POPULATION STRUCTURE AND GENETIC DIVERSITY OF INDIGENOUS CHICKEN POPULATIONS (*GALLUS GALLUS DOMESTICUS*) USING MHC-LINKED MICROSATELLITE LEI0258 IN SOUTH KIVU, EASTERN DEMOCRATIC REPUBLIC OF CONGO

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2024

Population Structure and Genetic Diversity of Indigenous Chicken Populations (*Gallus Gallus Domesticus*) using MHC-Linked Microsatellite LEI0258 in South Kivu, Eastern Democratic Republic of Congo

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Molecular Biology and Bioinformatics of the Jomo Kenyatta University of Agriculture and Technology

2024

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this work to my lovely wife Fadhili Mirindi Marceline and my children Aganze Emmanuel Bienvenu, Aganze Ntavigwa Bienfait, Aganze Murhula Bienveillant and Aganze Chirhuza Bienheureux who were on my side to give more courage.

ACKNOWLEDGEMENT

First and foremost, I am thankful to the Lord Almighty for the gift of life and for granting me an opportunity in education to better my family life.

I would like to express my heartfelt thanks to my supervisors Dr. Steven Ger Nyanjom and Dr. Roger PELLE. I greatly appreciate their meticulous guidance, patience, encouragement and leadership that I learnt from them during the time I was conducting this work.

I would like to greatly acknowledge:

Dr.Domelevo Entfellner Jean Baka and Martina Kyalo for their scientific and technical assistance during data analysis.

All BecA-ILRI Hub family for financial, scientific and technical support and the warm working environment during my lab work they created to smoothly accomplish my work. The laboratory part of the project was supported by the BecA-ILRI Hub through the African Biosciences Challenge Funds (ABCF) program. The ABCF program is funded by the Australian Department for Foreign Affairs and Trade (DFAT) through the BecA-CSIRO partnership; the Syngenta Foundation for Sustainable Agriculture (SFSA); the Bill & Melinda Gates Foundation (BMGF); the UK Department for International Development (DFID); and the Swedish International Development Cooperation Agency (SIDA).

I wish to convey my sincere thanks and acknowledgements to Fond Adrien Bauchau and the university of Namur for the financial supports without which the study would not have been possible in Kenya, and the field sampling work (in DRC) and the analysis of the project (in Kenya). I express my gratitude to Professors Pierre DEVOS, Michel Jadot, Robert and Simone Wattiaux for their supports. ISP/Bukavu and all the smallholder farmers are warmly acknowledged for their field supports.

I am thankful to my family members, friends and classmates for their encouragement, moral support and tolerance during my studies.

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ACRONYMS AND ABBREVIATIONS

AMOVA	Analysis of Molecular Variance
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphates
DRC	Democratic Republic of Congo
EDTA	Ethylene Diamine Tetra Acetic acid
Indels	Insertion / Deletion
ISP/BUKAVU	Institut Supérieur Pédagogique de Bukavu
MHC	Major Histocompatibility Complex
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
VNTR	Variable Number of Tandem Repeats

ABSTRACT

Chickens are the most important and widely distributed of all livestock in Africa. They play very significant socio-cultural and economic roles in African societies. In the Democratic Republic of Congo, chickens are generally kept by many poor small farmers for their meat and eggs. They provide a source of nutrition and income in rural and peri-urban areas. The availability of information on chicken population structure and diversity is important in the conservation of genetic resources which is critical in the selection of breeds that are important in diverse production systems. This study aimed to determine allelic variability, genetic diversity, and genetic relationships of the indigenous chicken populations from the South Kivu region to support breeding programs and genetic resource conservations. The *LEI0258* microsatellite marker within the major histocompatibility complex gene region was used for genotyping. 2-5 mL of blood sample was collected by venipuncture of the jugular vein from each bird and stored onto Whatman FTA filter paper. Three punches of dried blood spot per sample were used to isolate the total genomic DNA using Invitrogen DNA extraction Kit and boiling method. Then PCR amplification was performed followed by Sanger sequencing. The number of R13 and R12 repeats varied from 1 to 21 and 3 to 21, respectively; whereas several combinations of indels and single-nucleotide polymorphisms (SNPs) were observed in the microsatellite flanking regions. In total, 45 different *LEI0258* alleles ranging from 193 to 473 bp were determined, including 14 private alleles (Np). Expected heterozygosity (He) varied from 0.864 (Mwenga) to 0.938 (Bukavu) with a mean of 0.911, and observed heterozygosity (Ho) ranged from 0.417 (Uvira) to 0.667 (Mwenga), with a mean of 0.519. The analysis of molecular variance (AMOVA) revealed higher genetic variation within individuals (56%) than among individuals (43%) and among chicken populations (1%). Clustering into three admixed gene pools (K=3) showed the relationships among the chicken populations. The present study showed the existence of high genetic diversity in chicken populations from South Kivu. This study provides information useful for better conservation and breeding strategies of indigenous chicken populations in South Kivu.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Globally, chickens are the most widely distributed of all livestock species and most abundant species of domestic birds in the world (Scherf,, 2000). They play very significant socio-cultural and economic roles in most African societies (Mugumaarhahama *et al*. 2016). Indigenous chickens "*Gallus gallus domesticus*" in the Democratic Republic of Congo (D.R. Congo) are generally kept in traditional management backyard systems and scattered throughout the country. In South-Kivu, indigenous chickens play an important role in the livelihoods of smallholder families and contribute significantly to poverty alleviation, women empowerment, and food security as the main source of income, meat, egg, social and ritual values (Guèye 2002; Moula *et al.* 2012; Keambou *et al.* 2014). Despite their importance, little is known about their genetic diversity, different phenotypic data, local population sizes, production performances, adult body phaneroptic and measurements in Africa (Keambou *et al*. 2009).

The major histocompatibility complex B (MHC-B) is known for its implication in disease resistance and its susceptibility to numerous pathogens including in chickens (Fulton *et al*. 2006; Han *et al*. 2013). The MHC-B genes are used as a model for studying how genetic diversity is maintained in chicken populations (Spurgin and Richardson, 2010). These genes play a central role in the immune system where they code for molecules that bind to self-peptides and non-self- peptides involved in peptide antigens presentation to T-cells (Fulton *et al*. 2006; Chazara *et al*. 2013). The MHC molecules bind peptides with anchor amino acids at specific positions that fit exactly to their binding pocket thereby triggering a cascade of immune responses (Hako Touko *et al*. 2015). Genes of MHC provide the most promising opportunity for studying the genetic diversity of populations and how it operates to maintain variation in species (Lwelamira *et al*. 2008; Han *et al*. 2013; Keambou *et al*. 2014).

The microsatellite marker *LEI0258* is physically located within the MHC, between the erythrocyte antigen B encoded class IV (BG; MHC class 4) and erythrocyte antigen B encoded class I (BF; MHC class 1) regions and commonly defined by serological reactions between red blood cell antibodies specific to erythrocyte antigen B encoded class I (BF) and the highly polymorphic BG antigens, on chromosome 16. The marker is a highly polymorphic tendem repeat identified in chicken MHC haplotypes (Izadi *et al*. 2011; Fulton *et al*. 2006). Recent studies have reported that *LEI0258* microsatellite marker maps to chromosome 16 and is useful in determining heterozygosity and estimating genetic distances among closely related species including avians (Izadi *et al*. 2011). This marker is investigated as a genetic indicator for MHC haplotypes (Fulton *et al*., 2006). It is also useful in measuring the number of defective alleles and measuring suitably the polymorphism information content in populations. It has become a standard method to estimate genetic diversity indices in all livestock and avian species (Olowofeso *et al*. 2005; Chen *et al*. 2015).

The indigenous chickens exhibit high genetic diversity because they are raised in diversified ecological habitats (Ngeno *et al*. 2015). This maintains species by enabling them suitably to adapt to environmental changes and challengers. This diversification gives them a better ability to survive and reproduce (Sinoya, 2017).

However, studies on chicken diversity have used the reference marker *LEI0258* for the chicken MHC identified by McConnel *et al*. 1999, Kaufman *et al*.1999; described by Fulton et al. 2006 and validated as a predictor of MHC genotypes by Chazara *et al*. 2011. The *LEI0258* microsatellite marker has been used for single nucleotide polymorphisms (SNPs) and shows high polymorphism with a large number of alleles and a large range in allele size (Fulton *et al*. 2006; Chazara *et al*. 2013; Han *et al*. 2013). The *LEI0258* marker genotype is reported to give a good indication of the variability of the MHC region in different chicken populations and has been widely used in genetic diversity studies in many regions with different chicken breeds (Fulton *et al*. 2006; Lwelamira *et al*. 2008; Chazara et *al. 2013*; Hako Touko *et al*. 2015).

In this study, *the LEI0258* marker was used to identify existing and new MHC-B microsatellite haplotypes, the variability of alleles for the tandem repeat, genetic diversity and relationships between indigenous chicken populations from South-Kivu in the eastern part of the D.R. Congo.

1.2 Statement of the Problem

Farmers in South Kivu haven't exploited genetic information to improve local chickens as they still practice the traditional way of keeping chickens. The traditional chicken farming methods are associated with slow growth and maturity rate, poor feeding and high chicks' mortality rate (Katunga *et al*. 2020). As a result the farmers cannot benefit the full potential of local chicken farming since they lack information from genetic studies that would help to improve the local chicken traits, come up with measures and pertinent strategies to promote dissemination and uptake technologies to boost productivity of their meat and eggs higher and incomes.

