Genetic variation among Zulu sheep sub-populations of South

Africa assessed by Microsatellites and Mitochondrial DNA

(mtDNA)

By

Mokhethi Matthews Selepe (BSc. Hons. Animal Science)

Dissertation submitted in fulfilment of the requirements for the

Degree of Master of Science in Agriculture (Animal Science)

Department of Agriculture

Faculty of Science and Agriculture

University of Zululand

Supervisor: Prof N.W Kunene

Co-Supervisor: Dr E Lasagna

February 2018



Declaration

I **(Mokhethi Matthews Selepe)** declare that this dissertation is, save for the supervisory guidance received, the product of my own work and effort. I have, to the best of my knowledge and belief, complied with the University's Plagiarism Policy and acknowledged all sources of information in line with normal academic convections.

Signed on the _____ day of _____, 2018

Abstract

Human history has been completely transformed by the domestication of animals and plants over the past 10,000 years. Their domestication had a critical influence on demographic trends and was a requirement for the rise and development of civilisation. In regard to Zulu sheep, the Nguni people brought the ancestors of this breed to the east coast of South Africa (KwaZulu Natal) between 200 and 400 AD. The Zulu sheep are characterised by having either thin or fat tail (carrot shaped), multicolours, and a coat of either wool or hair. The Zulu sheep can be distinguished from other Nguni breeds by their small mouse ears, and they appear to be more woolly. In addition, the dominant colours are brown and white, black and brown, and a unique fawn colour. However, the population of Zulu sheep is reported to be declining, due to crossbreeding with exotic breeds, especially with the Dorper and Merino sheep breeds with the aim of increasing body weight.

The study used two molecular markers (microsatellites and mitochondrial DNA) to investigate the genetic diversity of Zulu sheep.

In experiment 1, 26 microsatellite markers were used; (i) to confirm or disconfirm the structuration among eight Zulu sheep populations (Eshowe, Jozini, Makhathini research station, Mtubatuba, Nongoma, Nquthu, Ulundi, University of Zululand) revealed by morphological analysis, (ii) to assess the phenomenon of crossbreeding, in particular with Dorper and Merino sheep. The Damara, Dorper and South African Merino breed were included in the experiment to investigate the genetic relationship between these breeds and the Zulu sheep. The results showed that there is considerable genetic diversity among the Zulu sheep populations (expected heterozygosity ranging from 0.57 to 0.69) and the level of inbreeding was not remarkable (ranging from -0.01 to 0.16). The structure analysis results revealed that Makhathini Research Station and UNIZULU research station share common genetic structure, while three populations (Nongoma, Ulundi and Nquthu) had some admixture with the exotic Dorper breed. Thus, there is a need for sustainable breeding and conservation programmes to control the gene flow, in order to stop possible genetic dilution of the Zulu sheep.

Experiment 2 assessed the genetic diversity and origin of Zulu sheep using mitochondrial DNA (D-loop). The results showed overall haplotype and nucleotide diversity

ii

of 0. 8113 and 0. 0115, respectively. Phylogenetic analysis showed two haplogroups (A and B). Haplogroup B lineage predominates among Zulu sheep with a frequency of 93%, while A lineage had a frequency of 7%. The analysis of mtDNA showed a high level of genetic diversity among Zulu sheep. The molecular information obtained in the present study will serve as a guideline for management and breeding strategies (reducing inbreeding and crossbreeding) for better utilisation and conservation of Zulu sheep.

Key words: Animal genetic resources, Conservation, Genetic variation, microsatellites, mitochondrial DNA, Zulu sheep, DNA

Acknowledgements

Firstly, I would like to thank Almighty GOD for being with me throughout the journey of completing this dissertation.

I would like extend my sincere gratitude to my supervisor Prof N.W Kunene and cosupervisor Dr. E Lasagna for their guidance, support, motivation and patience. This dissertation achieved completion through their fruitful ideas and criticism.

Dr S Ceccobelli played a big role by mentoring and training me from PCR till genotyping. Today I'm able to do all of this because of him and I really appreciate it and am truly thankful.

I would also like to thank Mr S.R Hlophe for his assistance during DNA extraction, whenever I needed extra hands.

Special thanks to the Department of Agriculture lab technicians S Moloi and E Maupa for their support throughout my lab work.

The National Research Foundation (NRF) supported financially towards this project.

Lastly but not least I would like to thank my family and friends for their spiritual support; you guys always gave me strength whenever I was down.

Dedication

To my mom Sesi Selepe; late aunty (God mother) Mbhele Matseleng.

Siblings; Ntombi, Lerato and Kamo.

Table of Contents

Declarationi
Abstractii
Acknowledgementsiv
Dedicationv
List of tablesix
List of figuresx
Abbreviations xi
CHAPTER 11
GENERAL INTRODUCTION1
1.1 Background1
1.2 Objectives
1.3 Dissertation outline3
CHAPTER 25
Literature Review
2.1 Characteristics of Zulu sheep breed5
2.2 Genetic Variation6
2.2.1 Importance of Genetic variation7
2.2.2 Effects of loss of Genetic Variation7
2.3 Animal genetic resource (AnGR)8
2.3.1 Conservation of Animal genetic resource9
2.3.2 Conservation approach of Animal genetic resources10
2.4 Molecular Markers12
2.4.1 Microsatellites13
2.4.1.1 Importance of Microsatellites13
2.4.2 Mitochondrial DNA14
2.4.3 Y chromosome15

2.4.4 Single Nucleotide Polymorphisms (SNPs)16
CHAPTER 3
Genetic structure of South African Nguni (Zulu) sheep populations reveals admixture with
exotic breeds18
Abstract18
3.1 Introduction19
3.2 Materials and methods21
3.2.1 Ethics Statement21
3.2.2 Animal sampling21
3.2.3 Molecular analysis22
3.2.4 Statistical Analysis22
3.3 Results25
3.3.1 Genetic variation25
3.3.3 Genetic differentiation, distance and phylogeny27
3.3.3 Genetic structure and admixture analysis29
3.4 Discussion
3.5 Conclusion
CHAPTER 4
Origin and genetic diversity of Zulu sheep assessed by Mitochondrial DNA D-Loop
sequence
Abstract
4.1 Introduction
4.2 Materials and Methods40
4.2.1 Animal sampling40
4.2.2 PCR amplification and Statistical analysis40
4.3 Results41
4.3.1 Mitochondrial DNA haplotypes and diversity41
4.3.2 Mitochondrial DNA phylogeny42

4.4 Discussion	43
4.5 Conclusion	44
CHAPTER 5	46
5.1 General discussion	46
5.2 Conclusions	48
5.3 Recommendations	49
6. References	49
Appendices	64
Appendix 1: Images of Zulu sheep	64
Appendix 2: Details of 11 studied sheep populations	65
Appendix 3: Microsatellites PCR conditions according to Kunene et al. (2014)	66

List of tables

Table 3. 1 Microsatellites loci, chromosome position (Chr), size range (S.R), number of
alleles observed (Na) at each locus, mean PIC (Polymorphism Information Content) pe
locus in 11 studied sheep populations and number of populations derived from HEW2
Table 3. 2 Genetic diversity of the studied sheep breeds obtained from the analysis of 2
microsatellites loci2
Table 3. 3 Pairwise genetic differentiation among the studied breeds/populations (with
confidence intervals at 95%)20
Table 3. 4 Results from AMOVA analysis of only Zulu sheep populations

List of figures

Figure 3. 1 Geographical location of the sampling sites for the studied South African
populations24
Figure 3. 2 Genetic relationship among 11 sheep populations using Reynolds genetic
distance according to neighbour-joining algorithm29
Figure 3. 3 Generic clustering of 11 sheep populations with STRUCTURE
Figure 3. 4 Delta K plots of STRUCTURE analysis averaged over ten repetitions at K= 1-13
Figure 3. 5 Representation of sample size (indicated by pies) and of relative admixture
distribution inferred by STRUCTURE at K=432
Figure 3. 6 Assignment of individuals to 9 clusters based on DAPC analysis(a). Scatterplot
of the first two pricnciples components of DAPC using populations as posteriori as a
posteriori cluster (b)

Abbreviations

- AMOVA = Analysis of molecular variance
- AnGR = Animal genetic resource
- BIC = Bayesian information criterion
- Chr = Chromosomal position
- DA = Damara
- DAPC = Discriminant analysis of principal component
- D-loop = Displacement loop
- DNA = Deoxyribonucleic acid
- dNTP = Nucleoside triphosphate
- DO = Dorper
- EDTA = Ethylenediaminetetraacetic acid
- ES = Eshowe
- Fis = Inbreeding coefficient
- FST = Pairwise genetic differentiation
- H_E = Expected heterozygosity
- H_O = Observed heterozygosity
- HWE = Hardy-Weinberg equilibrium
- HWE Pop = Hardy-Weinberg equilibrium per locus
- JO = Jozini
- K = number of clusters
- ME = South African Merino sheep
- MgCl₂ = Magnesium chloride
- MNA = Mean number of alleles
- MS = Makhathini research station
- MT = Mtubatuba
- mtDNA = Mitochondrial DNA
- N = sample size
- Na = number of alleles observed
- NG = Nongoma
- nh = number of unique haplotype
- np = nucleotide position
- NQ = Nquthu
- PCR = Polymerase chain reaction

PIC = Polymorphic information content

- PR = Private allelic richness
- R = allelic richness
- S = number of polymorphic site
- SNPs = Single nucleotide polymorphism
- SR = size range
- SSRSs = Simple sequence repeats
- STRs = Short tandem repeat
- UL = Ulundi
- UZ/ UNIZULU = University of Zululand
- Va = Variation within populations
- Vb = Variation among individuals within populations
- Vc = Variation within individuals
- w = Nucleotide weight
- π = Nucleotide diversity

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

The domestication of animals by man is said to have been forced by the climatic fluctuation, which followed the end of the glacial period some 14 000 years ago (Plug and Badenhorst, 2001). Since animal husbandry, humans have been in control over the reproduction, diet and protection of animals. The domestication of animals and plants has increased the stability of human subsistence and fueled population growth and expansion (Kijas et al., 2012). This has been a significant threshold in the human history (Zeder, 2008). Moreover, humans first domesticated goats and sheep, immediately followed by cattle (Taberlet et al., 2011).

Possible sheep domestication is reported by archaeological and molecular evidence to have occurred in the Fertile Crescent region crossing between Central Anatolia to the north of the Zagros mountains (Tapio, 2006). In addition, sheep were the first meat animals to be domesticated (Plug and Badenhorst, 2001). Domestication of animals from the wild has modified the morphology, behaviour and genetics of the animals involved, for example, changes to coat pigmentation and horn morphology (Kijas et al., 2012). Domestication of sheep has resulted in a decrease in body size, a decrease in horn size and changes from a hairy moulting fleece to a white woolly fleece (Devendra and McLeroy, 1982). The Southern African KhoiKhoi's domestication was based on the captive management of sheep, cattle and dogs (Horsburgh, 2008).

