indigenous chickens were kept under the same condition at the University of Rwanda.

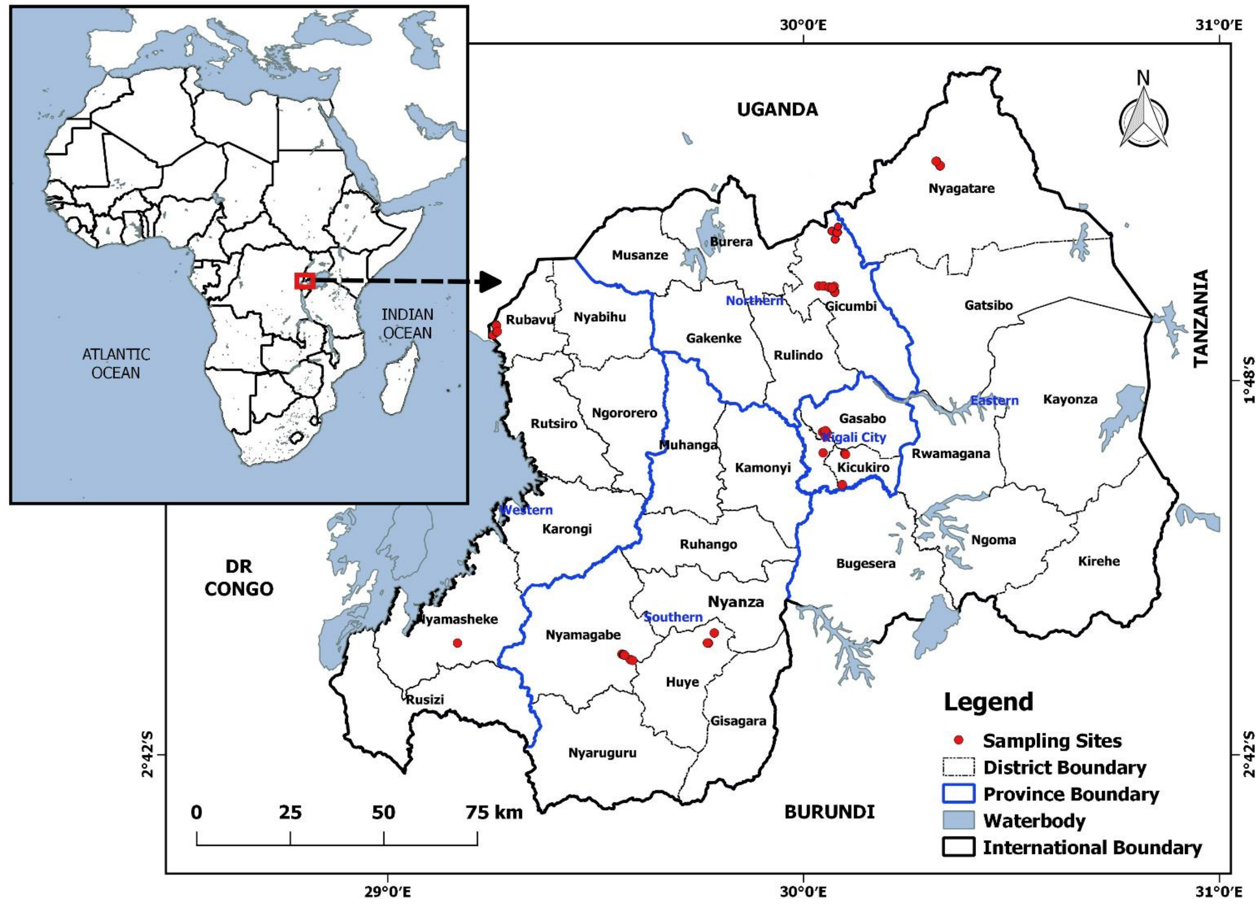


Figure 5.1: Map showing the sampling sites where the indigenous chicken were sampled

5.3.2 DNA isolation and genotyping

Blood samples were collected from 150 IC into an EDTA tube. This study used Promega DNA extraction kit to extract the DNA from the blood. Evaluation of genomic DNA quality and concentration was done to confirm if they meet the standard requirements for genotyping using NanoDrop™ 2000 spectrophotometer and electrophoresis of gel (1% agarose) (Lu, *et al,* 2016). The study obtained the raw reads by the genotyping-by-sequencing approach (GBS) (Elshire *et al.,* 2011).

5.3.3 Raw reads mapping and calling of the SNPs

Trimming of the raw reads was done using a sickle software and then aligned to the Galgal4 (chicken reference genome) using the Burrows-Alignment tool (BWA v0.7.17). The removal of duplicated raw reads and calling of the reads was done using the SAMtools v1.3.1 (Li *et al.,* 2008).

The SNPs that were obtained were thereafter subjected to the standard filtering procedures using Plink v1.07 software (Purcell *et al.,* 2007); minimum SNP quality of 20, 5% missing SNP genotypes, Hardy–Weinberg equilibrium (P < 10−6), call rate > 95%, heterozygosity > 0.4, and minor allele frequency > 0.05.