1.3 Justification

In DRC, reports on the diversity of indigenous chickens are restricted to phenotypic data and morphobiometric studies (Moula *et al*. 2012, Mugumaarhahama *et al*. 2016). The lack of chicken population studies in the D.R. Congo has led to difficulties in determining genetic diversity that can be used to improve local chicken productivity. Such studies can be explored to improve the local chicken breeds to produce more eggs, mature faster and meet market weight faster and make them potential source of income (Kamau *et al*., 2018).

In order to associate disease resistance and performance with the genotype, recent studies on chicken diversity have used functionally important protein coding genes such as major histocompatibility complex (MHC), which is known to have a very strong association to disease resistance and susceptibility to numerous pathogens (Fulton *et al*. 2006; Han *et al*. 2013). To capture this within the local chicken from South Kivu will be informative for future decision makers and breeders and even to farmers on the fattening of the hens.

In this study, the LEI0258 marker was used to identify established and new MHC-B microsatellite haplotypes, the variability of alleles for the tandem repeat, genetic diversity and genetic relationships of indigenous chicken populations from South-Kivu in the eastern part of the D.R. Congo. This will lead to chicken selections of hens for the future benefit to farmers

The preliminary study on genetic diversity of indigenous chicken in South-Kivu has the potential to guide the breeders to improve their strains and the policymakers to optimize conservation by utilizing adequate strategies.

1.4 Research Questions

We are focused to respond to the questions below:

1. What is the genetic variability in the MHC populations of indigenous chickens from South Kivu?
2. Are the microsatellite LEI0258 markers and SNPs in specific MHC gene loci in indigenous population of chickens from South Kivu?
3. Are populations of indigenous chicken in South Kivu genetically distant?

1.5 Hypothesis

1.5.1 The Null Hypothesis

Indigenous chicken from South Kivu eastern DRC are not diverse.

1.6 Objectives

1.6.1 General Objective

To determine population structure and genetic diversity of indigenous chicken populations (*Gallus gallus domesticus*) using MHC-linked microsatellite *LEI0258* marker in South Kivu, eastern D.R. Congo.

1.6.2 Specific Objectives

The specific objectives of this study are:

1. To determine the genetic makeup in the MHC populations of indigenous chickens from South Kivu
2. To identify the specific gene locus with the microsatellite *LEI0258* markers and the SNP positions within local chickens populations in South Kivu
3. To determine genetic distance and relationships in chickens populations in South Kivu.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin of Chickens in Africa

Chickens are believed to have descended from the four species of the jungle fowl: the red jungle fowl (*Gallus gallus*), the grey jungle fowl (*Gallus sonnerati*), the Ceylon jungle fowl (*Gallus lafayettei*) and the green jungle fowl (*Gallus various*) (Khobondo *et al*. 2014). The archeological discoveries done by West and Zhou (1988), and the protein polymorphisms and morphological characteristics done by Moiseyeva *et al*. (2003) suggested that domestic chickens were derived from the red jungle fowl. Studies done by Akishinonomiya *et al*. (1996) have reported that chicken from South Asia, South East Asia, Japan and Europe, on their 400 base pairs of mtDNA D-loop region show that the domestic chickens are derived from a single continental population of *G. g. gallus*.

The genetic integration of species in the genus Gallus and other subspecies of the red jungle fowl demonstrated and suggested that several species might have contributed to origin of modern chicken (Nishibori *et al*. 2005). The route and dates by which the chicken entered in Africa continent remain poorly understood (Khobondo *et al*. 2014). Maghote *et al*. (2012) suggests that the introduction of chicken in Africa was through Egypt from the South-Western Asia via the Middle East. The movement of the populations in Sub-Sahara Africa affected the spread of chickens in all areas of the continent because domestic chickens are closely associated with human interactions and they rely entirely upon humans for their dispersal and survival (Mwacharo *et al*. 2013). However, the route and how *Gallus gallus* moved and spread to D.R. Congo is not well documented.

2.2. Some Attributes of Indigenous Chickens Ecotypes in Africa

In Egypt, native chickens have been domesticated for many centuries and mostly kept as egg-layers. The breeds are known for their excellent resistance to diseases. Four native chicken types namely the Fayoumi, the Dandarawi, the Baladi Beheri

and the Sinai are well known in Egypt (Moula *et al*. 2012). The Fayoumi breed (bred for egg production) is well adapted to the African rural poultry system and climate. They are hardy, very precocious, early maturing and have excellent adapted flying abilities. They are hard to catch and once captured, they scream. They are also known as "Biggawi or Romand" in Egypt. Their eggs have unique features such as strong eggshell and low in cholesterol (Moula *et al*. 2012). The Dandarawi are found in Upper Egypt with a single comb and buttercup comb. They are reported to be used as stable chickens. The Baladi Beheri originated from the north region and the Delta of Egypt and are also Marek's disease resistant. The Sina are thought to be generated from the natural crossing between unknown native chickens and some foreign breeds since earlier time (Moula *et al*. 2012).

South-Africa is reported to have four native chicken breeds namely: the Potchefstroom koekoek, the veda, the ovambo and the naked neck. The Potchefstroom koekoek (the Potchefstroom cuckoo) was bred at the Potchefstroom agriculture college during the 1950s. The name koekoek refers to the color pattern of the bird and they are very popular and preferred for their suit eggs and meat as well as their ability to hatch their own offspring (Moula *et al.* 2012). The Venda was identified in the Venda area of Limpopo Province in 1979. Later on, similar chickens were identified in the Southern Cape. The Ovambo breed is reported to have originated from Namibia district of Ovamboland in 1975. The neked neck breeds are thought to have been introduced in South-Africa by the traders from Malaysia during their journey around the continent. Actually they are recognized as indigenous chicken breeds in South-Africa (Moula *et al*. 2012).

Distinct indigenous chicken ecotypes have been identified and named in Kenya: the names are coming from their phenotypic description of the birds. Generally the most common phenotypes are frizzled feathered, naked neck, barred feathered, feathered shanks, bearded and dwarf sized. These chickens vary in body size, conformation, plumage color and performance. They are heterogen population with no standardized characteristics and performance (Kigori *et al*. 2010). Indigenous chickens in Kenya vary in size, plumage color and skin color. The plumage color varies widely with black, brown or red dominating. Rare color patterns such as light, orange, yellow,

gray and white laced and mottled are seen. There is also variation in comb type and color of wattles; earlobes and beaks (Kigori *et al*. 2010).

Tanzania has eight local chicken breeds named ching'wekwe, Mbeya, Morogoro medium (frizzled), N'zenzegere (frizzled), Singamagazi, Pemba, Tanga and Unguja with small size. Recent studies have reported a chicken named Kuchi to have developed a higher level of resistance to diseases in Mwanza region (Moula *et al*. 2012). In the Republic of Central Africa, five main types of native chickens (feathering) are reported namely normal feathering, crested type, naked neck, feathered tarsus and frizzle type (Bembide *et al*. 2013). In Cameroon, we can find the Dzaye (with white feathers), the Dongwe (with black feathers), the Tsabatha (with mixed coloured feathers grey, black and white), the Zarwa (layer strain) and a foot feathered called "poule Brahman" (Moula *et al*. 2012).

Some phenotypic attributes used in the study as indigenous chicken ecotypes from South Kivu are shown in Figure 2.1.

Medium body size with multiple feather colors (majority red and black). 	Large body size with feather colors dominated by white. 
Large body size with multiple feather colors (majority red and black). 	Small body size with black feather color. 
Small body size with multiple feather colors (majority brown). 	Medium body size with multiple feather colors (majority brown). 

Figure 2.1: Some of the Indigenous Chicken Ecotypes Found in South Kivu
Source: (Researcher)

2.3. Poultry Farming System in DRC and South Kivu

The first and the most common form of chicken farming system in the DRC is the traditional system and represent 95% of local animals (Katunga *et al*. 2020). In this traditional system, indigenous breed are the most used and their housing is where the hens, which are counted on average between one and fifty per breeder, live with the

cock in the same house (Katunga *et al.* 2020). Their laying ranges from 8 to 25 eggs per clutch. The rate of mortality due to New Castle disease is very high and can even reach 100% since veterinary care is almost non-existent. Chickens are free range at 96% and hens are weaned from 4 to 5 months on average and are usually bred at 8 months. Each hen has 3 to 4 spawning cycles per year (Katunga *et al*. 2020; Mugumaarhahama *et al*. 2016).

The limitation of phenotypic attributes to determine population structure and diversity is focused on visual appearance and linear body measurements of the animals by determining the morphobiometric characteristics in traditional way (Bembide *et al*. 2013). Phenotypic characterization focused on qualitative phenotypic traits such as observation of feather morphology, feather distribution, plumage coloration, skin color, shank color, shape of crests, mumps and barbel color, tarsi and ear-lobe color, and comb type. On the other hand, quantitative traits focus on body weight, body length, circumference of the tarsus, and length of the tarsus, length of the wing, length of beak, thoracic circumference and egg weights. (Bembide *et al*. 2013; Mugumaarhahama *et al*. 2016; Katunga *et al*. 2020). Looking at genetic diversity, researchers are able to screen the genome of the animal (Groenen *et al.* 2000) and detect potential genes that are involved in breeding strategies and improvement in performance and productivity of the animals and increase their tolerance to diseases. The MHC-B region believed to control disease resistance, immune responses and other productive and reproductive traits in chickens (Sinoya, 2017).

The traditional system, also called the family poultry sector, is the most applied form in the DRC. In this system, the most common type of housing is where the chickens live with the humans in the same house or in the kitchen but in straying.

The semi-intensive system is the second form of chicken farming system in the DRC and represents 4% where both indigenous and laying hens are kept. Other forms of farming system are intensive (0.95%) and industrial (0.05%) with broiler chickens (Katunga *et al.* 2020).