The genetic resources of sub-Saharan African domestic sheep are extensive with an estimated 170 breeds. About 80% of these sheep are classified as indigenous populations and they are maintained under the traditional farming system (Kemp et al., 2007). In South Africa, about 590 000 km² of land is occupied by sheep and goat farming, where sheep are found in large numbers in more arid regions. Generally, in South Africa sheep are reared mainly for food and meat production (NDA, 2004).

Sheep are classified as small ruminant animals (Kunene and Fossey, 2006; Pollott and Wilson, 2009). Small ruminants (sheep and goats) require small investment in contrast to large ruminants, due to their shorter production cycle, faster growth rates and better environmental adaptability (Tibbo, 2006). Furthermore, small ruminants can improve soil and vegetation cover. They do these by their grazing preferences permitting them to

consume biomass, which may supply the fuel for bush fire, by limiting shrub growth and spreading seeds through their hooves and manure (Steinfeld et al., 2006).

Sheep play an essential role in the socio-economic lives of people around the world by providing food (meat, milk), income, socio-cultural wealth, and clothing (wool. (Ramsay et al., 2000; Pollott and Wilson, 2009). Furthermore, sheep are reared for their relatively ease of handling (Loukovitis et al., 2016). Indigenous sheep are a valuable source of genetic material, because of their adaptation to harsh environmental conditions, nutritional fluctuations and resistance to diseases and parasites (Kunene et al., 2007). In South Africa, animal production provides a livelihood for the majority of populations in the rural communities (Mavule et al., 2016). In Africa as a whole, agriculture provides about fifty percent of household food requirements and about fifty percent of household incomes. The majority of the income is produced by dairy cows, beef cattle, goats, sheep and chickens. About ninety two percent of the total revenue from livestock in Africa is generated from these four animal species (Rust and Rust, 2013).

The local breeds have been crossbred with commercial breeds for the past decades in order to improve their productive performance in meat, wool, milk yield and quality (Buduram, 2004). This crossbreeding has led to replacement and genetic dilution of local breeds with exotic breeds (Ceccobelli et al., 2016). The FAO (2007a), reported that the indigenous genetic resources of Africa and the world at large are threatened with extinction. The study by Muigai et al. (2009) also revealed that in sub-Saharan Africa it has been estimated that about thirty percent of the indigenous genetic resources are at risk of becoming extinct before they are characterised and documented. In South Africa, very little attention has been given to indigenous Zulu sheep compared to other sheep breeds (Mavule et al., 2013). Moreover, it is reported that the existence of the Zulu sheep is threatened and its place is being taken by exotic breeds, either by replacement or crossbreeding (Kunene et al., 2009; Mavule et al., 2013; Mavule et al., 2013a). It is therefore important that the genetic variation of indigenous sheep in Africa is assessed and documented (Ghazy et al., 2013).

The proposed method for decreasing the loss of indigenous breeds' diversity through extinction is by conservation of indigenous genetic resources (Parés Casanova et al., 2013). Conservation of indigenous animal resources plays a vital role in meeting present socioeconomic demand (source of income for poor rural communities), renewing population after disease outbreaks, saving rare or endangered breeds, and retaining indigenous animal gene pool diversity (Ruto et al., 2008). These emphasise that the understanding of the actual genetic resources can be improved by studying the molecular genetics of the population diversity and structure (Tolone et al., 2012). In the early 1900s genetic markers became more popular for identification or investigation of structure and genetic variation in animal populations (Groeneveld et al., 2010).

Genetic and phenotypic characterisation are the first essential step towards conservation and utilisation of animal genetic resources, principally the indigenous types that are ignored due to their lower production potential compared to commercial breeds (FAO, 2000). The FAO (2007a), suggested that a good understanding of breed characteristics is essential for guiding decision making in animal development and breeding programmes. Phenotypic characterisation is the procedure of classifying livestock populations and describing their external and production characteristics in a given environment and under given management, considering social and economic factors that affect them (FAO, 2012a). On the other hand, genetic characterisation is describing and classifying animal breeds and species at molecular level using techniques for analysis of DNA (Rao et al., 1996).

1.2 Objectives

The objectives of the current study were:

1.2.1 To assess the genetic variation within Zulu sheep populations using microsatellites and mitochondrial DNA;

1.2.2 To confirm or disconfirm the structuration among Zulu sheep populations revealed by morphological analysis using microsatellites;

1.2.3 To assess the phenomenon of crossbreeding, in particular with Dorper and Merino sheep using microsatellites and

1.2.4 To investigate the origin of Zulu sheep using mitochondrial DNA.

1.3 Dissertation outline

Chapter 1 presents the general introduction and the objectives of the dissertation. Chapter 2 covers the literature review of relevant journal articles. In this chapter, highlighted topics are the characteristics of the studied indigenous Zulu sheep, genetic variation and its importance, and why should it be maintained. Furthermore, Animal genetic resources and

approaches to their conservation are reviewed. Lastly, different molecular markers (microsatellites, mitochondrial DNA, Y-chromosome, single nucleotide polymorphisms) as molecular tools for mapping genes of interest are reviewed. Chapter 3 discusses the genetic structure and genetic diversity revealed by microsatellites analysis of 8 Zulu sheep populations (EShowe, Jozini, Makhathini research station, Mtubatuba, Nongoma, Nguthu, Ulundi, University of Zululand). The Damara, Dorper and South African Merino sheep breed were included to assess the admixture with the Zulu sheep. This chapter is a paper that has been published on the PLOS One Journal. Chapter 4 presents the maternal origin and genetic diversity of Zulu sheep revealed by mitochondrial DNA. In this chapter 8 Zulu sheep populations were assessed. This chapter will help to answer questions concerning the maternal origin of sheep brought into the African continent, since only a few studies have assessed the phylogenetic (evolutionary) history of African sheep breeds. A paper written from this chapter is to be submitted to the Journal of Genetic Engineering and Biotechnology. Chapter 5 covers the general discussion and conclusion on the molecular information obtained in this study. Moreover, recommendations are made for future studies on Zulu sheep genetic diversity.

CHAPTER 2

Literature Review

2.1 Characteristics of Zulu sheep breed

The Zulu sheep breed is one of four ecotypes of the Nguni sheep breed, that includes Landim, Pedi and Swazi (Kunene et al., 2009; Mavule et al., 2013). The Nguni people brought the ancestors of this breed to the east coast of South Africa, which is Kwa-Zulu Natal province, between 200 and 400 AD (Du Toit, 2008). The largest population of this breed is found in the northern region in Jozini, Msinga and Nongoma (Mavule et al., 2013; Kunene et al., 2014). Zulu sheep are normally owned by rural farmers and even though their production might not be high compared to the exotic breeds, they represent the lifeline of rural farmers (Kunene et al., 2011).

The Zulu sheep are characterised by having either a thin or fat tail, multicolours, and a coat of either wool or hair. Within the breed there is a variation of ear length, from ear buds and short prick ears to long pendulous ears (Kunene et al., 2009). Adults with brown or dark brown colour are born as black lambs and the colour gradually changes to brown or dark brown as the lambs mature (Kunene et al., 2007). Furthermore, Zulu sheep are mainly known by their high adaptation to harsh environmental conditions and their resistance to disease (tick-borne) and parasites (external and gastro-intestinal). This indigenous Nguni breed is also reported to have the ability to walk long distances searching for feed and water (Mavule et al., 2013), utilise low quality forage, and have lower levels of health care requirements and high mothering ability (Kunene et al., 2011).

Indigenous Zulu sheep serve as a source of livelihood and utilise marginal environments not suitable for cultivation (Mavule et al., 2013). The rural farmers also use these sheep for payment of penalties in tribal courts, but they are not used for marital payments (lobola) or cultural ceremonies (Kunene and Fossey, 2006). The sheep are reared under the extensive production system (Kunene and Fossey, 2006; Mavule, 2012). The study by Mavule et al. (2013) showed that Zulu sheep are owned in small numbers by farmers in Kwa-Zulu Natal after cattle and goats.

ARC-All (2001), reported that the documented Zulu sheep populations are the flocks that have been established at the Makhathini Research Station, which is located south of

Pongola river dam, and at the University of Zululand, near Empangeni in northern Kwa-Zulu Natal. The study by Kunene et al. (2011) reported that there is a potential extinction threat of Zulu sheep due to their declining number. Once the animal genetic diversity is lost it cannot be replaced (FAO, 2000). Hence, there is a need for conservation of Zulu sheep, since they are under threat (Ramsay, 2000; Kunene et al, 2014) and they may become extinct if interventions are not put into action (Kunene et al., 2014).

2.2 Genetic Variation

The potential of an animal population for existence depends on the quantity of genetic variation (Furlan et al., 2012). Genetic variation is referred to as differences between the DNA sequences of individual genomes (Alberts et al., 2013). Genetic variation normally results from the introduction of new alleles by random and natural processes of mutation, since the frequency of occurrence of an allele changes regularly as an effect of mutation, genetic drift and selection (Agaviezor et al., 2012). In addition, Marsjan and Oldenbroek (2006) reported that DNA variations are mutations deriving from substitution of single nucleotides, insertion or deletion of DNA fragments of dissimilar lengths, or duplication or inversion of fragments of DNA. Genetic diversity is normally assessed by the frequency of genotypes and alleles, proportion of polymorphic loci, and observed and expected heterozygosity or allelic diversity (Toro et al., 2009). Generally, genetic variation appears as an allelic diversity and heterozygosity over the genome (Toro et al., 2011).

The study by El Nahas et al. (2008) reported that genetic variability is structured by past population process and affects the sustainability of species and populations in the future. Under natural conditions, gene migration is influenced by the extent to which landscape topography facilitates interactions among populations. While under domesticates, gene migration is influenced by human socio-cultural and economic networks (Elbeltagy et al., 2015). Moreover, populations exposed to selection might encounter decreased genetic and phenotypic variability of desired trait, because selection has an influence on gene frequency in population by increasing the frequency of the advantageous alleles with addictive effect (Ghafouri-Kesbi et al., 2008). Generally, a wide diversity of breed has been created by domestication, migration, selection and adaptation (Groeneveld et al., 2010). Therefore, these demonstrate that the most accurate approach to conservation of breed is to evaluate the current state of population diversity (Kunene et al., 2014). This is because, the results of analysed data could then be used to decide whether actions are needed to safeguard the maintenance of variation within a population (Toro et al., 2011).

Conservation of genetic diversity also includes mating or breeding strategies to optimise genetic response in the long-term. Hence, conservation of genetic diversity is an essential component of recovery of threatened and endangered breeds (McFarlane et al., 2006).