5.3.4. Data analysis

5.3.4.1 Effective population size (Ne)

Linkage disequilibrium between the pairs of SNPs within each chromosome in each IC population were measured by estimation of a pair-wise correlated coefficient $(r)^2$ using version PLINK software for SNPs (Purcell *et al.,* 2007). Estimation of Ne was calculated using the formula shown below (Sved1971). Estimation of the effective population size will be calculated using the following formula (Sved1971):

$$E(r^2) = \frac{1}{(1+4Nec)} + (\frac{1}{n})$$

The effective population size, Ne will be calculated as:

$$Ne = \frac{1}{4c}\left(\frac{1}{E(r^2 - 1/n)}\right)$$

where n is the chromosome size; E (r^2) is the expected LD, c is distance between one locus and the other; N_e is the effective population size (Mbole *et al.,* 2014). Using the above formula, *Ne* for the generation $t=1/2c$ was estimated by counting backward from the generation that was genotyped (Hayes *et al.,* 2003). Thereafter, the trends in Ne for each of the indigenous chicken population were determined by the number of generations, and visualized in a graph.

5.3.4.2 Estimation of rates and levels of inbreeding

The inbreeding rate per generation per IC population was estimated as:

$$\Delta F = \frac{1}{2Ne}$$

where ΔF is the rate of inbreeding. This was done to test whether there was a significant increase or decrease in inbreeding rate as generations increased. The levels of inbreeding for IC from each agro-ecological zone was estimated using the following formula: $F_t = 1 - (1 - \left(\frac{1}{2Ne}\right))\text{^} - t$

where F_t is the level of inbreeding after t generations, Ne is the effective population size.

5.4 Results

5.4.1 LD-based estimation of Ne for the generations ago

The graph in Figure 2 is a plot of the estimated Ne at t generations ago for the IC from each agro-ecological zone. The estimated Ne for Northern and central regions overlapped from 297 to 267 and from 660 to 758 generations (Figure 2).

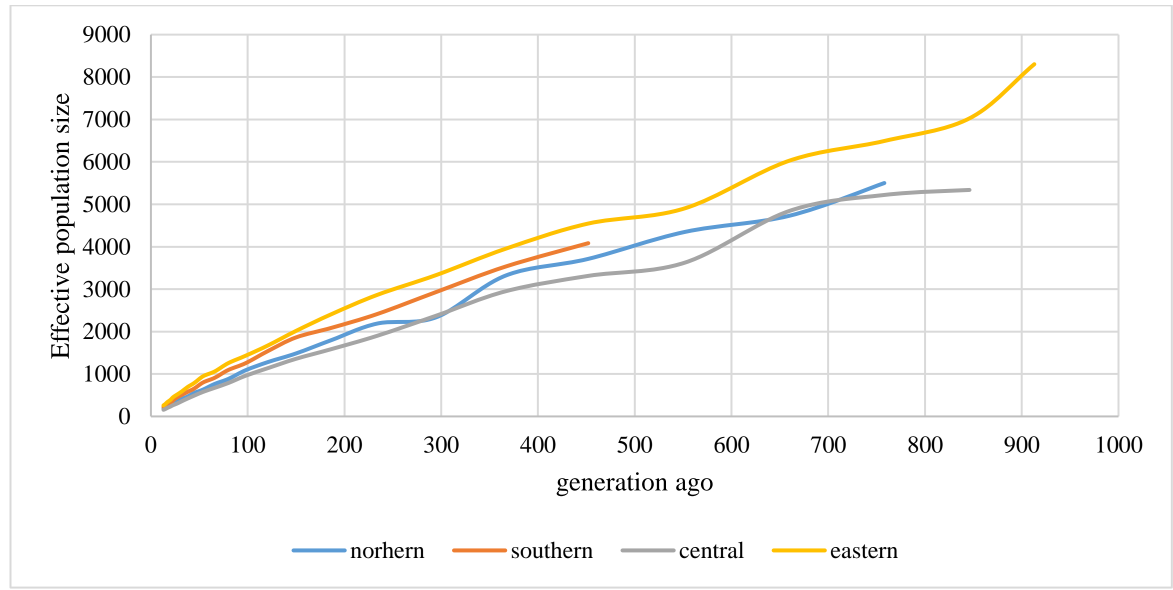


Figure 5.2: Trends in effective population size of indigenous chicken obtained from four agro-ecological zones in Rwanda

5.4.2 Rate and levels of inbreeding

The average rate of inbreeding for northern, southern, central, and eastern IC population are as follows 0. 087%, 0. 080%, 0. 095%, and 0. 057%, respectively. Average rate of inbreeding in the all the population was 0.08%. The rate of inbreeding for the IC from Eastern agro-ecological zone was significantly lower than the other three populations ($P<0.05$). The inbreeding rate decreased as the number of generations increased and remained constant at the 400th generation for all the IC from the four agro-ecological zones (Figure 3). At the current generation (13th), the level of inbreeding was high with central having the highest rate at 0.31%. The average current levels of inbreeding of Northern, southern, central and Eastern agro ecological zones were as follows 0.32%, 0.32%, 0.34%, and 0.34%, respectively.