2.4 Methods that Have Been Used to Study Population Structure and Genetic Diversity of Indigenous Chickens

Many studies have used different methods to study population structure and genetic diversity of indigenous chickens. The SSR and ISSR markers have been used in assessing genetic diversity in *Gallus gallus* domesticus (Mauricio *et al*. 2020). RFLP has been used in genetic identification of chicken in Bangladesh (Alam et al. 2016). The BCDO2 and mt-DNA D-loop have been used in chicken genetic diversity of Jiangxi in China (Yu-shi *et al*. 2017). 102 indels (insertion/deletion) markers have been used to study genetic diversity and population structure in native chicken in Thailand and Laos (Maw *et al*. 2015). Recent studies on structure and genetic diversity have focused on using microsatellite markers in genetic diversity of five local Swedish chicken breeds (Abiye *et al*. 2015). The LEI0258 marker is currently used in such studies (Mc Cornnell *et al*. 1999, Fulton *et al*. 2006, Chazara *et al*. 2011, Chazara *et al*. 2013).

2.5. Description of the Chromosome 16 and the B Region

The chicken karyotype comprises 39 chromosomes pairs as 10 pairs of large autosomes (chromosome 1-10), 28 pairs of microchromosomes (chromosome 11-38) and a pair of sex chromosome (chromosome W and Z). The chicken reference Genome assembly attributes the INSDC accession CM000108.3 and the total length (including gaps) 535,270 as the length of the chromosome 16 present in Galgal4 (Schmid *et al*. 2015).

There are two highly polymorphic blood group systems in chicken which are: the B region and the Y region reported by Briles *et al*. (1950) in 1940s on chromosome 16. The B region is composed of 19 family genes including the *LEI0258* marker (Miller *et al*. 2004, Fulton *et al*. 2006). The marker described by Mc Connell *et al*. (1999) and validated by Chazara *et al*. (2011) is reported to map the microchromosome 16, B-region and it is successfully used in several diversity studies and in many other diseases associations studies (Miller *et al*. 2004).

In chickens, the MHC occurs on chromosome 16, between the genetic marker *LEI0258* and *MCW0371* (10,560bp) and contains 19 genes spanning 92Kb. This marker is also found in a clone of the chicken MHC (GenBank, AL023516) (Guillemot *et al*. 1986, Mc Cornnell *et al*. 1999, Fulton *et al*. 2006, Chazara *et al*. 2011). The *LEI0258* microsatellite marker, physically located to genes of the MHC, was investigated as genetic indicator for the MHC haplotypes (Chazara *et al*. 2013). The microsatellite *LEI0258* is reported to amplify the *LEI0258* gene within the B region (Briles *et al*. 1982, Kaufman *et al*. 1999, Miller *et al*. 2004, Fulton et al. 2006, Chazara *et al*. 2011). The two levels of polymorphism for the *LEI0258* marker are the number of tandem repeats whose lengths are R12 bp (TTCCTTCTTTCT) and R13 bp (ATGTCTTCTTTCT) respectively (Fulton *et al*. 2006; Chazara *et al*. 2013).

The major histocompatibility complex (MHC) is a highly polymorphic blood group system known as "B region" and containing multiple alleles at the locus because it is responsible for graft rejection, or tissue compatibility (Lwelamira *et al*. 2008; Walker *et al.* 2011; Chazara *et al*. 2013).

MHC genes families are found in all vertebrates though they vary widely and provide the most opportunity for studying how balancing selection operates to maintain genetic variation in populations. The driving forces maintaining diversity at MHC loci have been suggested to derive from pathogen-mediated selection and, thus the MHC regulates the cell-mediated adaptive immune response (Spurgin, & Richardson, 2010).

The immune system is constantly monitoring the surfaces of cells and the MHC-presented peptides help immune cells to discriminate between normal antigens on the surface of all cells, and those that are foreign and potentially dangerous. This monitoring of the immune system offers IC long-term adaptation in response to varied agro-ecological zones and the permit to survival and production under the harsh climatic, nutritional, and management conditions (Mwacharo *et al*. 2006) 2.6 Microsatellite Markers

For assessing chicken diversity, autosomal microsatellites have now been isolated in large numbers from most livestock species. In chickens, the microsatellite markers

used in population structure and genetic diversity studies are the one proposed by the Food and Agriculture Organization and the International Society of Animal Genetics (Habimana et al., 2004) that recommended thirty microsatellite markers to determine within and between population genetic diversity as listed below: ADL0268, MCW0206, LEI0166, MCW0295, MCW0081, MCW0014, MCW0183, ADL0278, MCW0067, MCW0104, MCW0123, MCW0330, MCW0165, MCW0069, MCW0248, MCW0111,MCW0020, MCW0034, LEI0234, MCW0103, MCW0222, MCW0016, MCW0037, MCW0098, LEI0094, MCW0284, MCW0078, LEI0192, ADL0112 and MCW0216 (Habimana et al.,, 2004)

Many macrosatellites markers have been mapped in chicken genome such as LEI0144, LEI0229, in which LEI0166, LEI0234, LEI0094 and LEI0192 have been cites specifically by Habimana et al. (2004) in genetic diversity studies based on their high heterozygosity, chromosomal location, wide range of alleles and ease of amplification in multiplex polymerase chain reaction (Chen *et al.*2008). LEI0192 is confirmed to be located at the end of chromosome 6 (unlinked in East Lansing population), LE0144 has been mapped on chromosome 4 and chromosome Z while LEI0299 was mapped both to chromosome Z (East lansing population) and the W chromosome (Compton) (Groenem *et al*. 2000). Unfortunately, these markers are not informative in term of disease resistance and improved productivity of the chickens yet LEI0258 is. For historical reasons, the microchromosome containing the MHC is named chromosome 16. Thus, the LEI0258 microsatellite marker is preferred in characterization work because it showed high polymorphism and is the most variable and useful in reflecting the variability of the MHC region (Chazara *et al.* 2013) and therefore, the indigenous chicken populations at large (Chazara *et al*. 2011). The LEI0258 marker is characterized by the repetition of two tandem and conserved short sequences of 12 bp and 13 bp (Fulton *et al*. 2006; Chazara *et al*. 2011). Besides, it revealed several sequence polymorphisms (Indels and SNPs) in the flanking regions (Fulton *et al*. 2006; Chazara *et al*. 2013). It is the combination of the motifs and indels that determines the allele size and the polymorphs.

The LEI0258 marker has been used in genetic diversity studies of more than 1,600 chickens from more than 80 different populations (Khobondo *et al.* 2014). The study

provided a picture of worldwide diversity and categorized chicken MHC haplotypes (Chazara *et al.*, 2013). In Kenya, Ngeno *et al*. (2015) confirmed that the indigenous chickens host many and highly diverse LEI0258 alleles showing genetic variability. However, in that study only the allele (fragment) sizes were used to infer population clustering (Ngeno *et al*., 2015).

Diversity studies has been used to improve chicken productivity in genetic engineering to develop chicken with high egg production or high improved meat quality and chicken with tolerance to diseases. Knowledge on genetic diversity have been used also to help new technologies and support research to develop genetic improvement for enhanced production, maintenance and management plans (Sinoya, 2017). The new technology also might be useful to sustain chicken with reduced environmental impact such as reducing phosphorus emission in their wastes, utilization and conservation of the breeds. The present study will incorporate sequence polymorphism (SNP and indels) to infer population diversity and clustering.

CHAPTER THREE

METHODOLOGY

3.1 Study Sites

South Kivu is situated in eastern DRC at an elevation of 1134.04 meters above sea level at 2°30'S 28°52'E coordinates. It shares borders with Rwanda, Burundi and Tanzania. South Kivu has internal borders with three provinces: North Kivu to the north, Maniema to the west and Katanga to the south. Bukavu is the capital city of the province (former official name: Costermansville in French). The province is administratively divided into eight territories: Fizi, Idjwi, Kabare, Kalehe, Mwenga, Shabunda and Walungu. The official language one French and four national languages: swahili, lingala, chiluba and kikongo. The city's average yearly temperature is 20.02°C and the average precipitation per year is 494.6mm. It has a tropical wet and dry or savanna climate (classification Koppen-Geiger). The vegetation is composed of upland forests, Savannah grasslands, Bamboo woods, dense forests and the climate is tropical.

South Kivu, my home province, faces to malnutrition of his population and the importation of food is very high. Phenotypic characterization and survey studies have been implemented on indigenous chickens in South Kivu seeking to increase yield in production but genetic diversity information on indigenous chickens was still lacking.

3.2 Sample Collection

3.2.1 Sample Size Calculation

Charan and Biswas (2013) proposed to calculate the sample size as: $n = \frac{z^2 p(1-p)}{d^2}$.

This formula is similar to Cochran formula. It was used to estimate the sample size in this study. In this formula, - **n** is sample size

- **z** is standard normal variant at 95% ($p<0.05$) where p values are considered significant below 0.05 hence 1.96 is used in formula.
- **P** is expected proportion in population (in this study, we assume to cover 15% of the entire population)
- **d** is absolute error or precision.

The calculation of the sample size is $n = \frac{1.96^2 * 0.15(1-0.15)}{(0.05)^2}$ = 196. (Table 3.1)

3.2.2 Sampling Method

An animal was recognized as adult for a hen after the first laying and the rooster after starting crowing. This information was confirmed by the farmers before each animal sampling.

Initially, two hundred adult indigenous chickens both sex were randomly sampled across five districts of the South-Kivu/DRC (Bukavu, Kabare, Mwenga, Uvira, and Walungu) according to sample calculation estimated by (Charan and Biswas, 2013).