2.2.1 Importance of Genetic variation

Genetic variation or diversity plays an essential role in the long-term survival of species (Al-Atiyat et al., 2014). Moreover, genetic variation is necessary to allow animals to adapt to changing environmental conditions (Boettcher et al., 2010; Pilling and Hoffmann, 2011; Agaviezor et al., 2012; Al-Atiyat et al., 2014), poor quality forage or diets, and to become resistant and tolerant to diseases (Boettcher et al., 2010; Pilling and Hoffmann, 2011). The study by Agaviezor et al. (2012) showed that the Yankasa sheep had preferable adaptability to more agro-ecological areas in Nigeria compared to other sheep due to their high genetic variation (high number of observed alleles).

The record on genetic variability within and among breeds yields information for management, breeding strategy (inbreeding management), and as an aid to identifying breeds that may contain desired genotypes due to local adaptation and, therefore, worthy of being conserved (Loukovitis et al., 2016). Conservation of genetic diversity is essential in animal breeding schemes, since heterozygosity and allelic diversity can be lost from small, closed, selected populations at a rapid rate (Sheikhlou and Abbasi, 2016). This has been supported by many studies (Elbeltagy et al., 2015; Loukovitis et al., 2016; Sheikhlou and Abbasi, 2016), where data covering molecular genetic diversity of breeds was evaluated to assist breeding programmes.

2.2.2 Effects of loss of Genetic Variation

Loss of genetic variation negatively affects population fitness, with increased juvenile mortality, increased susceptibility to disease and parasites (Furlan et al., 2012) and difficulty in adapting to changing environmental conditions (Alberts et al., 2013). Moreover, lower genetic variation leads to a high risk of inbreeding effects, through the uncovering of deleterious recessive alleles (McFarlane et al., 2006). The gathering of deleterious recessive alleles might threaten the fitness of the population (Gandini et al., 2004). Hence, inbreeding depression leads to a lower chance of breed survival, due to decreased fitness (Pilling and Hoffmann, 2011). This results from faster changes in frequencies of genes, high rates of gene loss, and genetic constitutions (haplotypes) (Buduram, 2004).

Populations with small size generally suffer from inbreeding and the appearance of deleterious genetic defects (Bhatia and Arora, 2005). Breeding of individuals sharing common ancestors tends to lower the allelic variation rate in the next generation, thereby lowering the genetic variation of the population (FAO, 2007b). Generally, populations with lower genetic variation exhibit an increased rate of extinction (Furlan et al., 2012; Snyman et al., 2013). The majority of breeds at risk are found in developing countries (FAO, 2007a). The FAO (2009) reported that 740 breeds were documented as extinct, with 1 335 (32%) being considered at high risk of extinction. If insufficient action is taken concerning the erosion of animal genetic diversity, it is estimated that more than 2 000 livestock breeds will be lost within the next two decades (FAO, 2009).

2.3 Animal genetic resource (AnGR)

Animal genetic resource (AnGR) are referred as those livestock that are utilised, or may be utilised, for the production of food or for agriculture (FAO, 2007b). Globally more than 8 000 livestock breeds contribute the animal genetic resources (AnGR) (FAO, 2011b). In addition, it is estimated that 90% or even more of AnGR comes from pig, chicken, cattle, sheep, goat, buffalo, horse, ass and dromedary distributed globally (Cardellino and Boyazoglu, 2009). There is an increased demand for livestock products due to economic development and population growth (Ligda and Zjalic, 2011). A large quantity of global AnGR is found in developing countries (Boettcher et al., 2010), where sheep genetic resources are playing an essential role (Sassi-Zaidy et al., 2014). AnGR are a key to economic development, especially in developing countries (FAO, 2007a).

Animal genetic resource (AnGR) are necessary to satisfy the basic need of humans for food and livelihood security. They contribute to human needs by yielding meat, milk (dairy products), fibre, clothes, a resource for temporary and permanent shelter, and manure (fertilizer and fuel) (FAO, 2007a, 2011a). However, the study by Mavule et al. (2013) revealed that 38,5% of Zulu sheep farmers around Kwa-Zulu Natal had no knowledge of a global call for conservation of AnGR. As a consequence, loss of AnGR has become a major concern (Sassi-Zaidy et al., 2014). A report on the *Status and trends of animal genetic resource*- 2010, revealed that about 8% of breeds have become extinct and another 21% are considered to be at risk of extinction (FAO, 2011a). Furthermore, the situation is unknown for 35% of livestock breeds, the majority of which are reared in developing countries (FAO, 2011a). An evaluation of genetic variation in domestic sheep is a first step

towards the conservation of genetic resources for sustaining breeding programmes (Ghazy et al., 2013).

2.3.1 Conservation of Animal genetic resource

Suitable strategies should be put in measures for halting the genetic erosion of animal genetic resources (AnGR), principally in breeds with high genetic diversity which have the ability to adapt to ever-changing environmental conditions (Sassi-Zaidy et al., 2014). The motive behind conservation of AnGR is that humankind may need to preserve this specific genetic variability in order to face unexpected future challenges, such as change in demand for livestock products, the spread of new diseases, declining environmental impact, and climate change (Cardellino and Boyazoglu, 2009). However, AnGR are conserved for many different reasons (FAO, 2013). In developing countries AnGR are preserved for tradition and cultural values. Consequently, this encourages the development of conservation measures for breeds at risk and stimulates the emergence of niche markets for animal products. Whereas, in developed countries AnGR are conserved for food security and economic development (FAO, 2013). The dispersal of potentially valuable quantitative trait locus alleles between universal farm animal breeds is the key question in the management of livestock genetic diversity (Notter, 1999).

The effective conservation of endangered indigenous breeds requires a comprehensive knowledge of the breed's characteristics (Qwabe et al., 2012) for successful management programmes and formation of directives in animal development (Loukovitis et al., 2016). Hence, documentation on the current status of animal genetic resources is essential, including the description of the population phenotypic characteristics, performance and genetic uniqueness (Gornas et al., 2011). Furthermore, understanding data on population size, structure, geographical distribution and the production system in which they are kept is necessary for conservation of AnGR (Pilling and Hoffmann, 2011; Tolone et al., 2012). Thus, in order to halt the loss of genetic variability there is a need to implement actions that promote conservation and sustainable use of AnGR (Qwabe et al., 2012). The specific priorities for conservation and sustainable use of AnGR are set out in the *Global Plan of Action for Animal Genetic Resource (Global Plan of Action)* (FAO, 2007a).

The main aims of the *Global Plan of Action for Animal Genetic Resources* are: to encourage the development of AnGR for food security, sustainable agriculture and human

well-being; to ensure conservation of essential AnGR diversity for present and future generations and to stop loss of essential AnGR; to satisfy the needs of pastoralist and farmers; to encourage activities aimed at raising public awareness; and to provide for fair sharing of the benefits arising from their use (FAO, 2007a; Mäki-Tanila and Hiemstra, 2010). Generally, the implementation of the strategic priorities contained within the *Global Plan of Action for AnGR* will make a crucial contribution to ensuring long-term food security, enhancing self-sustainability of indigenous breeds, and the maintenance of genetic variation (Mäki-Tanila and Hiemstra, 2010; FAO, 2011a). Moreover, conservation measures have been implemented to decrease the risk of genetic erosion and extinction of breed populations (Pilling and Hoffmann, 2011).

2.3.2 Conservation approach of Animal genetic resources

The conservation approach of animal genetic resources (AnGR) can be categorised as either *in situ* or *ex situ* conservation (FAO, 2007b; Ligda and Zjalic, 2011; FAO, 2012b, 2013). *Ex situ* conservation is further subdivided into *ex situ in vivo* and *ex situ in vitro* conservation (Köhler-Rollefson, 2003; FAO, 2004, 2013).

2.3.2.1 In situ conservation

In situ conservation is the preservation of AnGR in their native or adaptive production environment (Ligda and Zjalic, 2011; FAO, 2012b, 2013). *In situ* conservation is the preferred conservation approach in most developing countries, because animals continue to evolve in their native environment (FAO, 2004, 2007a). The conservation measures of *in situ* are based on ecosystem management and use for sustainable food and agricultural production (FAO, 2012b). Thus, action plans of conservation should not only preserve the live animals within their original environment but also make them self-sustainable under the given agro-ecosystem (Bhatia and Arora, 2005). Furthermore, *in situ* conservation is principally the active breeding of livestock populations for food and agricultural production so that genetic diversity is best utilised in the short-term and conserved in the long-term (FAO, 2013). The conservation of *in situ* comprises activities such as performance recording and development of breeding programmes with special importance attached to maintaining genetic diversity within breed (FAO, 2012b, 2013).

2.3.2.2 Ex situ conservation

Ex situ conservation is the conservation of AnGR outside their native production environment (Ligda and Zjalic, 2011; FAO, 2013). This can either be *ex situ in vivo* or *ex situ in vitro* (Köhler-Rollefson, 2003; FAO, 2004, 2013).

2.3.2.2.1 Ex situ in vivo conservation

This category of conservation is the preservation of live animal populations in an environment that is not native to their management conditions (FAO, 2012b). In *ex situ in vivo* conservation live animals are conserved in the form of an organised herd maintained in research institutions, governmental farms, zoological parks and breed safaris (Bhatia and Arora, 2005; FAO, 2012b). Generally, livestock are often kept in very limited numbers due to financial and practical reasons. Since livestock are kept outside their original production environments in small numbers, natural selection is normally no longer effective in its role of ensuring the adaptation of the livestock in these environments (FAO, 2013). Therefore, the major restriction of *ex situ in vivo* conservation is population size, avoiding the negative effects that may results from inbreeding. Hence, effective population size is a major consideration and depends upon male to female ratio under different selection systems (Bhatia and Arora, 2005). It is highly recommended that *ex situ in vivo* and *ex situ in vitro* be complementary (FAO, 2012b).

2.3.2.2.2 Ex situ in vitro conservation

This category of conservation is the storage of genetic materials that can be used for future breeding, in order to increase or introduce diversity into the live populations (Ligda and Zjalic, 2011). Generally, this type of conservation consists of the collection and deep freezing of DNA, semen, cells or tissue. *Ex situ in vitro* conservation is also known as cryo-conservation (FAO, 2004; Bhatia and Arora, 2005; FAO, 2012b, 2013). Nevertheless, deoxyribose nucleic acid (DNA) is the easiest and most inexpensive approach, since it can be used as a source of single genes for livestock improvement (FAO, 2004). The main objectives of cryo-conservation are regeneration of endangered breeds, development of new breeds, supporting the *ex situ in vivo* populations, and aiding DNA studies and genome mapping (Bhatia and Arora, 2005). However, scientists are concerned whether the facilities and expertise necessary for sample collection can be financed and put in place (FAO, 2012b, 2013). This leads to the key question concerning *ex situ in vivo* conservation of

whether or not long period financial commitment is available to sustain generations of livestock to the levels required for successful conservation (FAO, 2013).