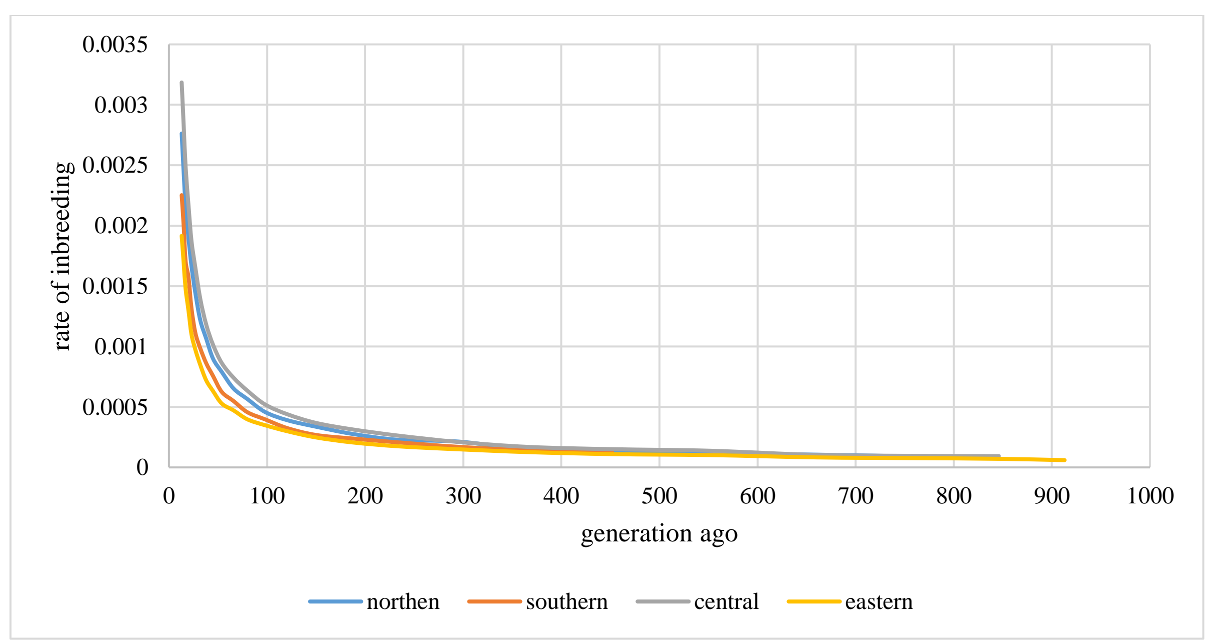


Figure 5.3: The rate of inbreeding per generation for the IC from the four agro-ecological zones

5.5 Discussion

Currently, there are limited studies on genetic parameters that shape the genetic structure of Indigenous chicken populations especially in the tropical African. There are several factors that make the estimation of Ne and other crucial population genetic parameters difficult; firstly, is the absence of pedigree data (Addis & Aschalew, 2014; Wright, 1938). Secondly, the lack of organization and proper record-keeping has been a hindrance in the estimation of Ne and inbreeding. Previously, most research focused on the variation of genetic materials and diversity of indigenous chickens mainly using microsatellite markers (Habimana *et al*., 2020; Mtileni et al., 2011b; Muchadeyi *et al.,* 2007). Although microsatellite markers are informative, they are too scarce over the genome to offer accurate information on the estimations of genetic parameters in a population. Recently there are several studies on indigenous chicken that have successfully used high-density SNP chips to characterize and estimate LD-based effective population size (Groenen *et al*., 2008; Nie *et al.,* 2019; Qanbari *et al.,* 2010) but not in Rwandan IC. Our study used SNPs to analyse Ne, inbreeding rates, and levels in indigenous chicken populations.

Analysis of trends in Ne of indigenous chicken in Rwanda suggested low Ne. The low Nes are consistent with the results found in both conserved and village chicken populations in South

Africa (Khanyile *et al*., 2015). Another study on Chinese local chicken population showed that the effective population is decreasing (Zhang *et al.,* 2020). The low trends in effective population size in study could be due to poor management of the indigenous chicken and could also be due to his could be due to a combination of founder effects in the IC flocks and also reduced gene flow among the IC population. The results in this study revealed a reduction in genetic variation over years which could be due to improper supervision of IC breeding and inbreeding which could be as a consequence of population being sub-structured within population bottlenecks is likely to have been done during the development of the populations. Shrinking in the Ne will disturb genetic progress in the indigenous chicken genetic improvement programs. Overlapping of northern and southern agro ecological zone generations may result in mating of closely related IC, thus this an increase in inbreeding levels. The highest historically Ne was found for northern and the lowest for central agro-ecological region. For the most recent Ne, the values varied from 181 (Northern) to 514 (Eastern) for the five generations ago. In overall, the differences for recent Ne between the agro-ecological regions were rather small.

5.5.1 Rate and levels of inbreeding

This study reported a lower rate of inbreeding than that found in Belgian chicken that ranged from 0.03% to 0.94% per generation (Lariviere *et al.,* 2011). A study on South African village chicken reported rate of inbreeding to be 0.119% (Zulu *et al*., 2015). The results showed that though the rates were decreasing as generations increased, the rates did not surpass the recommended rate of inbreeding of 0.1%-1% (FAO, 1998; Franklin & Frankham, 1998). A study on indigenous chicken of Indonesia calculated the rate of inbreeding and found it to be 0.024 (0.24%) signifying that this breed was not at danger of being extinct (Rusfidra *et al.,* 2016). The results observed in our study might be due to small flock size, use of few cocks for breeding and also due to absence of pedigree record. The consequences of inbreeding are potentially severe with the potential effects including population decline and possible extinction, high rates of inbreeding can also reduce genetic variance. However, one of the vital steps to decrease the inbreeding rate in these IC population is to implement a balanced breeding programme that monitors rate and levels of inbreeding in order to avoid its accumulation and its detrimental effects, this will be achieved by bringing of DNA materials from more superior and greater indigenous chicken from bordering societies or other superior IC ecotypes (Lariviere *et al.,* 2011). The results of this study

revealed the loss of genetic diversity in Rwandan IC contradicting results done using microsatellite by Habimana *et al.* (2020) which revealed that the Rwandan IC have high genetic variability. Microsatellites are more variable but suffer from ascertainment bias, homoplasy and amplification variation of primers (Tian *et al.,* 2008). Single nucleotide polymorphisms are more plentiful and evenly spread across the genome hence more informative (Nie *et al.,* 2019; Wollstein *et al.,* 2010).