Thirty-seven samples did not amplify during lab experiments. We proceeded with the remaining 163 samples from adult chicken which generated 525 sequences. Sequences in accordance with Hong and Park (2012) assuming that the Testing a Single Nucleotide Polymorphism (SNP) marker requires 248 cases. Within subpopulation, Nazareno et al. 2017 suggest that, in general, relatively small sample sizes are likely to be sufficient. Specifically, six to eight individuals should be sufficient to recover within-population genetic diversity estimates, even when monomorphic loci were included in the data set (Figure 3.1).

Figure 3.1: A Map of South-Kivu Showing Sampling Sites of the Indigenous Chicken Populations (QGAS)

Samples were assigned into five populations based on their geographical origin (Table 3.1).

Table 3.1: Geographical Origins of Indigenous Chicken Populations in South-Kivu

Population	Sampling site	Sample size
Bukavu	11localities	63
Kabare	3 localities	43
Uvira	5 localities	24
Walungu	6 localities	18
Mwenga	2 localities	15
5 populations	27 locations	163

3.3 DNA Extraction, PCR Amplification, and Sequencing

Whole blood sample was obtained by venipuncture of the jugular vein from each bird and stored onto Whatman FTA filter paper (Whatman WB12 0205; Smith and Burgoyne, 2004). Total DNA was extracted using the Genomic DNA Mini Kit (Invitrogen PureLink, USA) as per the manufacturer's instructions. Using a micro punch, 3 punches of dried blood spot (3mm in size) were made in a sterile 1.5ml

microcentrifuge tube. Then 180µl of purelink genomic digestion buffer was added to 20µl of proteinase K in a tube and mixed well. The tube was incubated at 55°C in the water bath with occasional vortexing every 20 minutes for 1 hour and centrifuged at 14000rpm for 3 minutes at room temperature to pellet paper fibers. The sample was transferred to a clean sterile 1.5ml micro-centrifuge tube. 20µl RNase A were added to the lysate and mixed well by brief vortexing, and incubated at room temperature for 2 min. 200µl of purelink genomic Lysis/Binding buffer were added and mixed well by vortexing to obtain homogenous solution. Then 200µl of absolute ethanol were added to the lysete and mixed well by vortexing for 5 seconds to obtain homogenous solution. The lysate was transferred to the purelink spin column and centrifuged at 13,000 rpm in a microcentrifuge for 1 min at the room temperature. The collection tube containing the flow through was discarded and placed the spin column into clean purelink collection tube supplied with the Kit. To wash the sample, 500µl of Wash buffer 1 were added into the column and spanned at 13,000 rpm at room temperature for 1 min. The collection tube was discarded, placed in a new clean spin column collection tube supplied with the Kit. Then 500µl Wash Buffer2 was added to the column and spun at 13,000 rpm at room temperature for 1 minute.

The column containing the DNA was transferred to sterile 1.5ml Eppendorf tube and 50µl of nuclease free water to the center of the column and left at room temperature for 5 min and then spined at 41,000 rpm for 1 min to elute the DNA. The purified DNA was conserved as stock concentration at -20°C for further analyses. (**QIAGEN manufacture's procedures**)

The concentration and purity of extracted DNA were assessed using spectrophotometry (Nanodrop1000 spectrophotometer, Thermo fisher scientific, Waltham, MA).

Total DNA was also extracted using the boiling method which entailed heating, in a water bath 90°C, 3 to 5 punches 1.2 mm discs from the FTA sample in 1 mL of 100 mM (1 M Tris HCl/0.1% SDS, pH 8) to preserve the DNA from degradation and 500

µL of guanidinium thiocyanate to lyse the cell membranes (Smith and Burgoyne, 2004; Sarma *et al*. 2014).

The selected primer pair LEI0258-F: 5'-CACGCAGCAGAACTTGGTAAGG-3' and LEI0258-R: 5'-AGCTGTGCTCAGTCCTCAGTGC-3', flanks the chicken *LEI0258* loci (Mc Connell et al. 1999, Fulton *et al*. 2006, Chazara *et al*. 2013, Han *et al*. 2013). All the PCR amplification mixture was performed in a total volume of 20 µL containing 14uL AccuPower ProFi Taq PCR PreMix (Bioneer K-2631), 2 µM of forward primer, 2 µM of reverse primer mixed with 2 µL of genomic DNA 50 ng/µL Amplifications was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) using the following PCR cycling conditions: 94°C for 5 min; followed by 40 cycles at 94°C for 60 sec, 65°C for 45 sec, 72°C for 1 min with a final extension at 72°C for 10 min. The PCR products were fragment separated on a 4% agarose gel pre-stained with 0.25X GelRed nucleic acid gel stain (Biotium, USA) and visualized under UV light. The PCR fragment sizes were estimated using Thermo-Scientific O'GeneRuler 100 bp DNA Ladder. The Qiagen QIAquick gel extraction and PCR purification kits (USA) were used to purifying PCR amplicons. Briefly, the DNA fragments were excised from the agarose gel with a clean, sharp scalpel and weigh the gel slice in a colorless tube. Then 3 volumes Buffer QG were added to 1 volume of gel (100mg gel ~ 100µl) and incubated at 50°C until the gel slice has completely dissolved and vortexed every 3 min to help dissolve gel. If the mixture was yellow we proceed but if the mixture was not yellow (orange or violet), it was recommended that 10µl 3M sodium acetate, pH5.0 to be added and mixed to turn the mixture yellow.

One volume of isopropanol was added to the sample and placed into Qiaquick spin column in a provided 2ml collection tube (or into a vacuum manifold). To bind the DNA, the sample was applied to the Qiaquick column and centrifuged for 1 with the flow-through discarded. The Qiaquick column was placed back into the same tube. To wash, 750µl of Buffer PE ware added to Qiaquick column and the column stood for 3 minutes at room temperature, centrifuged for 1 min and the flow-through discarded. The Qiaquick column was placed back into the same tube, placed into a clean 1.5ml micro-centrifuge. DNA was eluted by adding 50µl water to the center of

the Qiaquick membrane and the column centrifuged for 1 minute. (this step was done twice to increase the yield) and samples sent to **Sanger sequencing** lab at SEGOLIP (BecA-ILRI hub, Nairobi, Kenya).

Sanger Sequencing Protocol

The primers used in sequencing to amplify the MHC-linked LEI0258 region were LEI0258 forward tagged with T7 promoter (underlined): 5'-TAATACGA CTCACTATAGGGCACGCAGCAGAACTTGGTAAGG-3'; LEI0258 reverse was tagged with SP6 (underlined): 5'-ATTTAGGTGACACTATAGAGCTG TGCTCA GTCCTCAGTGC-3'. Tagging was done to increase specificity. The sequences were generated for the entire region encompassed by the LEI0258 primers. The PCR products of homologous individuals were selected and cleaned. The PCR products of heterogous were selected and the separation of the two alleles on 4% agarose gel and purified by the QIAquick PCR purification Kit (QIAGEN), following the manufacture's protocol for heterozygous and then sequenced. In fact, the sequencing PCR amplification was performed in a final volume of 10 µL with 2µL of the PCR product, 2µL of sequencing buffer (5x), 1µL of BigDye terminator premix, 2µL of each separated forward and reverse primers, and 3µL of nuclease free water. Each DNA sample was sequenced in both forward and reverse direction. The PCR cycles consisted of 35 cycles of 96°C for 10 seconds, 55°C for 10s and 60°C for 4 minutes, and a final extension at 72°C for 10 min. The PCR products were isolated by alcohol precipitation, rinsed twice in ethanol, and resuspend in HiDi formamide then sent for direct Sanger sequencing analyzer at SEGOLIP (BecA-ILRI Hub) ABI 3130 XL genetic analyzer (Chazara *et al*. 2013; Khobondo *et al*. 2015).

3.4 Data Analysis

3.4.1 Analysis of Genetic Diversity

The raw Sanger sequences were trimmed using CLC Main Workbench software 6.9.1. The total number of alleles (N), different alleles per locus (Na), effective alleles per locus (Ne), private alleles (Np), observed heterozygosity (Ho), expected heterozygosity (He), and Shannon's Information Index (I) were computed for each

population using GenAlEx version 6.41 software (Peakall and Smouse, 2006). Analysis of Molecular Variance (AMOVA) using GenoDiva version 3.05 (Meirmans, 2020), genetic distances (Nei *et al*. 1983) and Principal Component Analysis (PCoA), population differentiation, and gene flow were computed using GenAlEx 6.41 software. Phylogenetic analyses were conducted using the Neighbor-joining algorithm to generate the tree using MEGA 6 software (Tamura *et al*. 2013).

3.4.2 Analysis of Population Structure

Population structure was inferred using a Bayesian model-based clustering algorithm implemented in STRUCTURE version 2.3.4 (Pritchard *et al*. 2000) using the admixture model with correlated allele frequencies. A burn-in period of 10,000 was used in each run, and data were collected over 100,000 Markov Chain Monte Carlo (MCMC) replications from K = 1 to 10 with 10 iterations. The most probable K-value was determined by Structure Harvester (Earl and Vonholdt, 2012) and individual Q matrix and a population Q matrix generated in CLUMPAK (Kopelman *et al*. 2015). The bar plot was plotted using STRUCTURE version 2.3.4 (Pritchard *et al*. 2000). The genetic distances between pairs of accessions were calculated using GenAlEx v6.41 (Peakall and Smouse, 2012), from which a principal coordinate analysis (PCoA) was conducted. To build the haplotype network, the data were prepared by running the motifs R12 and R13 by perl scripts and the subsequences were imported into R software version 3.4.2 with pegas packages (Paradis *et al*. 2018).

3.5 Ethical Clearance Statement

The research protocols followed the guidelines stated by IACUC No. 2014.19.