2.4 Molecular Markers

Molecular markers are essential tools that are used to determine genetic variability and biodiversity with high levels of accuracy and reproducibility, through comparing the genotypes at a number of polymorphic loci (Arif and Khan, 2009). The FAO (2006), revealed that genetic markers act as Mendelian traits, meaning they pursue the laws of segregation and independent assortment, which was first reported by Mendel. With the use of molecular or genetic markers it is easier to understand the evolutionary (phylogenetic) history of animal breeds (Lancioni et al., 2013). Molecular characterisation also assists in the genetic management of small populations to avoid excessive inbreeding (FAO, 2006). In addition, genetic markers are very important for mapping genes of interest (Hayashi et al., 2004). Kumar et al (2009) reported that DNA markers may vary with respect to essential features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements, and financial investment.

The first molecular markers to be used in livestock were protein polymorphisms. However, the lower level of polymorphism observed in proteins has made DNA-based polymorphism the marker of choice for molecular based assessments of genetic diversity (FAO, 2005). DNA molecular markers are divided into two classes, nuclear DNA and uniparental (mitochondrial DNA or Y-chromosome) markers (Kumar et al., 2009). The nuclear DNA markers are bi-parental inherited. On the other hand, mitochondrial DNA (mtDNA) markers are maternally inherited, whereas Y-chromosome markers are paternally inherited (Liu and Cordes, 2004; Kumar et al., 2009).

Nuclear DNA evolves five to ten times more slowly than mtDNA and its variation is affected by demographic forces (bottleneck). For this reason, nuclear genetic markers can detect more recent genetic evolution that influences the extant divergence of livestock (Pariset et al., 2011). However, since mtDNA evolves faster than nuclear genome it can be used in degraded or old samples (Arif and Khan, 2009). Therefore, the combination of nuclear DNA and mtDNA can expand the information obtained (Pariset et al., 2011). In addition, application of both nuclear DNA and mtDNA may provide additional accuracy and a comprehensive understanding of a species' background (Pariset et al., 2011).

2.4.1 Microsatellites

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), denote a specific DNA sequence consisting of 1 to 6 bp (base pairs) in length (Ellegren, 2004; Sharma et al., 2007; Miah et al., 2013; Abdul-Muneer, 2014). They are short, tandemly arrayed mono-, di-, tri-, tetra-, penta or hexa-nucleotide repeats (Ellegren, 2004; Chistiakov et al., 2006; Sharma et al., 2007; Simpson, 2012; Miah et al., 2013; Abdul-Muneer, 2014). Mono-, di-, tri and tetra-nucleotide repeats are the most common types of microsatellites (Abdul-Muneer, 2014). Microsatellites occur in both coding and non-coding regions (Sharma et al., 2007; Simpson, 2012; Miah et al., 2013). Furthermore, microsatellites are highly distributed throughout the genome, are co-dominant inheritance, are Mendelian inheritance, are hyper-variable, have high mutation rate and a high level of polymorphism (Kevorkian et al., 2010; Simpson, 2012; Ghazy et al., 2013; Miah et al., 2013; Abdul-Muneer, 2014; Al-Atiyat et al., 2014). These characteristics have led to microsatellite markers being widely applied in the studies of genetic variation, genetic relationship and population structure of sheep breeds (Al-Ativat et al., 2014; do Amaral Crispim et al., 2014; Kunene et al., 2014; Sassi-Zaidy et al., 2014; Gaouar et al., 2016; Naqvi et al., 2017). All these studies were aimed at getting results which will aid in conservation strategies and utilisation of genetic diversity.

2.4.1.1 Importance of Microsatellites

The high mutation rate and co-dominant nature of microsatellites permit the estimation of genetic diversity within and between breeds, even if they are closely related (Marsjan and Oldenbroek, 2006; Ahmed et al., 2014). Hence, with microsatellite markers the heterozygotes can be distinguished from homozygotes, due to their co-dominant transmission (Abdul-Muneer, 2014). Generally, genetic variability is distinguished by high heterozygosity and the presence of multiple alleles (Qwabe et al., 2012; Sassi-Zaidy et al., 2014).

The high mutability at microsatellite loci play an essential part in genome evolution by generating genetic variation within a gene pool. This variation arises principally by slipped strand, mis-paring and subsequent errors during replication of DNA, repair or recombination, generating tandem arrays repeated a varying number of times (variable number of tandem repeats) (Sharma et al., 2007). Moreover, microsatellite markers are useful instruments for tracking alleles through a population (Nanekarani et al., 2011). For example, they frequently

show multiples of alleles at loci that vary from each other in the numbers of the repeats, due to their hyper-variability (Marsjan and Oldenbroek, 2006; Ghazy et al., 2013).

Microsatellites can be easily amplified by polymerase chain reaction (PCR), due to their small size (Tapio et al., 2010). Where alleles at each loci can be amplified from small DNA samples and then separated under gel electrophoresis and thus used for analysing genetic variability within and between populations (Simpson, 2012). With regard to the PCR assays associated with the high polymorphic content of microsatellites it is feasible to determine the identity of individuals based on the assessments obtained from allelic frequencies (do Amaral Crispim et al., 2014). Furthermore, microsatellite markers are capable of creating information for the planning of crossing and selection of genotypes in genetic breeding schemes (do Amaral Crispim et al., 2014). An exciting and important feature of this marker is that it is easy to analyse (Tapio et al., 2010; Ghazy et al., 2013; Sassi-Zaidy et al., 2014).

2.4.2 Mitochondrial DNA

The haploid mitochondrial DNA (mtDNA) is found in mitochondria that are located in the cell cytoplasm (Bailey et al., 2002). The mitochondrial genome of mammals is a closed circular supercoiled molecule of approximately 16kb comprising genes for rRNAs, tRNAs, and mRNAs distributed between the two DNA strands. Mitochondrial DNA (mtDNA) of sheep is about 16-58kb (Wood and Phua, 1996). Mitochondrial DNA (mtDNA) is usually used in the studies of phylogenetic and genetic diversity (Naderi et al., 2007; Galtier et al., 2009; Dancause et al., 2011; Di Lorenzo et al., 2015). This is due to its numerous characteristics that include maternal inheritance, high mutation rate, lack of recombination, and fast evolution rate (Bailey et al., 2002; Gissi et al., 2008; Galtier et al., 2009; Dancause et al., 2011; Di Lorenzo et al., 2015). The property of maternal inheritance means that individuals inherit the mtDNA from their dams, but not from their sires. Therefore, only ewes (females) will pass the mtDNA on to the next generations (Marsjan and Oldenbroek, 2006). In addition, maternal inheritance means that the complete genome behaves as a single and thus shares a common genealogy (Galtier et al., 2009).

Phylogenetic analysis of domestic sheep mtDNA has revealed five maternal lineages (haplogroups A to E) (Pereira et al., 2006; Meadows et al., 2011). However, haplogroup D and E were identified most recently and are scarcest (Meadows et al., 2011b). The first study to examine mtDNA variation in sheep was conducted by Wood and Phau (1998). This study assessed the control region sequence of New Zealand sheep and identified two major

haplogroups, lineage A and B. The haplogroups (A-E) are found, or predominate, in different areas. The major haplogroups A and B predominate in Asia and Europe, respectively. Haplogroups C, D and E originated in the Near East (Pereira et al., 2006; Meadows et al., 2011; Arora et al., 2013). During molecular studies the control region of mtDNA is usually examined for phylogenetic and genetic diversity (Pereira et al., 2006; Meadows et al., 2011b).

2.4.2.1 Importance of mitochondrial DNA

Mitochondrial DNA (mtDNA) is an appropriate DNA maker to track back the origin of livestock (Groeneveld et al., 2010; Dancause et al., 2011; Arora et al., 2013), since it has a direct lineage to the ancestral mother (FAO, 2006; Di Lorenzo et al., 2015). The control region of mtDNA is usually used for determining genetic polymorphism, since it is variable, structured sufficiently throughout the geographical range of the species, and evolves at a constant rate (Naderi et al., 2007). The high mutation rate of mtDNA has enabled it to generate a signal concerning population history over a short period (Galtier et al., 2009). Hence, biologists have used this marker to restructure phylogenetic relationships between and within species by evaluating the patterns of mutations in mtDNA (FAO, 2006; Dancause et al., 2011).

During DNA sequencing (phylogenetic analysis) the most examined mtDNA control region is displacement loop (D-loop) (Di Lorenzo et al., 2015). D-loop is a non-coding region and shows a high level of variation relative to coding regions such as the cytochrome b (Liu and Cordes, 2004; FAO, 2006). The D-Loop control region in mammals is located between the proline (PRO) and phenylalanine (PHE) t-RNA genes (Wood and Phua, 1996). Moreover, polymorphism in the D-Loop sequence has contributed considerably to the identification of the wild ancestors of domesticated species (Bruford et al., 2003) and its rate of nucleotide substitution is 5 to 10 times faster than that of nuclear DNA (Mburu and Hanotte, 2005). The mitochondrial gene is strongly preserved across animal species, because of insufficient duplication, no intron, and extremely short intergenic regions (Gissi et al., 2008).

2.4.3 Y chromosome

The Y chromosome is a non-recombining haploid unit of male (Ling et al., 2010). The paternal inheritance of Y chromosome does not undergo recombination at meiosis, thus

providing a marker to examine male dispersal (Pérez et al., 2011). In addition, the Y chromosome genetic marker yields essential information about genetic origins and the diversity of animals from the paternal lineage, which is complementary to those discovered by autosomal and mitochondrial variations (Zhang et al., 2014). The patterns of malemediated introgressions during breed development have been discovered through the analysis of the non-recombining region of the Y chromosome (Pariset et al., 2011; Wang et al., 2015). This is because examination of haploid state alleles in the absence of X-Y recombination permits haplotypes that describe the individual male lineages to be identified (Meadows et al., 2006). Wang et al. (2015), reported that the male imbalance impacts the male specific gene flow between geographic regions, male mediated events, and influences (changes) that occurred during sheep domestication.

Evaluation of male lineages is of great value in domesticated animals, where controlled breeding results in a small number of males contributing a disproportionately large number of offspring to subsequent generations (Meadows et al., 2006). Nevertheless, low genetic variability are present in Y chromosome genetic makers in sheep studies, hence most studies are based on mtDNA (Zhang et al., 2014; Wang et al., 2015). Meadows et al. (2006), revealed a total of 18 haplotypes and two main lineages with specific geographic patterns of frequency dispersal. Furthermore, Niemi et al. (2013) reported that little information is available about Y chromosome polymorphism in ancient domestic animals, partially because of the challenges in the Y chromosome genetic marker typing and because of the insufficiency of the polymorphic markers currently available for characterisation.