5.6 Conclusion

This study has confirmed that Ne of indigenous chickens is decreasing whereas the inbreeding rate is increasing as the number of generations increase. The rate and levels of inbreeding seem to be in line with low Ne thus leading to loss of diversity in the Rwanda indigenous chicken. To conserve and improve the IC genetic diversity, it may be advisable that the breeders should design and implement a separate, but connected conservation and genetic improvement programmes. When designing a breeding programme, the breeders should put emphasis on the following; improving the fitness of Indigenous Chicken population and also ensure there is simultaneous improvement of disease resistance and immune traits. It might also be beneficial to improve the existing conservation indigenous chicken by advising farmers to swap cockerels and hens with their neighbouring societies and countries for the re-establishment of lost genetic diversity and reduce the effects of Ne and inbreeding.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion

Chicken are mostly selected by humans because they have important economic traits that benefit man (Desta, 2015). However, there are different chicken breeds used for food production but only few are selected for their high food out-put thus leaving other breeds not developed hence exposing these breeds to danger of extinction (Eding & Bennewitz, 2007). Indigenous chicken have multiple traits of economic importance alongside them being hardy and resistant to diseases and hence needs better characterization for future utilization, conservation and sustainability (Ngeno *et al.,* 2014). Indigenous chicken have undergone natural selection over years through their scavenging nature hence making them robust and increase their resistance to disease causing pathogens thus increasing their survivability to village conditions than the exotic breeds (Besbes *et al.,* 2007). However, their production is minimal as compared to the commercial exotic chicken hence making them have a small impact to the smallholder farmer's livelihood (Mahoro *et al.,* 2017a). Several breeding programs including crossbreeding indigenous chicken and the exotic chickens have been started in the tropical countries to improve the performance of indigenous chicken but some of them failed (Dana *et al.,* 2011; MINAGRI, 2012; Okeno *et al*., 2012).

To properly improve and utilize indigenous chickens in the tropics their phenotype and genotype diversity must be properly and well examined for future improvement (Muchadeyi *et al*., 2008). In the tropics few and non-exhaustive studies have been done to characterize the genomic capabilities of indigenous chicken using genetic technologies. Never the less, phenotyping of an individual animal from sequenced data is feasible with the aid of genome wide variants and the advancements in bioinformatics analyses (Alderborn, 2000; Gheyas *et al.,* 2015; Khoo, 2017; Schmid *et al.,* 2015).

The study aimed to examine the immunogenetic variation, population structure of ICs at both whole-genome level and MHC region in chromosome 16, secondly, it aimed to identify the deleterious impacts of mutation with relation to disease resistance and undertaking functional characterization of deleterious variants of indigenous chicken. Lastly, the study aimed to estimate Linkage Disequilibrium-based effective population size, rate and levels of inbreeding of IC. The

findings in this thesis provided insight understanding on the immunogenetic and genomic diversity of IC from four agro-ecological environments in Rwanda.

In the first chapter, emphasis was on the problems that justify this work and provide background information and objectives of the study. Chapter two expansively reviewed indigenous chicken origin, purpose, production, genetic improvement attempts, disease resistance, their phenotype and their genetic diversity. This chapter also comprehensively reviewed on the molecular methods that examine the genomic variations of chicken. In line with the previous studies (Cendron *et al.,* 2020; Dar *et al*., 2018; Habimana *et al.,* 2020; Kingori *et al*., 2010; Lee *et al.,* 2011; Lindahl *et al.,* 2019; McLaren *et al.,* 2016; Moges *et al.,* 2010; Mwamachi *et al.,* 2000; Okeno *et al.,* 2012;; Oloo *et al*., 2018; Uimari & Tapio, 2011;Vali, 2008; Zekarias & Rebel , 2014), this review evidences the high phenotypic and genotypic diversity of indigenous chickens in the tropics. Chapter three examined; immunogenetic variations based on Newcastle disease antibody titers, phenotypic variations (body weights) and genomic diversity at MHC region and at whole genome level. Results revealed that both phenotypic (antibody titer and bodyweight) and genotypic characterization using whole genome data grouped the IC into two groups, providing a deeper understanding of the structure of IC population to supplement the use of phenotypes for IC selection (Chiwanga *et al.,* 2020). At MHC region, the genetic diversity analysis revealed one genetic group. These results indicate that at the MHC region, there is significantly less genetic variation than the variation within the whole genome of a population.