CHAPTER FOUR

RESULTS

4.1 Polymorphism and Genetic Diversity

Of the 163 samples analyzed, a total of 252 LEI058 sequences were obtained which were grouped into 45 different alleles ranging in size from 193 bp to 473 bp (Table 4.1). The sequencing data revealed that for some LEI0258 PCR products had more than one allele: there were 2 alleles each for 259, 261, 295, 307, 309, 319, 321, 345, 357 bp and 3 alleles for the 249 bp LEI058 microsatellite size markers. The result of the Agarose gel electrophoresis of the LEI0258 PCR products showed that most samples contained two DNA bands of different size that were labeled the large band "a" and the small band "b" (Figure 4.1). This implies that most chickens were heterozygotes, however, for small number of samples we were able to sequence only one of the two alleles. The most frequent allele was 205 (11.9%, 30/252) and the alleles 205, 249, 259, 285 and 432 were common to all the five chicken populations.

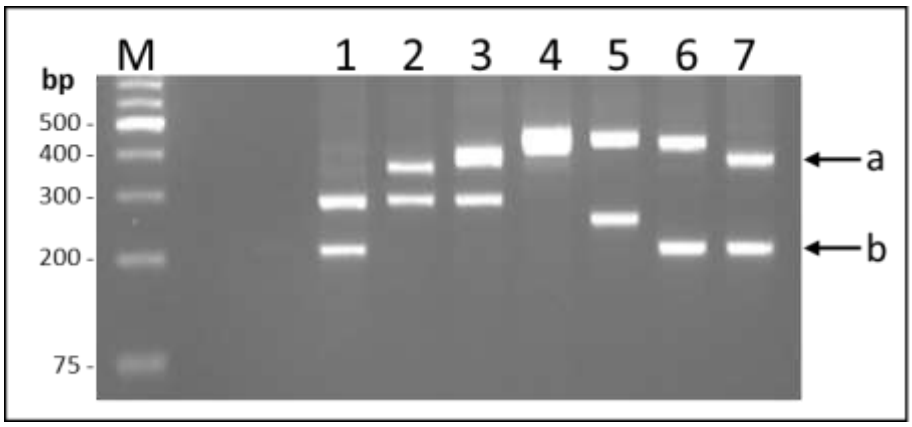

Figure 4.1: 4% Agarose Gel Electrophoresis of LEI0258 PCR Products. Seven samples (Lanes 1-7) were analyzed. Lane M is the Thermo Fisher Scientific GeneRuler 1 kb Plus DNA Ladder.

For samples with two bands, large band is labeled "a" while small band is labeled "b". In lane 4, the two bands are too close to be separated.

Across all the chicken ecotypes, 14 private alleles were detected with the number of private alleles ranging from 1 to 4 (Table 4.1).

Polymorphism was revealed as two repeat motifs i.e. R12 and R13 with different sizes (R12: TTCCTTCTTTCT) and (R13: ATGTCTTCTTTCT), SNPs, and indels flanking the LEI0258 locus in the upstream and the downstream regions. The upstream sequence started from -79 bp to -1 bp while the downstream sequence was +1 bp to +73 bp. In the upstream region, the sequence information revealed three SNPs at position -2 bp, -12 bp, and -30 bp and the TT deletion were observed at position -31 bp, - 32 bp before the R13 repeat. In the downstream region, the sequence information revealed three SNPs at positions +1 bp, +25 bp and +32 bp, one long deletion of 8 bp (ATTTTGAG) was located at position +9 bp to +16 bp and one insertion of 1 bp (A) appeared at position +19 bp. Depending on the allele sizes, the number of the repeat units or motifs, R13 appeared 1 to 21 times whereas R12 appeared 3 to 21 times (Table 4.1).

Table 4.1: Polymorphisms Identified by the LEI0258 Alleles in South Kivu Indigenous Chicken Population with their Frequencies

Allele name		Allele size (bp)	Number and frequency (%)				Upstream		Repeats		Downstream			Populations
			-32 - 31 TT/ Δ	-30 G/A	-12 G/A	-2 C/G	R13	R12	+1 T/C	+9 to +16 ATTTTGAG/Δ	+19 Δ /A	+25 A/T	+32 T/A	
193	193	7 (2.78)					1	3		Δ				BKV19b,BKV25b,BKV26b,BKV61b,KBR71b,KBR72b,KBR74b
205	205	30 (11.90)					1	4		Δ				BKV7b,BKV9b,BKV22b,BKV24b,BKV31b,BKV37b,BKV39b,KBR44b,KBR52b,KBR55b,BKV64b,BKV66b,BKV69b,KBR77b,KBR85b,KBR86b,KBR88b,KBR98b,WLG104b,WLG108b,MWG122b,MWG125,MWG126b,MWG129b,MWG130b,UVR160b,BKV182b,BKV187b,BKV192b,MWG198b
206	206	5 (1.98)			A		1	4		Δ	A			BKV57,BKV59b,UVR154b,BKV183b,BKV193a
217	217	11 (4.37)					1	5		Δ				KBR40b,KBR73b,KBR83b,WLG101b,MWG124b,WLG137b,UVR142b,UVR147b,UVR151b,KBR177b,KBR181b
235	235	3 (1.19)	Δ				1	6					A	UVR141,UVR148b,UVR167a
241	241	8 (3.17)					1	7		Δ				BKV15,BKV20b,BKV23b,BKV35b,BKV58b,BKV59a,KBR100b,MWG126a
249A	249	8 (3.17)					1	7					A	BKV11b,BKV17b,BKV18b,BKV38b,WLG107b,BKV110b,WLG115b,KBR179b
249B	249	2 (0.79)					1	7						BKV16,WLG105
249C	249	12 (4.76)					1	7				T		BKV22a,BKV28b,BKV29,BKV36b,BKV37a,KBR55a,BKV60,BKV65b,MWG123b,MWG130a,UVR138b,BKV194a
259A	259	2 (0.79)	Δ				1	8					A	BKV7a,KBR177a
259B	259	6 (2.38)	Δ				1	8	C				A	BKV70b,KBR82,MWG128b,WLG135b,UVR152b,BKV183a
261A	261	4 (1.59)					1	8					A	BKV5b,BKV21b,KBR92b,UVR171b
261B	261	2 (0.79)					1	8	C				A	KBR44a,MWG129a
273	273	15 (5.95)					1	9					A	BKV2b,BKV6b,BKV28a,BKV35a,KBR41b,BKV62b,KBR73a,KBR80b,KBR95b,KBR97b,MWG119b,MWG124a,MWG133b,UVR

Allele name		Allele size (bp)	Number and frequency (%)				Upstream		Repeats		Downstream			Populations
			-32 - 31 TT/ Δ	-30 G/A	-12 G/A	-2 C/G	R13	R12	+1 T/C	+9 to +16 ATTTTGAG/Δ	+19 Δ /A	+25 A/T	+32 T/A	
														168,BKV182a
283	283	1 (0.40)	Δ				1	10					A	UVR145b
285	285	10 (3.97)					1	10					A	BKV56b,KBR86a,WLG114,WLG118b,UVR140b,UVR144b,BKV196a,BKV197a,MWG198a,BKV200b
295A	295	2 (0.79)	Δ				1	11						BKV1b,KBR75b
295B	295	3 (1.19)	Δ	A			1	11						BKV11a,BKV12,BKV14b
307A	307	2 (0.79)	Δ			G	1	12					A	BKV27b,KBR78b
307B	307	8 (3.17)	Δ	A			1	12						BKV38a,KBR40a,KBR81,KBR96,WLG111b,WLG115a,MWG122a,BKV184
309A	309	17 (6.75)					1	12				T		BKV4b,BKV8b,BKV21a,BKV32b,BKV39a,KBR42b,KBR45b,KBR79b,KBR84,KBR90,MWG120a,MWG127b,MWG131b,MWG133a,UVR149b,KBR178b,KBR179a
309B	309	1(0.40)					1	12						BKV63
319A	319	2 (0.79)	Δ	A			1	13					A	BKV5a,BKV191b
319B	319	4 (1.59)	Δ	A			1	13						KBR49b,KBR50b,WLG106b,WLG175b
321A	321	1 (0.40)					1	13						UVR143b
321B	321	1 (0.40)					1	13				T		BKV20a
333	333	1 (0.40)					1	14				T		MWG128a
345A	345	5 (1.98)					1	15				T		BKV13b,BKV14a,BKV17a,BKV18a,KBR97a
345B	345	2 (0.79)					1	15					A	UVR144a,BKV190b
357A	357	7 (2.78)					1	16					A	BKV1a,BKV3,BKV8a,BKV31a,WLG135a,UVR138a,BKV197b
357B	357	6 (2.38)					1	16				T		KBR71a,WLG118a,WLG134b,UVR146b,UVR158,BKV186b
369	369	1 (0.40)					1	17					A	KBR54b
379	379	6 (2.38)	Δ	A			1	18						BKV19a,WLG109b,UVR140a,UVR149a,BKV192a,BKV196b
381	381	7 (2.78)					1	18				T		BKV6a,BKV32a,BKV33b,WLG102b,WLG111a,UVR164b,UVR169
391	391	1 (0.40)	Δ	A			1	19						KBR53b