2.4.4 Single Nucleotide Polymorphisms (SNPs)

The process of building the human genetic blueprint has led to the development of different methodologies and technologies, which have been directly carried into the studies of domestic animal genomics (Fan et al., 2010). Moreover, numerous complete genome studies have led to the advent of single nucleotide polymorphisms (SNPs) as modern DNA (genetic) markers (Pariset et al., 2006). Single nucleotide polymorphisms (SNPs) are normally found in non-coding regions (FAO, 2006).

SNPs are characterised as bi-allelic genetic markers (Fan et al., 2010), are widely distributed throughout the mammalian genome (Pariset et al., 2006; Fan et al., 2010; Miller et al., 2011) and have a slower rate of mutation, and thus a lower level of homoplasy (Miller

et al., 2011). In addition, the FAO (2006) reported that SNPs are variations at single nucleotide, which do not change the complete length of the sequence of the DNA in the region. The high density SNPs arrays were designed for essential farm animals, initially for those with reference genomes latterly also for those without reference genomes with the emergence and application of huge parallel sequencing technologies (Fan et al., 2010). SNPs are rapidly becoming the genetic marker of choice for assessing a wide variety of evolutionary and population genetic queries (Miller et al., 2011).

2.4.4.1 Importance of Single Nucleotide Polymorphism (SNPs)

Single nucleotide polymorphisms (SNPs) are able to evaluate the pattern of linkage disequilibrium, the dissection of quantitative trait loci, the consequence of selection, and genome wide selection as a technique to hasten genetic gain in animals (Kijas et al., 2009). Moreover, SNPs allow direct comparison and dual analysis of different studies (Miller et al., 2011). The first genome-wide experiment which was done on domestic animals revealed that SNPs can be used to map Mendelian traits (Kijas et al., 2009). In addition, SNPs have been successfully used for assessing relationships, paternity testing, and tracing back the geographic origins of livestock (Pariset et al., 2006; Fan et al., 2010). SNPs, due to their high density have been extensively used to detect copy number variations (CNVs) in livestock. Copy number variations are known to influence gene expression, phenotypic diversity and animal adaptation by disrupting genes and changing gene dosage (Liu et al., 2013). In brief, SNPs can easily be used to assess genotypes(Fan et al., 2010; Miller et al., 2011).

CHAPTER 3

Genetic structure of South African Nguni (Zulu) sheep populations reveals admixture with exotic breeds

Mokhethi Matthews Selepe¹ Simone Ceccobelli², Emiliano Lasagna², Nokuthula Winfred Kunene¹

¹Department of Agriculture, University of Zululand, Kwadlangezwa, South Africa ²Department of Agricultural, Food and Environmental Sciences, University of Perugia, Perugia,

Italy

(This chapter has been published on PLOS One Journal)

Abstract

The population of Zulu sheep is reported to have declined by 7.4% between 2007 and 2011 due to crossbreeding. There is insufficient information on the genetic diversity of the Zulu sheep populations in the different area of KwaZulu Natal where they are reared. The study investigated genetic variation and genetic structure within and among eight Zulu sheep populations using 26 microsatellite markers. In addition, Damara, Dorper and South African Merino breed were included to assess the genetic relationship between these breeds and the Zulu sheep. The results showed that there is considerable genetic diversity among the Zulu sheep populations (expected heterozygosity ranging from 0.57 to 0.69) and the level of inbreeding was not remarkable. The structure analysis results revealed that Makhathini Research Station and UNIZULU research station share common genetic structure, while three populations (Nongoma, Ulundi and Nquthu) had some admixture with the exotic Dorper breed. Thus, there is a need for sustainable breeding and conservation programmes to control the gene flow, in order to stop possible genetic dilution of the Zulu sheep.

Key words: Biodiversity, Crossbreed, Microsatellites, South African indigenous sheep breeds

3.1 Introduction

Human history has been completely transformed by the domestication of animals and plants over the past 10,000 years (Dobney and Larson, 2006). Their domestication had a critical influence on demographic trends and was a requirement for the rise and development of civilisation (Clutton-Brock, 1999; Diamond, 2002). Archaeozoologists have stated that the domestication of animals according to the criterion of body size reduction is said to have begun with goats and then sheep about 10,000 to 9,500 B.P ago, approximately 1,000 years after plant domestication (Zeder, 2008). The domestic stocks in Southern Africa were initially acquired by nomadic people (KhoiKhoi) in Botswana around 2300 B.P. The main southward dispersal route of animals to Southern Africa was through the Kalahari desert, the Orange River, or either the route that runs parallel to the western coast of Namibia (Pleurdeau et al., 2012). The KhoiKhoi people moved their livestock around searching for enough grazing for them and they migrated according to the season (Sadr, 2015).

Sheep play an essential role in the livelihood of people around the world as they are a source of meat, milk, wool, hide and manure, especially in developing countries (Ramsay et al., 2000; Pollott et al., 2009). In South Africa, wool production plays an important economic role as an earner of foreign exchange. This is because as an export product, more than 90% of the total production is exported either as greasy wool or in semi-processed form as scoured and wool top. South African wool production is mainly Merino and Karakul, but coarse and coloured types are also produced and marketed on a limited scale (DAFF, 2015). In particular, local breeds can be considered as reservoirs of genetic diversity. Indeed, they have evolved over centuries managed by traditional pastoralists, enabling the emergence of a wide diversity (Taberlet et al., 2008), of strong adaptations to harsh conditions, nutritional fluctuations, and resistance to diseases and parasites (Mukhongo et al., 2014; Wei et al., 2015). Human socio-cultural and economic networks have had a marked impact on their genetic makeup.

Zulu sheep are one of four ecotypes of the Nguni sheep breed (Landim, Pedi, Swazi and Zulu) (Kunene et al., 2009; Mavule et al., 2013). The Nguni people brought the ancestors of this breed to the east coast of South Africa (KwaZulu Natal) between 200 and 400 AD (Du Toit, 2008). In their southward migration, Nguni people migrated along the eastern part of Southern Africa from Central and East Africa during the 16th and 17th centuries. Some of the migrants settled along the way, while the other group travelled

further. This gave rise to the division of Nguni people. The Zulu people, who took their name from their first ruler or king, "Shaka Zulu", settled in the green plains of KwaZulu Natal (Erwee and Young, 2008). The farmers in the rural communities of KwaZulu Natal keep Zulu sheep as a source of protein and for sale (Kunene and Fossey, 2006). This Nguni sheep ecotype is characterised by having either thin or fat tail (carrot shaped), multicolours, and a coat of either wool or hair (Kunene et al., 2009). Moreover, the Zulu sheep have acquired high adaptation to harsh environmental conditions, resistance against diseases and parasites and, the ability to walk long distances. The Zulu sheep can be distinguished from other Nguni breeds by their small mouse ears, and they appear to be more woolly. In addition, the dominant colours are brown and white, black and brown, and a unique fawn colour (Kunene and Fossey, 2006; Mavule et al., 2013). However, the population of Zulu sheep is reported to be declining, due to cross-breeding with exotic breeds, especially with the Dorper and Merino sheep breeds (Mavule et al., 2013; Kunene et al., 2014; Mavule et al., 2016) with the aim of increasing body weight (Mavule et al., 2013). Uncontrolled mating strategies may result in genetic erosion of Zulu sheep, leading to their eventual extinction (Mavule, 2012).

There is currently a gap in information available on the genetic variation among Zulu sheep populations. The research published so far by Kunene et al. (2014) and Hlophe (2011) on genetic characterisation covered a few populations of Zulu sheep. In these studies, the authors concluded that there is a notable level of inbreeding among Zulu sheep and thus that a ram exchange programme should be implemented. Furthermore, the authors recommended that more Zulu sheep populations should be analysed in comparison with Dorper, Damara and Merino, since crossbreeding was suspected in some areas. According to Mavule et al. (2013a) the spread of Zulu sheep into different areas of KwaZulu Natal has fractured the sheep into isolated subpopulations occupying different ecological, social-cultural and management environments. However, this study was based on morphological features, thus assessment at molecular level is required.

The aims of this study were two-fold: (i) to confirm or disconfirm the structuration among eight Zulu sheep populations revealed by morphological analysis, using molecular tools; (ii) to assess the phenomenon of crossbreeding, in particular with Dorper and Merino sheep, using microsatellites. Microsatellites are characterised as co-dominant inheritance, highly distributed throughout the genome, showing a high mutation rate and a high level of polymorphism (Kevorkian et al., 2010; Ghazy et al., 2013; Al-Atiyat et al., 2014). They are one of widely used markers to assess genetic variation, genetic relationship, and population structure of sheep breeds (Ceccobelli et al., 2015; Elbeltagy et al., 2015; Gaouar et al., 2016; Loukovitis et al., 2016; Sharma et al., 2016). The molecular information obtained in the present study will serve as a guideline for management and breeding strategies (reducing inbreeding and crossbreeding) for better utilisation and conservation of Zulu sheep.

3.2 Materials and methods

3.2.1 Ethics Statement

All experimental procedures were reviewed and approved by the University of Zululand Research Ethics Committee, Reg No: UZREC 171110-030 PGM 2015/227.

3.2.2 Animal sampling

A total of 207 blood samples of Zulu sheep were randomly collected from Eshowe (ES) n=19, Makhathini research station (MS) n=33, Mtubatuba (MT) n=29, Jozini (JO) n=30, Nongoma (NG) n=30, Nquthu (NQ) n=22, Ulundi (UL) n=23 and UNIZULU research station (UZ) n=21 (Fig 3.1). Data on the UZ and MS populations were obtained from a recent previous study that used the same microsatellite loci (Kunene et al., 2014). Blood samples were collected from each animal with the Vacutainer® system, in tubes with the addition of EDTA as anticoagulant, and stored at -20°C until analyses were performed. Analysed animals can be considered as a representative sample of the populations as they were chosen from different flocks, trying to avoid closely related individuals. The eight populations used in the study were selected based on the availability of Zulu sheep in these areas. The pictures (Zulu sheep) and details of the 11 studied sheep populations are reported in Appendix 1 and Appendix 2, respectively. Due to probable crossbreeding of Nguni Zulu populations with exotic breeds which are common in the areas of KwaZulu Natal where the Zulu sheep are found; a total of 53 Dorper (DO) and South African Merino breed (ME) individuals were included in the dataset. In addition, 29 Damara (DA) animals were included as out-group.

3.2.3 Molecular analysis

The GenElute Blood Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA) was used to extract the genomic DNA. Twenty-eight microsatellite loci (Table 3.1) were selected from the list of recommended markers for genotyping analyses in sheep breeds (FAO, 2004b). The markers were selected based on degree of polymorphism and their position in the sheep genome. The microsatellite markers were optimised for multiplex PCR amplification using an ABI ProFlex PCR system under the same conditions reported in a previous work by Kunene et al. (2014) (Appendix 3). The multiplex PCR products were pooled to allow the analysis of more microsatellites in each electrophoresis. The size of the fragments was determined using an automated DNA sequencer (ABI 3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) and GeneMapper version 5.0 software (Applied Biosystems, Foster City, CA, USA).