The fourth chapter focussed on identification and annotation of deleterious variants in IC genome. Results revealed six genes that are associated with immune and disease resistance traits which include; *FANCA, RBBP5, CRB1, RUFY3, FBXO38 and PDE3A*. Functional annotation linked the *FANCA* gene to the regulation of the following; cell proliferation, T cell differentiation, inflammatory response, and CD40 signaling pathway. The *RBBP5* gene was linked to the cellular response to DNA damage stimulus and the *CRB1* gene was linked to heterophilic adhesion of cell-cell through adhesion of the plasma membrane cell molecules and regulation of the synaptic vesicle exocytosis. The *RUFY3* gene was linked to positive regulation of cell migration. The *FBXO38* gene was linked to controlling T cell-mediated immune reaction to the cancer cell and finally and the *PDE3A* gene was linked to response to the drug. This chapter also presents a vital understanding of the role of MHC region in chromosome 16, which is associated with immune traits in the genetic diversity. Functional annotation of variants at MHC location revealed one gene

(ENSGALG00000046739) that is associated with non-deleterious missense variants. The study reported mutational load to be 0.59 which was higher than 0.018 reported in commercial chicken and 0.014 that was observed in African village chicken (Bosse *et al.,* 2018). In relation to the results mentioned above on chicken, it can be concluded that the mutational load of Rwanda's indigenous chicken is high and maybe attributed to domestication of these chicken species and artificial selection.

In chapter five, effective population size and inbreeding were examined. The effective population size of indigenous chickens in Rwanda is decreasing whereas the inbreeding rate is increasing as the number of generations increases. The rate and levels of inbreeding are in line with low Ne thus leading to loss of diversity in the Rwanda indigenous chicken.

6.2. Conclusions and recommendation

From the results of this study, the following conclusions can be drawn:

1. Phenotypic and genotypic clustering found 2 genetic clusters of Indigenous Chicken in Rwanda and also clustering based on chromosome 16 which is related to disease resistance and immune traits placed the IC as one population.
2. Six deleterious genes (*FANCA, RBBP5, CRB1, RUFY3, FBXO38,* and *PDE3A)* were revealed and have impact on immune traits of indigenous chickens.
3. All though the rates and the levels of inbreeding are lower than the recommended rate of 1% and also the effective population of indigenous chickens is decreasing whereas the inbreeding rate is increasing as the number of generations increase

6.3 Recommendations

1. Since 182 alleles were identified to be deleterious further, research is necessary to evaluate the other remaining genes influencing other traits other than immune traits
2. Chicken improvement programs ahead should consider the 2 genetic clusters revealed in this study.
3. When designing a breeding programme, the government should put emphasis on the following; improving the fitness of Indigenous Chicken population and also ensure there is simultaneous improvement of disease resistance and immune traits.

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8. APPENDICES

Appendix 1: Research permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 238395

Date of Issue: 18/March/2022

RESEARCH LICENSE

This is to Certify that Miss.. ESTHER KULUNDU MBAKAYA of Egerton University, has been licensed to conduct research in Nakuru on the topic: GENETIC EVALUATION OF IMMUNOGENETIC VARIATIONS, MUTATIONAL LOAD AND EFFECTIVE POPULATION SIZE IN INDIGENOUS CHICKEN for the period ending : 18/March/2023.

License No: NACOSTI/P/22/16316

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1. The License is valid for the proposed research, location and specified period
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Appendix 2: Ethical clearance

EGERTON **UNIVERSITY**

TEL: (051) 2217808
FAX: 051-2217942

P. O. BOX 536
EGERTON

EGERTON UNIVERSITY RESEARCH ETHICS COMMITTEE

EU/RE/DVC/009
Approval No. EUREC/APP/139/2021 **21st October, 2021**

Esther Kulundu Mbakaya
Animal Science Department
Egerton University
P.O. Box 536-20115
Egerton
Telephone: 0703280352
E-mail: esthermbakaya@gmail.com

Dear Esther,

RE: ETHICAL APPROVAL: GENETIC EVALUATION OF IMMUNOGENETIC VARIATIONS, MUTATIONAL LOAD AND EFFECTIVE POPULATION SIZE IN INDIGENOUS CHICKEN.

This is to inform you that ***Egerton University Research Ethics Committee*** has reviewed and approved your above research proposal. Your application approval number is ***EUREC/APP/139/2021.*** The approval period is ***21st October, 2021 –22nd October, 2022.***

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used.
ii. You are required to adhere Institutional Experimental Animals use and Care policy.
iii. All changes including (amendments, deviations, and violations) are submitted for review and approval by ***Egerton University Research Ethics Committee***.
iv. Death and life-threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to ***Egerton University Research Ethics Committee*** within 72 hours of notification
v. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to ***Egerton University Research Ethics Committee*** within 72 hours
vi. Clearance for Material Transfer of biological specimens must be obtained from relevant institutions.

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vii. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
viii. Submission of an executive summary report within 90 days upon completion of the study to ***Egerton University Research Ethics Committee***.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Yours sincerely.