Allele name		Allele size (bp)	Number and frequency (%)				Upstream		Repeats		Downstream			Populations
			-32 - 31 TT/ Δ	-30 G/A	-12 G/A	-2 C/G	R13	R12	+1 T/C	+9 to +16 ATTTTGAG/Δ	+19 Δ /A	+25 A/T	+32 T/A	
393	393	4 (1.59)					1	19				T		KBR93b,MWG132b,UVR145a, UVR155b
405	405	8 (3.17)					1	20				T		BKV2a,BKV4a,BKV9a,KBR46b, KBR48,KBR49a,KBR54a,KBR99b
417	417	1 (0.72)					1	21				T		KBR180
419	419	3 (1.19)					15	6						BKV24a,BKV64a,BKV190a
420	420	2 (0.79)					16	5						WLG136,KBR178a
432	432	16 (6.35)					16	6						BKV33a,KBR42a,KBR50a,KBR53a,KBR99a,WLG102a,WLG109a, WLG117,MWG127a,WLG134a, UVR151a,UVR155a,UVR159a, UVR162a,BKV193b,BKV195
443	443	9 (3.57)					15	8						BKV25a,KBR45a,KBR46a,KBR74a,KBR93a,MWG131a,WLG137a, UVR146a,BKV194b
445	445	3 (1.19)					17	6						KBR41a,UVR154a,BKV200a
461	461	2 (0.79)					21	3						BKV67,BKV70a
473	473	1 (0.40)					21	4						UVR171a
Total			**45**			**252 (100)**						**252 sequences**		

Abbreviations**:** BKV = Bukavu, MWG = Mwenga, UVR = Uvira, WLG = Walungu, KBR = Kabare

4.2. Population Genetic Diversity

In the entire populations, 45 different alleles were observed with a mean of 12.184. The effective alleles were higher in Bukavu population (16.004) and lower in Mwenga (7.377). The expected heterozygosity was higher than the observed heterozygosity frequencies in all the 5 chicken populations. The Bukavu ecotype showed the highest He (0.938) and Mwenga ecotype showed the lowest He (0.864). Mwenga ecotype showed the highest Ho (0.667) and Uvira ecotype showed the lowest (0.417). The highest Shannon's information index was observed in Bukavu (3.027) and the lowest were observed in Mwenga (2.248). The total mean of the entire population sampled was 0.911 whereas the expected was 0.519 (Table 4.2).

Table 4.2: Averaged Population Diversity Indices of the LEI0258 Microsatellite Loci

Pop	Locus	N	Na	Ne	I	Ho	He	F	uHe	% pop
Bukavu	LEI0258	63	28.000	16.004	3.027	0.603	0.938	0.357	0.945	100%
Kabare	LEI0258	43	26.000	13.902	2.907	0.488	0.928	0.474	0.939	100%
Mwenga	LEI0258	15	13.000	7.377	2.248	0.667	0.864	0.229	0.894	100%
Uvira	LEI0258	24	21.000	13.880	2.822	0.417	0.928	0.551	0.948	100%
Walungu	LEI0258	18	13.000	9.757	2.406	0.421	0.898	0.531	0.922	100%
All	Mean	32.600	20.200	12.184	2.682	0.519	0.911	0.428	0.930	100%
	SE	8.947	3.153	1.572	0.151	0.050	0.013	0.060	0.010	0%

N = number of samples; Na = number of alleles; Ne = number of effective alleles; I = Shannon's information index; Ho = observed heterozygosity, He = expected heterozygosity; F = fixation index, and % P = percentage of polymorphic loci, uHe= unbiased heterozygosity, SE = G-statistics for an alternative mean Fst estimate.

4.3. Population Structure Analysis

Structure analysis revealed three gene pools with admixture of populations. The proportion of the five populations is slightly distributed nearly equal in the three gene pools (Figure 4.2).

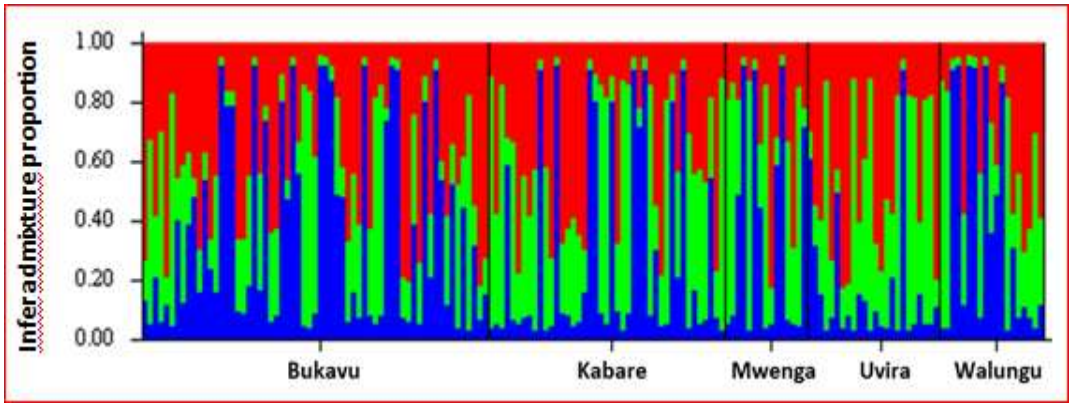

Figure 4.2: The Gene Pools with Admixture Model (Ancestry Model)

A graphical display of the genetic structure of 163 accessions of indigenous chickens forms three clusters (shown by different colors: red, blue and green) and exhibit different levels of admixture.

Individual accessions were arranged according to their assigned populations (Bukavu, Kabare, Mwenga, Uvira and Walungu comprising 63, 43, 15, 24 and 18 respectively.

The possible population group was determined by calculating the optimum Evanno K. The best Evanno K value was observed at K=3 (Delta K = 6.674) as shown in figure 4.3.

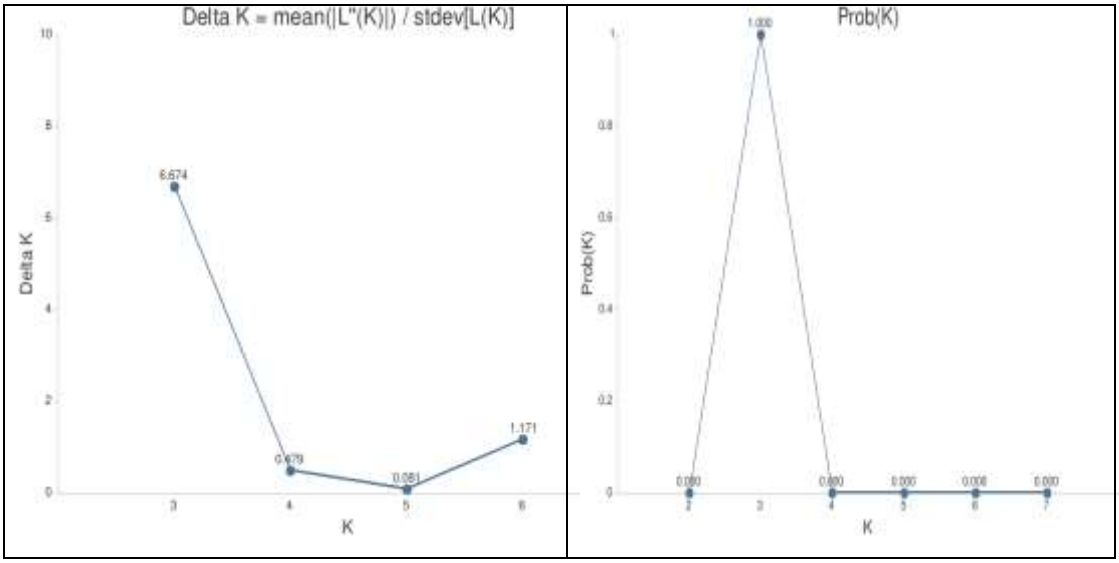

Figure 4.3: Structure Clustering of the Chicken Populations Obtained for K = 3 (the Best Delta K, was Selected Using the Evanno Method)

The rows show individual ancestry inferences estimated by structure outputs assuming the optimal K=3

4.4. Principal Coordinate Analysis

The PCoA clustered the study populations into three clusters without clear separation of the populations. Analysis of PCoA showed that the three axes explained 12.38%, 22.63% and 29.87% of the variation among population for axes 1, 2 and 3 respectively. Plotting principal coordinate analysis 2 versus 3 depicted similar results to that of 1 versus 2 (Figure 4.4). The samples from Bukavu and Kabare are scattered in all the three clusters. Analysis of PCoA

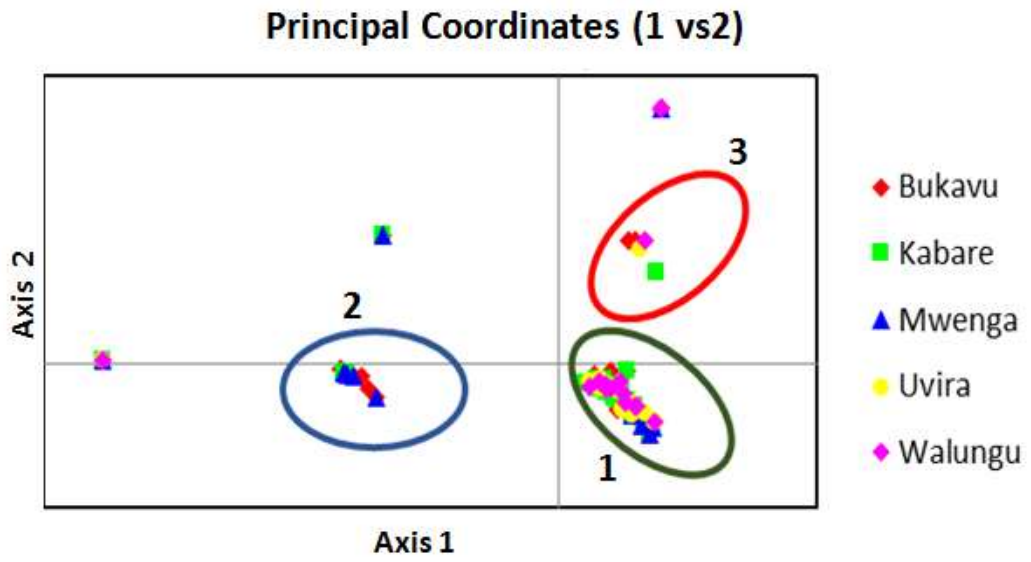

Figure 4.4: Bi-Plot of Principal Coordinates Analysis (PCoA) of the Chicken Samples from South Kivu Was Generated Depicting the Genetic Relationships among the 163 Indigenous Chickens' Accessions

A symbol of the same shape and colored represents accessions of the same population

4.5 Cluster Analysis

The samples were clustered into three distinct clusters namely I, II, and III, with admixtures of chicken populations in each cluster (Figure 4.6). The largest was cluster II consisted of 14 subclusters (1-14). The other clusters had 7 subclusters each. In all the clusters there were representative samples from all three populations as shown below in figure 4.6.