3.2.4 Statistical Analysis

Allele frequencies, mean number of alleles, polymorphic information content (PIC) for each microsatellite loci, and the observed and expected heterozygosity in the eleven populations were estimated using the MICROSATELLITE TOOLKIT (Park, 2001).

To calculate average allelic richness (R) and the richness of private alleles (PR) for each population, the rarefaction method (Kalinowski, 2004) implemented in HP-RARE version 1.0 software was used, adopting a sample of 12 individuals (Kalinowski, 2005). A test for departure from Hardy-Weinberg equilibrium (HWE) was done using the Markov Chain Monte Carlo method (20 batches, 5,000 iterations per batch and a dememorisation number of 10,000) implemented in GENEPOP version 4.0 software (Raymond and Rousset, 1995). Level of significance were adjusted using false discovery rate (FDR) procedure (Benjamini and Hochberg, 1995).

The F_{IS} for each population was calculated via bootstrapping using 1,000 replicates with GENETIX software version 4.05 (Benjamini et al., 2000). The extent of population subdivision was investigated by calculating the global multi-locus F_{ST} value. The index of pairwise F_{ST} of Weir and Cockerham (1984) between populations and their associated 95% confidence intervals was estimated using GDA software (Lewis and Zaykin, 1999). Bottleneck events in the Zulu populations were tested by the program BOTTLENECK version 1.2 (Lewis and Zaykin, 1999) utilising the Wilcoxon test for heterozygote excess, as well as the two-phase model (TPM) recommended by Weir and Cockerham (1984) and Peery et al. (2012).

The Reynolds' weighted genetic distance (Reynolds et al., 1983) among the populations was calculated and a neighbour-joining tree was reconstructed using the PHYLIP package version 3.6 (Felsenstein, 2005); the dendrogram was depicted using the software package TreeView version 1.6.6 (Page, 1996). Bootstrap values were obtained with 1,000 replicates over the loci.

The hierarchical analysis of molecular variance (AMOVA) was performed using ARLEQUIN software version 3.5 (Excoffier and Lischer, 2010) in order to quantify the degree of differentiation among breeds.

Population structure across the entire dataset was analysed using a Bayesian approach implemented in STRUCTURE software version 2.3.4 (Pritchard et al., 2000) to assess the most probable number of partitions in the dataset without the assumption of the breed identities. The assignment of individuals to populations considered an ancestry model with admixture, correlated allele frequencies, and defined sampling location for each individual. Ten independent runs with 500,000 MCMC (Markov Chain Monte Carlo) iterations and a burn-in of 200,000 steps were performed for $2 \le K \le 13$ (K= number of clusters) to estimate the most likely number of clusters present in the dataset. The algorithm of Evanno et al. (2005) was adopted in order to evaluate the most probable value of K. Moreover, in order to investigate population substructures, the most interesting cluster identified with STRUCTURE was re-analysed using the same settings and assuming K=2to K= n+3 (n being the number of populations included in each cluster). STRUCTURE HARVESTER (Earl, 2011), a web-based program, was used for collating the results generated by the program STRUCTURE. The clustering pattern was implemented in the CLUMPP program and visualised using the software DISTRUCT software version 1.1 (Rosenberg, 2004).

To further investigate the genetic structure of each breed when adopting an approach without assumptions about HWE or linkage disequilibrium, Discriminant Analysis of Principal Component (DAPC) was carried out with the method implemented in the ADEGENET
package (Jombart, 2008) within the statistical package R version 3.3.2 (Team, 2013). DAPC was conducted without *a posteriori* group assignments by inferring the most likely number of genetic clusters (K) using the *find.clusters* function in the ADEGENET package. This function utilises *K*-means clustering to calculate a Bayesian information criterion (BIC) value for each potential value of *K* (the most likely *K* has the lowest BIC value) and delineates individual group assignments for DAPC.



Figure 3. 1 Geographical location of the sampling sites for the studied 11 sheep populations Geographical co-ordinates: JO, Jozini: latitude: 27° 42' 94"S longitude: 32° 06' 57"E; MT, Mtubatuba latitude: 28° 40' 59"S longitude: 32° 21' 43"E; NG, Nongoma latitude: 27° 89' 43"S longitude: 31° 64' 54"E; ES, Eshowe latitude: 28° 89' 47"S longitude: 31° 46' 28"E; UL, Ulundi latitude: 28° 29' 97"S longitude: 31° 43' 42"E; NQ, Nquthu latitude: 28° 30' 08"S longitude: 30° 80' 39"E; UZ, UNIZULU research station latitude: 28° 85' 24"S longitude: 31° 84' 91"E; MS, Makhathini research station latitude: 27° 39' 53"S longitude: 32° 17' 64"E; DO, Dorper latitude: 29° 80' 00"S longitude: 30° 65' 00"E; DA, Damara latitude: 25° 16' 74"S longitude: 29° 39' 87"E; ME, South African Merino latitude: 29° 60' 06"S longitude: 30° 37' 94"E and latitude: 29° 98' 25" S longitude: 30° 92' 17"E

3.3 Results

3.3.1 Genetic variation

In total, 323 alleles were detected across the 28 microsatellites loci in the studied Zulu sheep populations and three exotic breeds with a mean of 11.54 alleles per locus (Table 3.1). The most polymorphic marker with the highest number of alleles per locus was HSC (19), whereas ETH10 had the lowest number of alleles per locus (3). The polymorphic information content (PIC) per locus ranged from 0.11 (ETH10) to 0.80 (HSC) (Table 3.1). PIC values revealed that all markers were informative with the exception of ETH10 which was thus excluded for further statistical analysis. As significant deviation from Hardy-Weinberg equilibrium detected in the populations studied, the locus TGLA126 was also excluded for further statistical analysis. The results of the genetic diversity, genetic distance and breed assignment are based on the 26 remaining microsatellite markers.

The mean number of observed alleles (MNA) ranged from 3.84 (UZ) to 6.64 (NQ) (Table 3.2). After adopting the rarefaction methodology, the mean allelic richness ranged from 3.53 (ES) to 6.29 (NQ) in a sample size of 12 individuals. Distribution of allelic richness and distribution of private allelic richness were significantly different (respectively: Kruskal-Wallis chi-squared= 42.15, df= 10, p-value= <0.0001; Kruskal-Wallis chi-squared= 38.10, df= 10, p-value= <0.0001) between populations.

The highest observed heterozygosity (H_o) was detected in ME (0.67), while JO showed the lowest (0.53). The highest expected heterozygosity (H_E) (with the exception of the exotic breeds) was observed in NQ (0.69), with the lowest (0.57) in ES (Table 3.2). Distribution of H_o was not significantly different between populations (Kruskal-Wallis chi-squared= 13.80, df= 10, p-value= 0.18), whereas distribution of H_E was significantly different between populations (Kruskal-Wallis chi-squared= 20.93, df= 10, p-value= 0.02). The inbreeding coefficient (*F*_{is}) estimated ranged from -0.01 (ES) to 0.16 (JO) (Table 3.2).

The two-phase mutation model under Wilcoxon sign rank tests was utilised to find out recent bottlenecks (heterozygosity excess) in the Zulu sheep populations. The heterozygosity excess obtained (data not shown) were significant (P<0.05) in ES (0.005), UZ (0.003) and MS (0.03).

Locus	Chr.	S.R. (bp)	Genebank accession number	References	Na	H _E	Ho	PIC	HWE Pop†
BM1824	1	169-177	G18394	[1]	7	0.66	0.65	0.59	3
BM6506	1	192-208	G18455	[1]	9	0.56	0.57	0.51	0
INRA006	1	101-141	X637951	[2]	12	0.60	0.59	0.53	1
OarFCB11	2	117-151	L01531	[3]	11	0.77	0.75	0.72	0
OarFCB20	2	106-130	L20004	[4]	14	0.78	0.78	0.72	1
D5S2*	5	166-192	Z22743	[5]	8	0.45	0.42	0.50	0
ETH10*	5	190-206	Z22739	[5]	3	0.13	0.09	0.11	1
RM006	5	205-211	-	[6]	11	0.67	0.63	0.60	0
McM527	5	113-141	L34277	[7]	13	0.75	0.71	0.69	1
ETH225	9	134-156	-	[8]	13	0.52	0.38	0.51	4
CSSM66	9	165-225	-	[9]	18	0.57	0.55	0.53	1
ILSTS11	9	201-288	L23485	[10]	10	0.48	0.42	0.50	2
INRA035	12	112-138	X68049	[2]	11	0.71	0.60	0.65	0
TGLA53	12	147-167	-	[11]	13	0.79	0.78	0.75	2
CSRD247*	14	213-259	EU009450	[12]	13	0.66	0.58	0.61	2
INRA063	14	157-195	X71507	[2]	12	0.60	0.49	0.54	2
SPS115	15	234-250	-	[13]	10	0.56	0.48	0.51	0
MAF65	15	128-144	M67437	[14]	10	0.72	0.78	0.66	3
TGLA126	16	119-147	-	[15]	17	0.77	0.44	0.72	8
MAF214	16	107-137	M88160	[16]	10	0.49	0.49	0.50	0
MAF209	17	108-140	M80358	[17]	13	0.74	0.64	0.68	1
BM8125	17	141-171	G18475	[1]	8	0.51	0.48	0.50	0
OarFCB48	17	138-174	M82875	[4]	10	0.45	0.33	0.51	4
OarFCB304	19	151-194	L01535	[3]	14	0.75	0.73	0.70	0
HSC	20	268-300	M90759	[18]	19	0.84	0.70	0.80	4
BM1818	20	256-284	G18391	[1]	14	0.73	0.72	0.67	2
OarCP20	21	68-94	-	[19]	9	0.61	0.52	0.52	0
BM6526	26	151-175		[1]	11	0.69	0.58	0.63	2
					11.54	0.63	0.57	0.59	

Table 3. 1 Microsatellites loci, chromosome position (Chr), size range (S.R), number of alleles observed (Na) at each locus, mean PIC (Polymorphism Information Content) per locus in 11 studied sheep populations of SA and number of populations derived from HEW

*: relative to cattle linkage map (not mapped in Ovis aries). HWE: Hardy-Weinberg Equilibrium per locus

†: after Benjamini and Hochberg (1995) correction

In grey, the markers excluded from the further analysis on the bases of PIC values and HWE equilibrium