EGERTON UNIVERSITY
Date. 21/10/2021

Prof. R. Ngure
CHAIRMAN, EGERTON UNIVERSITY RESEARCH ETHICS CTTEE
RMN/BK/

Appendix 3: Abstract of paper for objective one

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E Mbakaya
Department of Animal Sciences, Egerton University, P.O. Box 536-20115, Nakuru, Kenya

T Muasya
Department of Animal Sciences, Egerton University, P.O. Box 536-20115, Nakuru, Kenya

K Ngeno
Department of Animal Sciences, Egerton University, P.O. Box 536-20115, Nakuru, Kenya

Corresponding Author:
E Mbakaya
Department of Animal Sciences, Egerton University, P.O. Box 536-20115, Nakuru, Kenya

Immunogenetics and genetic variations in indigenous chicken in the tropics using SNP data

E Mbakaya, T Muasya and K Ngeno

DOI: https://doi.org/10.22271/veterinary.2022.v7.i4a.428

Abstract
This study used data from 150 indigenous chicken from four agro-ecological zones in Rwanda to provide deep insight of the population structure and variation of the immunogenetics using several approaches based on phenotypic and SNP data. The population structure of indigenous chicken was analysed using Principal Component Analysis (PCA), ADMIXTURE analysis, and phylogenetic relationships for the whole genome and at chromosome 16. The study used 65,945 SNPs from the collected chicken. Phenotypic analysis was done for the Newcastle disease titer (ab) alongside bodyweight at 20 weeks with the highest having 1.6 kg. The genome analysis was done using the genotyping-by-sequencing approach. The grouped the indigenous chicken into two genetic clusters, which was confirmed by ADMIXTURE analysis that revealed that the lowest cross-validation (CV) error (0.51) was at K=2. The analysis of Population structure at chromosome 16 showed that the population had the lowest CV error (0.50) at K=1. The mean body weight and antibody titer were 1673.61±237.14 g and .5±55.35, 1311.34±121.9 g and 8832.5±55.36, respectively, depicting an inverse relationship between bodyweight and antibody titers. The cluster means for body weight and antibody titers were significantly different ($P<0.001$) for body weight and antibody titers. The indigenous chicken genetic clusters in Rwanda have variation in antibody titers which can be attributed to varied selection pressure. The observed genetic diversity of the indigenous chicken for disease resistance should be well-thought-out when scheming a selection programme to ensure that the ICs population is sustainable, flexible and simultaneous improvement of this trait. Based on this study's findings government should implement strategies that conserve and maintain the genomic diversity of Rwanda indigenous chicken.

Keywords: Chromosome 16, immune traits, flexibility, MHC, sustainability

Introduction

Indigenous Chicken farming has been gradually shifting from subsistence to commercial chicken farming due to increased demand for IC meat and eggs (Magothe *et al.*, 2019) [25]. The increase in IC demand makes farmers house birds in large flocks at high stocking density thus the increased risk of diseases, and disease spreading (Mujyambere *et al.*, 2022) [26]. Newcastle disease is one of the common diseases affecting IC farming (Kapczynski *et al.*, 2013; Walugembe *et al.*, 2019) [20, 43]. Identifying the genes that control disease resistance would make the selection of IC for improved productive performance and enhanced disease resistance possible. A useful spinoff would reduce cost of production due to decreased use of drugs, as well as better product quality due to lowered drug residues (Jie & Liu ,2011; Dar *et al.*, 2018) [19, 9].

A number of efforts have been practiced worldwide to appreciate and improve resistance to disease in livestock through the application of immunogenetics. In pigs improvement of resistance to disease was applied using gram–negative bacteria (Zhao & Chen, 2012) [47] and in ruminants it was done using gastrointestinal nematodes (Sweeney & Good, 2016) [39]. In bovine, immunogenetics was applied to improve resistance to mastitis (sodeland *et al.*, 2011) [38]. Information on both immunology and genetics of animal would well describe the disease phenotype (Bishop, 2014) [6]. Immune capability related to a particular disease used can be to show indirect selection for resistance to disease because these traits can be assessed and measured in breathing animals (Luo *et al.*, 2013) [22]. Santos-Argumedo, 2012 showed that antibody titers are immulogical traits which can be inheritated in poultry thus making it easy to determine loci or a particular gene associated to immune-related traits.

~ 26 ~

Appendix 4: Abstract of conference paper for objective one

Tropentag 2021, September 15 - 17, hybrid conference, Germany

"Towards shifting paradigms in agriculture for a healthy and sustainable future"

Genomic and Immunogenic Variations of Indigenous Chicken in the Tropics

Esther Mbakaya, Kiplangat Ngeno, Thomas Muasya

Egerton University, Dept. of Animal Science, Kenya

Abstract

Indigenous chicken (IC) farmers in developing countries desire enhanced disease resistance alongside improvement of body weight and egg production. This study aimed at providing insight into the population structure and immunogenetic variability of the indigenous chicken using various methods. Population structure of IC was analysed through genotypic clustering, admixture analyses and phylogenetic relationship for the whole genome and at chromosome 16. A total of 150 IC sampled from five agro-ecological zones in Rwanda were phenotyped for Newcastle disease titer alongside body weight and genotyped with the genotyping-by-sequencing (GBS) method. After quality control procedures for SNP data, 65,945 SNPs were retained for analysis. Following PCA, the IC were grouped into two genetic clusters, which were confirmed by lowest CV error (0.51) at K = 2. Population structure assessments based on SNPs in the MHC region indicated that the population as one with lowest CV error (0.50) which was confirmed at K =1. Clusters one mean body weight and antibody titre of 1673.61 ±237.14g and 4912.5±55.35, respectively. Corresponding values for cluster 2 were 1311.34±121.9 g and 8832.5±55.36. The clusters differed significantly ($P < 0.001$) for body weight and antibody titer. The cluster with high mean in bodyweight (Cluster 1) and low mean in titer and vice versa. The IC genetic clusters in Rwanda have variation disease resistance, which can be attributed to varied selection pressure. The observed genetic diversity of IC for BW and their negative association should be considered when designing a selection programme to ensure sustainability, flexibility and simultaneous improvement of the two traits.