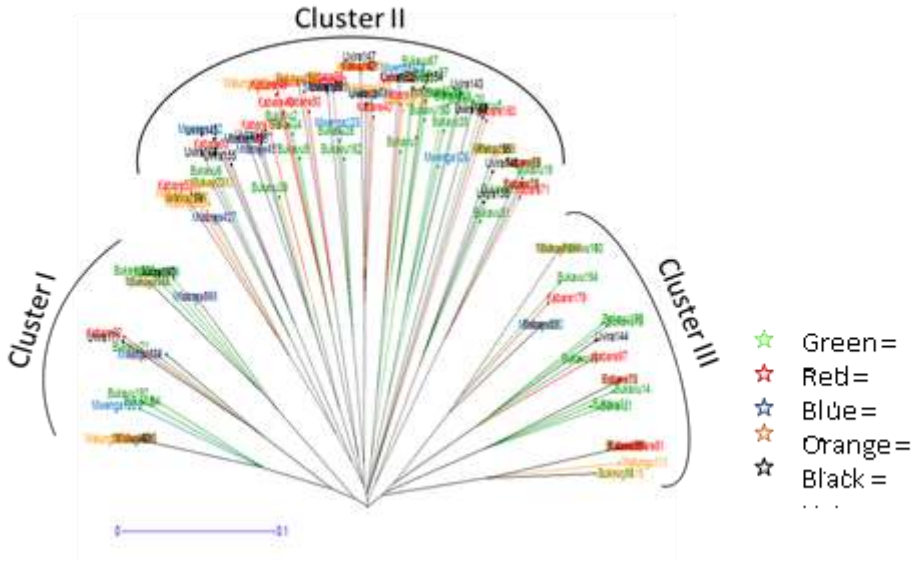

Figure 4.5: A Radiation Neighbor-Joining Dendrogram Constructed from Allele-Sharing Distances among the 163 Individual Chicken Populations Coming Together and Admixture

4.6. Genetic Distribution among Populations

The molecular variance analysis (AMOVA) results indicated 55.6% within individuals; 43.2% among individuals and 1.2% among populations (Figure 4.5). AMOVA was used to calculate the level of genetic differentiation among different populations (Bukavu, Kabare, Mwenga, Uvira and Walungu.

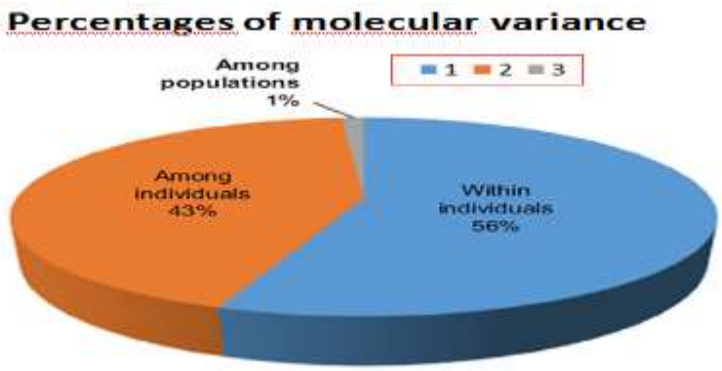

Figure 4.6: Analysis of Molecular Variance

4.7 Genetic Distance and Relationships

The standard genetic distance (D_A) and gene differentiation indices among the five chicken populations are summarized in Table 4.4. The D_A indices ranged from 0.000 for Kabare and Mwenga ecotypes to 0.742 Mwenga and Uvira. And the F_{ST} indices ranged from 0.011 for the Mwenga and Kabare ecotypes to 0.035 for the Mwenga and Walungu ecotypes.

Table 4.3: Pairwise Nei Unbiased Genetic Distance (Lower Diagonal) and Fixation Indices F_{st} Values (Upper Diagonal) between Indigenous Chicken Ecotypes of South Kivu

Bukavu	Kabare	Mwenga	Uvira	Walungu	
-	0.012	0.019	0.021	0.014	Bukavu
0.243	-	0.011	0.022	0.025	Kabare
0.175	0.000	-	0.037	0.035	Mwenga
0.646	0.632	0.742	-	0.020	Uvira
0.151	0.514	0.514	0.260	-	Walungu

According to Nei's unbiased genetic distance using the pairwise population matrix, chicken population from Kabare were genetically identical to those from Mwenga (0.000), and closely related to Bukavu ecotype (0.175) and Walungu ecotype (0.151). The most distant was Mwenga from Uvira (0.742), Kabare form Uvira (0.632) and Bukavu from Uvira (0.646).

4.8 Distinctiveness and Relationships of the Chicken Population

The analysis revealed that the coefficient of genetic differentiation (F_{ST}) was 0.034 and the estimated of gene flow (Nm) over all populations was 7.089 as shown in table 4.5 below

Table 4.4: F-Statistics and Estimates of Nm over All Populations for Each Locus

All Pops.	Locus	Fis	Fit	Fst	Nm
	LEI0258	**0.430**	**0.450**	**0.034**	**7.089**

Fis= inbreeding coefficient of an individual (I) relative to the sub-population (S), Fit= inbreeding coefficient of an individual (I) relative to the total population (T), Fst= genetic differentiation coefficient which is the effect to sub-population (S) compared to the total population (T) and Nm = gene flow

If the Fst value of the populations was between 0 and 0.05, it will indicate that there is no genetic differentiation among populations. And if Fst value is between 0.05 and 0.15, then it is moderately differentiated, and finally if Fst value is between 0.15 and 0.25, then it is highly differentiated Wright (1978). The result form this study, reveals that there is no genetic differentiation among populations Fst (0.034).

4.9 Haplotypes Network Analysis and Allele Relationships

For the network construction, the 45 alleles were used based on indels and repeats. The size of each node is proportional to the frequency of the allele, varying from 1 to 30. Numbers between one node to another represents mutations and the highest number of mutations was sixteen. The colors define the five different populations where the biggest node is represented by allele 205 with a frequency of 30 (Figure 4.7). The most common haplotypes were alleles 205, 249, 309, 432 and 273. There were no clear correlations between haplotypes, sampling sites and dominant haplotypes were quite widely shared across sampling locations. However, haplotypes 249C and B, 241, 257A and 345B were found predominantly in Bukavu.

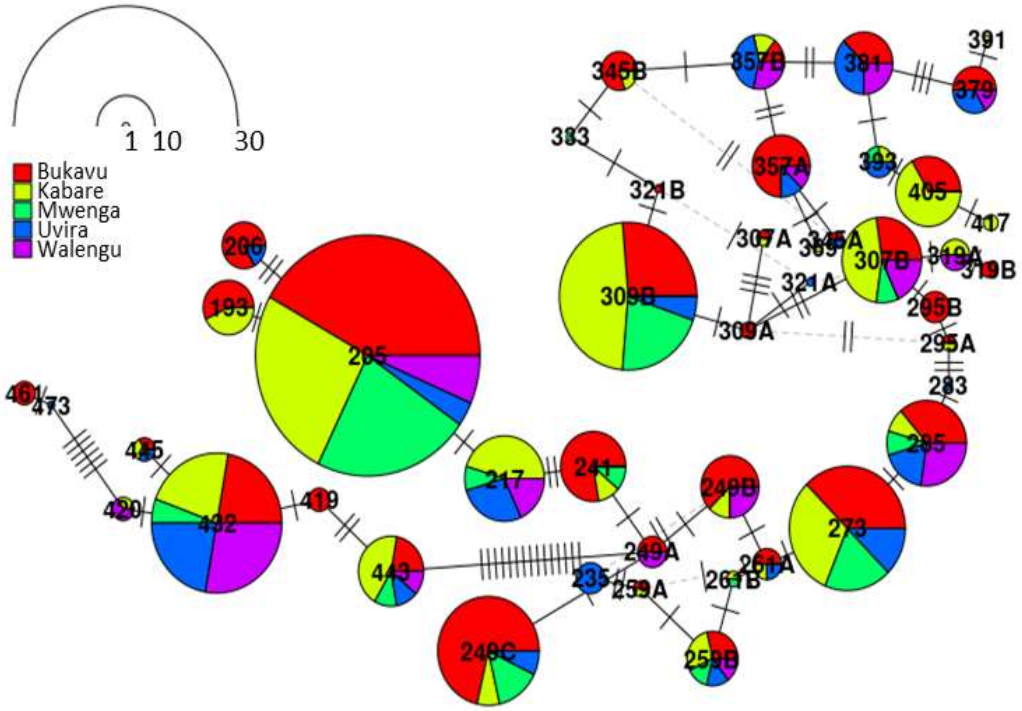

Figure 4.7: Haplotypes Network of the 45 Alleles Defined for the VNTR LEI0258.