Breed							
	Sample Size	MNA ± SD	R(PR)	$HO \pm SD$	$HE \pm SD$	F18 [IC95%]	
/population							
JO	30	5.69±1.87	3.94 (0.11)	0.53±0.02	0.63±0.03	0.16 [0.08-0.20]	
MT	29	5.58±1.77	3.91 (0.16)	0.57±0.02	0.62±0.04	0.09 [-0.00-0.13]	
NG	30	5.85±1.59	4.05 (0.19)	0.56±0.02	0.65±0.03	0.13 [0.04–0.17]	
ES	19	3.73±1.34	3.14 (0.04)	0.58±0.02	0.57±0.03	-0.01 [-0.10-0.02]	
UL	23	5.54±1.70	4.04 (0.24)	0.59±0.02	0.66±0.03	0.11 [-0.00-0.16]	
NQ	22	6.50±1.75	4.44 (0.25)	0.62±0.02	0.69±0.02	0.12 [0.05–0.13]	
UZ^1	21	4.00±1.57	3.35 (0.14)	0.58±0.02	0.60±0.03	0.03 [-0.09–0.09]	
MS^1	33	5.46±2.47	3.83 (0.29)	0.60 ± 0.02	0.64±0.03	0.06 [-0.02–0.12]	
DO	23	4.92±1.57	3.69 (0.17)	0.57±0.02	0.61±0.03	0.06 [-0.04–0.11]	
DA	29	6.12±1.73	4.24 (0.29)	0.63±0.02	0.67±0.04	0.07 [0.01–0.09]	
ME	30	6.08±1.92	4.31 (0.38)	0.67±0.02	0.70±0.03	0.04 [-0.01–0.06]	

Table 3. 2 Genetic diversity of the studied sheep breeds obtained from the analysis of 26 microsatellites loci

Population/breed acronyms, N: sample size of each breed, MNA: mean number of observed alleles, R: allelic richness, PR: private allelic richness, H₀: mean observed heterozygosity, $H_{E:}$ expected heterozygosity, $F_{IS:}$ inbreeding coefficient per breed.

¹Data taken from Kunene et al. (2014).

3.3.3 Genetic differentiation, distance and phylogeny

Pairwise genetic differentiation (F_{ST}) among populations is shown in Table 3.3. The F_{ST} genetic distance estimate values revealed that the closest populations were NQ and NG (0.056) and MS and UZ (0.070), while the longest distance (0.235) between the Zulu sheep populations was realised between ES and MS. DO was more genetically distant from UZ and MS (0.272 and 0.255, respectively) than to the other Zulu sheep populations. The NQ population was genetically the closest population to the DO sheep breed.

	IO	MT	NG	ES	UI.	NO	UZ	MS	DO	DA
МТ	0.110 [0.050-		110		01		02	1110		
IVII	0.181]									
NG	0.082 [0.046-	0.142 [0.082-								
110	0.122]	0.208]								
ES	0.159 [0.079-	0.209 [0.124-	0.095 [0.067-							
ĽS	0.253]	0.293]	0.132]							
Ш	0.146 [0.087-	0.180 [0.103-	0.072 [0.040-	0.101 [0.071-						
UL	0.220]	0.255]	0.110]	0.133]						
NO	0.093 [0.055-	0.142 [0.073-	0.056 [0.037-	0.118 [0.069-	0.081 [0.044-					
NQ	0.141]	0.209]	0.077]	0.185]	0.134]					
117	0.204 [0.134-	0.184 [0.109-	0.219 [0.153-	0.228 [0.128-	0.233 [0.162-	0.226 [0.162-				
UZ	0.285]	0.273]	0.293]	0.331]	0.301]	0.294]				
0.158 [0.09]	0.158 [0.093-	0.150 [0.085-	0.202 [0.143-	0.235 [0.154-	0.220 [0.146-	0.207 [0.141-	0.070 [0.038-			
MB	0.234]	0.228]	0.266]	0.316]	0.294]	0.273]	0.106]			
DO 0.131 0.	0.131 [0.085-	0.205 [0.136-	0.111 [0.077-	0.143 [0.105-	0.132 [0.103-	0.084 [0.060-	0.272 [0.206-	0.255 [0.187-		
	0.177]	0.284]	0.146]	0.182]	0.162]	0.110]	0.343]	0.326]		
D٨	0.144 [0.098-	0.132 [0.092-	0.153 [0.110-	0.203 [0.137-	0.168 [0.110-	0.167 [0.113-	0.147 [0.095-	0.110 [0.069-	0.220 [0.152-	
DA	0.201]	0.179]	0.196]	0.273]	0.243]	0.231]	0.212]	0.157]	0.220 [0.152- 0.291] 0.216 [0.162- 0.269]	
ME	0.161 [0.119-	0.159 [0.128-	0.170 [0.128-	0.215 [0.163-	0.173 [0.115-	0.162 [0.115-	0.185 [0.139-	0.155 [0.121-	0.216 [0.162-	0.090 [0.061-
IVIL	0.205]	0.195]	0.212]	0.268]	0.246]	0.214]	0.238]	0.190]	0.269]	0.118]

Table 3. 3 Pairwise genetic differentiation among the studied breeds/populations (with confidence intervals at 95%)

JO, Jozini; MT, Mtubatuba; NG, Nongoma; ES, Eshowe; UL, Ulundi; NQ, Nquthu; UZ, UNIZULU research station; MS, Makhathini research station; DO, Dorper; DA, Damara; ME, South African Merino.

The neighbor-joining tree obtained from Reynolds weighted genetic distance (Fig 3.2) showed the genetic relationship among 11 sheep populations. The phylogenetic tree revealed two clear clusters, the first cluster comprising MS and UZ, with the second comprising NQ, DO, UL, ES and NG. The remaining 4 populations (JO, MT, DA and ME) could not be grouped in any cluster.



Figure 3. 2 Genetic relationship among 11 sheep populations using Reynolds genetic distance according to neighbour-joining algorithm

JO, Jozini; MT, Mtubatuba; NG, Nongoma; ES, Eshowe; UL, Ulundi; NQ, Nquthu; UZ, UNIZULU research station; MS, Makhathini research station; DO, Dorper; DA, Damara; ME, South African Merino.

3.3.3 Genetic structure and admixture analysis

H0.01

The populations' structure (Fig 3.3) was analysed using Bayesian clustering analysis to determine the number of clusters (*K*) present in the populations, permitting the identification of differences among populations and hidden substructures within them. The highest ΔK value was detected at K = 4 (Fig 3.4a). The Q-matrix averaged over the most similar run for K = 4, was used to display in a map the distribution of membership coefficients according to population and geographical location (Fig 3.5). As shown in Figure 3.3a, at K = 2, two clusters were formed; UZ, MS, DA and ME clustered together, while JO, NG, ES,

UL, NQ and DO formed a second cluster. MT appeared as an admixture between the two clusters. At *K*=3, DA and ME clustered together separately. At *K*= 4, four different clusters were defined; the first cluster (JO and MT), the second cluster (NG, ES, UL, NQ and DO), the third cluster (UZ and MS) and the fourth cluster (DA and ME). However, DA and ME contained some individuals associated with other eight Zulu sheep populations (not well differentiated), while DO related to NG, ES, UL and NQ. The lower ΔK peak was detected for *K*= 9, showing that the Bayesian analysis was able to distinguish more substructure in the dataset. At this level JO and MT appear to be different, whereas NG and UL were still not distinguished and admixed, especially with NQ. The admixture between these groups (NG, UL, NQ and DO) was further investigated in a substructure analysis (Fig 3.3b), where UL appeared as an admixture with NQ and NG populations.



Figure 3. 3 Generic clustering of 11 sheep populations with STRUCTURE

(a) Analysis of the entire data set obtained from 10 runs for each number of assumed populations (K) value ranging from 2 to 9; (b) further analysis obtained from four populations (NG, UL, NQ and DO).

JO, Jozini; MT, Mtubatuba; NG, Nongoma; ES, Eshowe; UL, Ulundi; NQ, Nquthu; UZ, UNIZULU research station; MS, Makhathini research station; DO, Dorper; DA, Damara; ME, South African Merino.





(a) Bayesian information criterion (BIC) values plotted for number of clusters ranging from K = 1 to 40 derived from discriminant analysis of principal components (DAPC) (b) Delta *K* distribution of sub-STRUCTURE analysis obtained from four populations (NG, UL, NQ and DO).



Figure 3. 5 Representation of sample size (indicated by pies) and of relative admixture distribution inferred by STRUCTURE at K=4

JO, Jozini; MT, Mtubatuba; NG, Nongoma; ES, Eshowe; UL, Ulundi; NQ, Nquthu; UZ, UNIZULU research station; MS, Makhathini research station; DO, Dorper; DA, Damara; ME, South African Merino.

The Bayesian Information Criterion (BIC) statistic generated by Discriminant Analysis of Principal Components (DAPC) indicates that the optimal number of clusters in the data set is K=9 (Fig 3.4c), showing five more clusters generated by DAPC than those generated by STRUCTURE. In the DAPC analysis, 80 PCs of the PCA were retained as input to discriminant analysis, accounting for approximately 89% of the total genetic variability. The scatterplot of the first two components of the DA (Fig 3.6a and Fig 3.6b) showed extensive sharing of genetic variation among Zulu sheep. In particular, the plot showed that UZ and MS appeared clearly distinct from the other populations. The MT and JO populations showed genetic proximity and a particular affinity was observed among ES, UL, NQ, NG and DO. Using the grouping function obtained in the discriminant analysis, a high proportion of individuals were found to be correctly assigned to their original group: ES (100%), UZ (100%), MS (100%), MT (97%) UL (91%). The lowest scores were observed for JO (67%), NQ (64%) and NG (63%).

Moreover, the DAPC results are related to those of STRUCTURE at K= 9 presented in Figure 3.3.



Figure 3. 6 Assignment of individuals to 9 clusters based on DAPC analysis(a). Scatterplot of the first two pricnciples components of DAPC using populations as posteriori as a posteriori cluster (b).

In figure 3.6b, the individuals are assigned to populations *a posteriori*, that is, after determining the number of clusters by the programme, instead of forcing them into known populations. Populations are labeled inside their 95% inertia ellipses and dots represent individuals. The inset indicates the eigenvalues of the first four principal components and the variance explained by the PCA.

Analysis of molecular variance (AMOVA) was performed to assess the variation within and between only Zulu populations. AMOVA revealed a high variance component within individuals (87.58%), followed by among populations (8.99%), and among individuals within populations (3.43%) (Table 3.4).

Source of variation	df	Sum of squares	Variance components	Percentage of variation	
Among populations	7	67.147	0.15473 Va	8.99	
Among individuals within populations	199	323.418	0.05899 Vb*	3.43	
Within individuals	207	312.000	1.50725 Vc*	87.58	
Total	413	702.565	1.72097	100.00	
$F_{\rm IS} = 0.03^{\circ}$	766	$F_{\rm ST} = 0.08991^*$	$F_{\rm IT} = 0.12419^*$		

Table 3. 4 Results from AMOVA analysis of only Zulu sheep populations

**P*<0.01

The source of variation within populations (*Va*), among individuals within populations (*Vb*), within individuals (*Vc*) is given as a percentage for each comparison. F_{IS} , genetic variation among groups; F_{ST} , genetic variation among populations within groups; F_{ST} , overall genetic variation among these populations.