Keywords: Disease resistance, MHC, sustainability

Contact Address: Esther Mbakaya, Egerton University, Dept. of Animal Science, 536-20115, 20100 Nakuru, Kenya, e-mail: esthermbakaya@gmail.com

Appendix 5: Biological processes and functions of the disease related genes

Gene	Function		G.O terms	Molecular function	Biological processes
FANCA	protein_coding	46	GO:0007140	male meiotic nuclear division	A cell cycle process by which the cell nucleus divides as part of a meiotic cell cycle in the male germline.
FANCA	protein_coding	46	GO:2000348	regulation of CD40 signaling pathway	Any process that modulates the frequency, rate or extent of signaling via the CD40 signaling pathway.
FANCA	protein_coding	46	GO:0036297	interstrand cross-link repair	Removal of a DNA interstrand crosslink (a covalent attachment of DNA bases on opposite strands of the DNA) and restoration of the DNA. DNA interstrand crosslinks occur when both strands of duplex DNA are covalently tethered together (e.g. by an exogenous or endogenous agent), thus preventing the strand unwinding necessary for essential DNA functions such as transcription and replication.
RBBP5	protein_coding	44.71	GO:0006974	cellular response to DNA damage stimulus	Any process that results in a change in state or activity of a cell (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus indicating damage to its DNA from environmental insults or errors during metabolism.
RBBP5	protein_coding	44.71	GO:0051568	histone H3-K4 methylation	The modification of histone H3 by addition of one or more methyl groups to lysine at position 4 of the histone.
CRB1	protein_coding	39.26	GO:0045197	establishment or maintenance of epithelial cell apical/basal polarity	Any cellular process that results in the specification, formation or maintenance of the apicobasal polarity of an epithelial cell.
CRB1	protein_coding	39.26	GO:0007157	heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules	The attachment of an adhesion molecule in one cell to a nonidentical adhesion molecule in an adjacent cell.
CRB1	protein_coding	39.26	GO:0001974	blood vessel remodeling	The reorganization or renovation of existing blood vessels.
CRB1	protein_coding	39.26	GO:0007009	plasma membrane organization	A process that is carried out at the cellular level which results in the assembly, arrangement of constituent parts, or disassembly of the plasma membrane.
CRB1	protein_coding	39.26	GO:0007601	visual perception	The series of events required for an organism to receive a visual stimulus, convert it to a molecular signal, and recognize

					and characterize the signal. Visual stimuli are detected in the form of photons and are processed to form an image.
CRB1	protein_coding	39.26	GO:0010001	glial cell differentiation	The process in which a relatively unspecialized cell acquires the specialized features of a glial cell.
CRB1	protein_coding	39.26	GO:0010467	gene expression	The process in which a gene's sequence is converted into a mature gene product or products (proteins or RNA). This includes the production of an RNA transcript as well as any processing to produce a mature RNA product or an mRNA or circRNA (for protein-coding genes) and the translation of that mRNA or circRNA into protein. Protein maturation is included when required to form an active form of a product from an inactive precursor form.
CRB1	protein_coding	39.26	GO:0010842	retina layer formation	The process in which the vertebrate retina is organized into three laminae: the outer nuclear layer (ONL), which contains photoreceptor nuclei; the inner nuclear layer (INL), which contains amacrine, bipolar and horizontal cells; and the retinal ganglion cell (RGC) layer. Between the inner and outer nuclear layers, the outer plexiform layer (OPL) contains connections between the photoreceptors and bipolar and horizontal cells. The inner plexiform layer (IPL) is positioned between the INL and the ganglion cell layer and contains the dendrites of RGCs and processes of bipolar and amacrine cells. Spanning all layers of the retina are the radially oriented Mueller glia.
CRB1	protein_coding	39.26	GO:0034613	cellular protein localization	Any process in which a protein is transported to, and/or maintained in, a specific location at the level of a cell. Localization at the cellular level encompasses movement within the cell, from within the cell to the cell surface, or from one location to another at the surface of a cell.
CRB1	protein_coding	39.26	GO:0035845	photoreceptor cell outer segment organization	A process that is carried out at the cellular level and results in the assembly, arrangement of constituent parts, or disassembly of the outer segment of a photoreceptor cell, a sensory cell that reacts to the presence of light. The outer segment of the photoreceptor cell contains the light-absorbing materials.
CRB1	protein_coding	39.26	GO:0042462	eye photoreceptor cell development	Development of a photoreceptor, a sensory cell in the eye that reacts to the presence of light. They usually contain a pigment