CHAPTER FIVE

DISCUSSION

In this study, we aimed to determine the population structure and genetic diversity of the South Kivu indigenous chicken populations using molecular approaches focused on LEI0258 fragment size detection and Sanger sequencing analysis. Sequence data analysis revealed a total of 45 different alleles ranging in sizes from 193 to 473 bp. Previous studies had reported 26 alleles ranging from 182 to 552 bp in North America and European layer-type chickens (Fulton *et al*. 2006). 25 alleles were observed in Vietnam (Schou *et al*. 2007), 69 alleles ranging from 193 to 489 were found in 33 Chinese indigenous chickens with 21 novel alleles (Han *et al*. 2013), 30 distinct alleles ranging from 197 to 569 were found from 10 selected ecotypes in Tanzania (Mwambene *et al*. 2019), 22 and 23 alleles were identified in Kuchi and Medium Tanzanian chicken ecotypes (Lwelamira *et al.* 2008) and 19 and 15 alleles were identified in local chicken of Brazil (Lima-Rosa *et al.* 2005).

The number of 45 alleles in this study is higher than those reported in Kenya (Ngeno *et al*. 2015), in Tanzania (Lwelamira *et al*. 2008), in Brazil (Lima-Rosa *et al*. 2005), in Taiwan (Chang et al. 2012), and in Ethiopia (Addisu *et al*. 2020). Fourteen private alleles are reported in this study and this number is higher than four private alleles found in 10 selected ecotypes in Tanzania (*Mwambene et al 2019*), and lower than sixty found in Rwandan ecotypes (Habimana *et al*. 2020). The private alleles detected in this study are estimators of the MHC genetic diversity and allelic variability at the LEI0258 locus within and among chicken populations (Chazara *et al*. 2013). The private alleles bring out the uniqueness of a population. They are indicating whether the populations under study are genetically different or not. Low frequencies of private alleles imply that probably the ecotypes might be sharing the majority of the observed alleles or that the chicken populations might be genetically subjected to a similar evolutionary direction (Mwambene *et al*. 2019).

Allele 205 was the most abundant (30 out of 252 chicken sequences studied, 11.90%) and randomly distributed across the 5 districts studied. In a similar LEI0258 microsatellite-based study of Tanzanian chickens, Lwelamira *et al*. (2008) found that

allele 205 bp was positively associated with the elevated primary antibody responses against Newcastle Disease vaccine, while the allele 307 bp was negatively associated with this trait. Our result suggests that most chickens from South Kivu could have been exposed to Newcastle Disease virus infection and have developed resistance or tolerance to the disease. In contrast, allele 307 which is reported to be positively correlating with bodyweight traits was found in 10 chickens in Bukavu, Kabare, Mwenga and Walungu ecotypes, and the allele 357 serologically defined as B12 (Fulton *et al*. 2006) which is associated with Marek's disease resistance was found in Bukavu, Kabare, Uvira and Walungu chicken populations. The allele 276 bp was not found in this study whereas it is reported to be associated with some species of worms in Vietnamese local chickens (Schou *et al*. 2007). This 276 bp allele is also reported in sheep livestock species and is linked with performance (Bot *et al.* 2004; Kannaki *et al*. 2017). Bukavu and Uvira towns may be the best areas that domesticate birds with strong resistance may be due to high exchanges of commercial trades. The absence of allele 357 in Mwenga needs further investigation.

In terms of *LEI0258* allele composition, there is a symmetric indication of genetic similarity between chicken populations found in North America and Europe (Fulton *et al*. 2006, Chazara *et al*. 2013). This symmetric similarity may be associated with the fact that the birds might derive from the same ancestors (or from the same origin). Looking at the MHC region, the combination of R13 and R12, and the number of SNPs and Indels, the observation indicates similar dynamic evolution in genetic polymorphisms within and among chicken populations. Similar findings have been reported by Mwambene *et al* 2019 in Tanzania, and by Addisu *et al*. 2020 in Ethiopia.

The polymorphism is revealed as the repetition of the two different repeat units R12 and R13 with different sizes (R12 TTCCTTCTTTCT) and (R13 ATGTCTTCTTTCT) and as SNPs and Indels flanking the LEI0258 locus. The upstream sequence started from -79 bp to -1 bp while the downstream sequence was +1 bp to +73 bp as described by (Fulton *et al*. 2006). In the upstream region, the sequence information revealed three SNPs at position -2 bp, -12 bp, and -30 bp, and the TT deletion was observed at position -31bp and -32bp before the R13 repeat, this

result is similar to the previous findings reported by Chazara *et al*. 2013. In the downstream region, the sequence information revealed three SNPs at position +1 bp, +25 bp, and +32 bp, one long deletion of 8 bp (ATTTTGAG) was located at position +9 bp to +16 bp and one insertion of 1 bp (A) appeared at position +19 bp. These five polymorphic site positions agreed symmetrically with the previously reported studies which included the last R12 repeat due to the presence of one SNP at position 3 within the R12 motif (Chazara *et al*. 2013, Fulton *et al*. 2006). This finding is in line with similar results reported by Mwambene et al 2019 in Tanzanian ecotypes, and Chinese ecotypes (Han *et al*. 2013). High polymorphism at the MHC might increase the ability of indigenous chickens to respond to various disease antigens and hence chance of their survival in their environments (Lwelamira *et al*. 2008).

South Kivu indigenous chicken revealed lower genetic diversity with 56% within populations than that of 84 to 88% reported previously in Kenyan local breeds (Ngeno *et al*. 2015). This result is lower than 86.4 to 88.2 % reported by Lwelamira *et al*. 2008 in Kuchi and medium local chicken from eastern Tanzania, and 98% from the 10 selected ecotypes from the Southern highlands of Tanzania reported by Mwambene *et al*. 2019. Also Compared to Brazilian local chicken populations in which the genetic diversity ranged from 50 to 75% (Lima-Rosa *et al*. 2005). The value of 56% is seen among the different populations of indigenous chickens in South Kivu at the same microsatellite. This similarity might be explained by the fact that the ecotype had been exposed to similar environmental challenges enabling them to share similar LEI0258 forms as Brazilians. This agrees with Lwelamira *et al*. (2008) when they compared Brazilian ecotype to Tanzanian at the low degree of heterozygosity. This might be due to relatively low antigenic diversity prevailing in the environments in which chickens had evolved in.

Heterozygosity was calculated to determine the genetic variations. The value of the expected heterozygosity (He) ranged from 0.864 to 0.938 with a mean of 0.911 and the value of observed heterozygosity (Ho) ranged from 0.417 to 0.667 with a mean of 0.519 in South Kivu ecotypes. The average of the Ho was less than the expected heterozygosity in the South-Kivu chicken populations. This study findings are not similar to previously reported findings in Kenya with Ho 0.92 and He 0.83 (Ngeno *et*

al. 2015). In Ethiopia Ho ranged from 0.381 to 0.977 and He ranged from 0.04 to 0.97 (Mogesse H. 2007). The average Ho (0.519) was less than the He in each chicken population probably due to random mating or mating between relatives as explained by (Mwambene et al 2019) or due to high selection against heterozygote as explained by (Ohwojakpor *et al*. 2012). Similar observations were reported by Mogesse H. (2007) in chicken populations in Ethiopia, and by Kannaki *et al*. (2017) in Indian native chicken breeds. MHC heterozygosity is reported to be advantageous in MHC mediated disease resistance due to increased diversity of antigens capable of being presented to T cells. Emergence of higher frequency of heterozygosity at the MHC is driven by exposure to many kinds of infectious agents (Lwelamira *et al*. 2008).

The F statistics Fit, Fst and Fis 0.450, 0.034 and 0.430 respectively were different to to those found in Kenya indigenous chickens (0.093, 0.029 and 0.066 respectively (Sinoya, 2017), in Ethiopian chickens 0.095, 0.033 and 0.064 respectively (Dana *et al*. 2011), and in Cameroon chicken 0.13, 0.08 and 0.03 respectively (Keambou *et al.* 2014). Within-population inbreeding coefficient (Fis) found in Kenya ecotype 0.093 (Sinoya, 2017), 0.10 (Ngeno *et al*.2015) and in Camerronian chickens 0.095 (Keambou *et al.*2014) is lower than the one foun in South Kivu ecotype (0.430). At high level of inbreeding, we assume that chicken populations from South Kivu are experiencing slow inbreeding and may actually display higher within-population inbreeding depression. This is in accordance with the result found because such population is expected to be more heterozygous at loci under selection, and the animals were almost heterozygous in South Kivu ecotypes.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From the study, it was observed that:

1. There was the presence of various alleles that suggested higher allelic polymorphism at the LEI0258 marker within the MHC. The locus showed three gene pools with the optimal K=3 and 14 private alleles.
2. The LEI0258 microsatellite marker is highly informative in describing the MHC-B region gene loci in indigenous population of chickens from South Kivu. There exist significant association between some LEI0258 microsatellite alleles and antibody response against Newcastle disease and body weight
3. There is no genetic differentiation among indigenous chicken populations and the relationships among them are closer to each other

6.2 Recommendations

It was recommended further researchers to focus on:

1. Association between MHC haplotypes and phenotype should be carried out to explore other components that might map inside and outside the MHC region using the marker assisted selection to improve the South Kivu traits in selecting birds for breeding programs and genetic resource conservations.
2. Determination of the genetic variability in the MHC populations with other microsatellites which should be more informative in genetic study on looking at functional important protein coding genes to improve resistant chickens to New castle diseases.
3. Identification of new gene locus with the microsatellite markers and the SNP positions within local breed to improve their production such as more eggs, mature faster and meet market weight as potential source of income. This will

increase the benefit of the full potential of local chickens farming system and improve farmer’s life in the country.

4. Genetic distance and relationships in guiding policy makers to prevent extinction of indigenous chicken populations in South Kivu.

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