					that undergoes a chemical change when light is absorbed, thus stimulating a nerve.
CRB1	protein_coding	39.26	GO:0045494	photoreceptor cell maintenance	Any process preventing the degeneration of the photoreceptor, a specialized cell type that is sensitive to light.
CRB1	protein_coding	39.26	GO:0050908	detection of light stimulus involved in visual perception	The series of events involved in visual perception in which a light stimulus is received and converted into a molecular signal.
CRB1	protein_coding	39.26	GO:0060041	retina development in camera-type eye	The process whose specific outcome is the progression of the retina over time, from its formation to the mature structure. The retina is the innermost layer or coating at the back of the eyeball, which is sensitive to light and in which the optic nerve terminates.
CRB1	protein_coding	39.26	GO:0060042	retina morphogenesis in camera-type eye	The process in which the anatomical structure of the retina is generated and organized.
CRB1	protein_coding	39.26	GO:0061024	membrane organization	A process which results in the assembly, arrangement of constituent parts, or disassembly of a membrane. A membrane is a double layer of lipid molecules that encloses all cells, and, in eukaryotes, many organelles; may be a single or double lipid bilayer; also includes associated proteins.
CRB1	protein_coding	39.26	GO:0061159	establishment of bipolar cell polarity involved in cell morphogenesis	The specification and formation of bipolar intracellular organization or cell growth patterns that contribute to cell morphogenesis. Bipolar organization is the organization that is a mirror image along an axis from a plane.
CRB1	protein_coding	39.26	GO:0071482	cellular response to light stimulus	Any process that results in a change in state or activity of a cell (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a light stimulus, electromagnetic radiation of wavelengths classified as infrared, visible or ultraviolet light.
CHRNA5	protein_coding	39.37	GO:0035095	behavioral response to nicotine	Any process that results in a change in the behavior of an organism as a result of a nicotine stimulus.
CHRNA5	protein_coding	39.37	GO:0034220	ion transmembrane transport	A process in which an ion is transported across a membrane.
CHRNA5	protein_coding	39.37	GO:0006811	ion transport	The directed movement of charged atoms or small charged molecules into, out of or within a cell, or between cells, by means of some agent such as a transporter or pore.
CHRNA5	protein_coding	39.37	GO:0035094	response to nicotine	Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme

					production, gene expression, etc.) as a result of a nicotine stimulus.
CHRNA5	protein_coding	39.37	GO:2000300	regulation of synaptic vesicle exocytosis	Any process that modulates the frequency, rate or extent of synaptic vesicle exocytosis.
RUFY3	protein_coding	43.83	GO:0030335	positive regulation of cell migration	Any process that activates or increases the frequency, rate or extent of cell migration.
PDE3A	protein_coding	40.55	GO:0001556	oocyte maturation	A developmental process, independent of morphogenetic (shape) change, that is required for an oocyte to attain its fully functional state. Oocyte maturation commences after reinitiation of meiosis commonly starting with germinal vesicle breakdown, and continues up to the second meiotic arrest prior to fertilization.
PDE3A	protein_coding	40.55	GO:0019933	cAMP-mediated signaling	Any intracellular signal transduction in which the signal is passed on within the cell via cyclic AMP (cAMP). Includes production of cAMP, and downstream effectors that further transmit the signal within the cell.
PDE3A	protein_coding	40.55	GO:0040020	regulation of meiotic nuclear division	Any process that modulates the frequency, rate or extent of meiotic nuclear division, the process in which the nucleus of a diploid cell divides twice forming four haploid cells, one or more of which usually function as gametes.
PDE3A	protein_coding	40.55	GO:0042493	response to drug	Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a drug stimulus. A drug is a substance used in the diagnosis, treatment or prevention of a disease.
PDE3A	protein_coding	40.55	GO:0043116	negative regulation of vascular permeability	Any process that reduces the extent to which blood vessels can be pervaded by fluid.
PDE3A	protein_coding	40.55	GO:0043951	negative regulation of cAMP-mediated signaling	Any process which stops, prevents, or reduces the frequency, rate or extent of cAMP-mediated signaling, a series of molecular signals in which a cell uses cyclic AMP to convert an extracellular signal into a response.
PDE3A	protein_coding	40.55	GO:0060282	positive regulation of oocyte development	Any process that increases the rate or extent of the process whose specific outcome is the progression of an oocyte over time, from initial commitment of the cell to its specific fate, to the fully functional differentiated cell.
PDE3A	protein_coding	40.55	GO:0071321	cellular response to cGMP	Any process that results in a change in state or activity of a cell (in terms of movement, secretion, enzyme production,

					gene expression, etc.) as a result of a cGMP (cyclic GMP, guanosine 3',5'-cyclophosphate) stimulus.
PDE3A	protein_coding	40.55	GO:0007165	signal transduction	The cellular process in which a signal is conveyed to trigger a change in the activity or state of a cell. Signal transduction begins with reception of a signal (e.g. a ligand binding to a receptor or receptor activation by a stimulus such as light), or for signal transduction in the absence of ligand, signal-withdrawal or the activity of a constitutively active receptor. Signal transduction ends with regulation of a downstream cellular process, e.g. regulation of transcription or regulation of a metabolic process. Signal transduction covers signaling from receptors located on the surface of the cell and signaling via molecules located within the cell. For signaling between cells, signal transduction is restricted to events at and within the receiving cell.
FBXO38	protein_coding	37.26	GO:0002842	positive regulation of T cell mediated immune response to tumor cell	Any process that activates or increases the frequency, rate, or extent of a T cell mediated immune response to tumor cell.
FBXO38	protein_coding	37.26	GO:0031146	SCF-dependent proteasomal ubiquitin-dependent protein catabolic process	The chemical reactions and pathways resulting in the breakdown of a protein or peptide by hydrolysis of its peptide bonds, initiated by the covalent attachment of ubiquitin, with ubiquitin-protein ligation catalyzed by an SCF (Skp1/Cul1/F-box protein) complex, and mediated by the proteasome.
FBXO38	protein_coding	37.26	GO:0070936	protein K48-linked ubiquitination	A protein ubiquitination process in which a polymer of ubiquitin, formed by linkages between lysine residues at position 48 of the ubiquitin monomers, is added to a protein. K48-linked ubiquitination targets the substrate protein for degradation.