3.4 Discussion

A survey of Zulu sheep population size over a period of five years (2007 to 2011) revealed that population size decreased by 7.4% due to crossbreeding (Mavule et al., 2013). Consequently, critical attention should be paid to the population to stop their declining numbers. Official data on the census of the Zulu sheep is unavailable. The evaluation of genetic variation in Zulu sheep using a molecular approach is necessary for better understanding of the genetic diversity and structure of the sheep.

A total of 26 microsatellite loci revealed a recommended minimum number of alleles per locus (FAO, 2004). The mean value of MNA of Zulu sheep (5.28) is similar to the one revealed by Buduram (2004) in a study of South African sheep breeds (including Zulu sheep) assessed by 24 microsatellites loci. The mean values of H_o and H_E of the Zulu sheep were 0.58 and 0.63 respectively. In comparison, these values are closer to those revealed by Soma et al. (2012) and Kunene et al. (2014). The values for H_o and H_E were above 0.50 in all the populations, indicating that the populations analysed are characterised by a noticeable genetic variation. The NQ had the highest values of observed and expected heterozygosity of all the populations which could be attributable to the large numbers of alleles detected. The lower mean number of alleles observed in ES is probably due to a recently reduced effective population size highlighted in the bottleneck analysis, because there were few farmers in this area who owned Zulu sheep. A bottleneck effect was also

observed in UZ and MS, probably as a consequence of the small size of these two populations. The theory of genetics predicts that levels of genetic variation should increase with increasing effective population size, as bottlenecks entail genetic drift and inbreeding (Othman et al., 2016). In comparison to other African indigenous sheep breeds, the genetic diversity indices (MNA, Ho and H_E) of Zulu sheep were higher than Namaqua Afrikaner sheep (Qwabe et al., 2012). Whereas, Nigerian indigenous sheep (Agaviezor et al., 2013), Algerian indigenous sheep (Gaouar et al., 2015) and Egyptian indigenous sheep (Othman et al., 2016) had higher genetic diversity than the Zulu sheep.

The $F_{\rm IS}$ values for the majority of the populations were positive indicating some level of inbreeding. The JO and NG populations were relatively highly inbred compared to the rest of the Zulu sheep, followed by the NQ and UL. Similar findings were reported by Kunene et al. (2014), where the authors found that some of the Zulu sheep populations were more inbred (Msinga) than others. Mavule et al. (2013), reported that the Zulu sheep populations in 11 areas of KwaZulu Natal were genetically isolated; the authors revealed that 28% of the flocks did not interact with other flocks, but existed in isolation from neighbouring flocks and that at least 54% of the flocks interacted with 1 flock to a maximum of 3 flocks. Moreover, the majority of the farmers (66%) in these areas reported not practicing any form of inbreeding control (Mavule et al., 2013). Although the H_E and average number of alleles per locus were high in NQ, indicating a wide genetic base, the Fis value indicate that the individuals in the population were inbred. The negative value (F_{IS} = -0.00642) for the sheep at ES was not significantly different from 0, indicating that rather than having excess heterozygotes, the population was not inbred. However, heterozygosity excess is usually developed when a population experiences a reduction of its effective size [40]. Although not significantly different from 0, the inbreeding coefficient (F_{IS}) in the research stations (UZ, MS) was lower than that of the majority of the populations, indicating low inbreeding level. The Fis values were lower than those reported by Kunene et al. (2014), which were 0.0333 and 0.1178 for UZ and MS, respectively. The heterozygosity deficiency as a result of high level of inbreeding is not a threat only to Zulu sheep. The studies conducted on Moroccan (Gaouar et al., 2016) and Sudanese indigenous sheep (Gornas et al., 2011) showed similar $F_{\rm IS}$ mean values to Zulu sheep, while studies conducted on Nigerian (Agaviezor et al., 2012), Egyptian (Elbeltagy et al., 2015) and Tunisian indigenous sheep (Kdidi et al., 2015) had higher Fis mean values than in Zulu sheep. Consequently, these results reveal that the level

of inbreeding due to un-controlled mating strategies is a major problem in African local breeds.

The pair-wise F_{ST} values were computed to assess the level of genetic dilution in Zulu sheep due to crossbreeding with exotic sheep breeds. The lowest F_{ST} value (0.084) was found between the DO and NQ populations. Gaouar et al. (2016), reported that the gene flow has an effective role in reducing the genetic differentiation among the breeds, particularly among those reared within the same or close geographical location. Among the Zulu sheep population, NQ had very low F_{ST} values, along with NG and UL (0.056 and 0.081, respectively). This could be the result of uncontrolled crossbreeding due to their geographical location. The phenomenon of crossbreeding has also been reported in Algerian breeds by Gaouar et al. (2015), where Rembi and Taâdmit breeds were crossbreed with Ouled-Djellal.

The Reynolds' neighbor joining dendrogram showed that the populations ES, NG, UL and NQ had some considerable genetic influence from the Dorper breed. One of the purposes of the study was to investigate if there has been some introgression of the Zulu sheep with some of the exotic breeds. This was based on the survey conducted by Mavule et al. (2013), where 43% of the farmers reported a history of crossbreeding. The farmers at UL and NQ specifically reported a history of genetic influence of Dorper and Merino on their Zulu sheep (Mavule et al., 2013). In addition, Mavule et al. (2016) reported that the large size of the Nquthu sheep population is the result of crossbreeding with Dorper and Merino sheep. The Dorper is the second largest breed in South Africa. It is able to adapt to dry regions and produce mutton lambs in the harsh conditions in South Africa (Degen and Kam, 1992; Cloete et al., 2000). The breed is larger in size than the Zulu sheep, which may have been one reason some farmers crossbred it with Zulu sheep to produce more meat. Nevertheless, FAO (2015) reported that almost 100 livestock breeds became extinct between 2000 and 2014, where country data revealed that the main cause of genetic erosion is crossbreeding.

A common genetic structure between the MT and JO population (K= 4) is probably caused by the geographic location. Zulu sheep farmers in these areas buy sheep from each other. Moreover, Mtubatuba is one of the areas with Zulu sheep distribution near the Jozini area (Mavule et al., 2013). The Zulu sheep populations at the two research stations (UZ and

MS) formed one cluster and proved not to have any genetic introgression with any of the out-groups used in the study. The relationship between the populations in the research institutes was explained by Kunene et al. (2014) as having some of the founder sheep purchased from common areas and controlled breeding management (exchange of breeding rams). Furthermore, the result of the structure assignment test confirmed the introgression of the Dorper with the four populations NG, ES, UL, and NQ (K=4). The relationships between groups NG, UL, NQ and DO were further investigated (3.3.b), with UL appearing as an admixture with the NG population. These two populations seem to have some of the genetic material observed in the NQ population. The study by Mavule et al. (2016) showed that although NQ was larger in body measurements than the other Zulu sheep populations studied, due to crossbreeding, membership percentage of 11% of the population in NG could be classified as NQ using discriminant analysis. The current study may indicate that some of the genetic material found in NG could have been through the influence of crossbreeding. The close genetic relationship between the UL, NG and NQ may have been also caused by the effect of geographical location. The study by Mavule et al. (2013) indicated that farmers got their founder flocks in some of the neighboring areas where the Zulu sheep were reported to be available.

As previously enunciated, the optimal number of clusters was revealed as K= 9, generated by Discriminant Analysis of Principal Component (DAPC). These clusters highlighted the uniqueness of each Zulu sheep population, which is a reflection of the large gene pool reported by Ramsay et al. (2000). Even the morphological studies have shown differences in traits such as coat colour, presence of horns, tail type within the same Zulu sheep populations in the different areas, leading the authors to conclude that individual populations could not be singled out as separate types (Kunene et al., 2007; Mavule, 2012).

3.5 Conclusion

Our study has shown that Zulu sheep are threatened by crossbreeding with exotic breeds, especially with the Dorper breed. Although some of the studied populations were affected by the admixture phenomenon there is still genetic diversity among populations. It can also be concluded from this work that Zulu sheep have some uniqueness among populations. Thus, there is a need for sustainable breeding and conservation programs to control the gene flow, in order to stop their possible genetic dilution.

37

CHAPTER 4

Origin and genetic diversity of Zulu sheep assessed by Mitochondrial DNA D-Loop sequence

(This chapter to be submitted to Journal of Genetic Engineering and Biotechnology)

Abstract

Due to the lack of mtDNA analysis the origin of African sheep and details of their domestication are still something of a mystery. In addition, the maternal origin of the Zulu sheep population is unknown. The study evaluated the maternal origin and genetic diversity of Zulu sheep using mtDNA. A total of 118 mtDNA sequences of 110 individual Zulu sheep from 8 populations (Eshowe, Jozini, Makhathini research station, Mtubatuba, Nongoma, Nguthu, Ulundi, University of Zululand) were analysed to assess the genetic diversity and maternal origin. mtDNAs were amplified and sequenced from nucleotide position (np) 15,436 to np 157, which included the D-Loop. The mitochondrial sequences of 110 analysed Zulu sheep were aligned with the reference sequences of different haplogroups around Africa to identify the haplogroup lineages to which the analysed populations belong. The mean haplotype and nucleotide diversity were 0. 8113 and 0. 0115, respectively. Phylogenetic analysis showed two haplogroups (A and B). Haplogroup B predominates among Zulu sheep with a frequency of 93%, while the frequency of the A lineage stands at 7%. The analysis of mtDNA showed a high level of genetic diversity among Zulu sheep. It can be concluded that the indigenous Zulu sheep originate from haplogroup B. However, future studies should be conducted by evaluating more populations of Zulu sheep mtDNA.

Key words: Genetic diversity, Haplogroup, Mitochondrial DNA, Origin, Sheep

4.1 Introduction

Domestic sheep (*Ovis aries*) were first domesticated in the Near East about 9 000 years ago (Tapio et al., 2010). Domestic sheep are assumed to be derived from the wild mouflon in Europe and Asia (Meadows et al., 2011a; Brahi et al., 2015). Archaeological evidence reveals that African domestic sheep originated from the Sinai Peninsula, progressed via the Nile Delta, the eastern Sahara and the Red Sea Hills (between 7500 and 7000 years BP) (Muigai and Hanotte, 2013; Brahi et al., 2015) and entered Africa

approximately 3700 BP (Brahi et al., 2015). The routes of introduction of sheep into Africa, and their subsequent dispersal, was through the northeastern part and the horn of Africa (Muigai and Hanotte, 2013). Domestication of sheep in Southern Africa began approximately 2000 years BP (Horsburgh and Rhines, 2010; Pleurdeau et al., 2012). Neolithic pastoralists migrated southward to the savanna habitats of eastern and southern Africa due to climatic and demographic pressures (Wright, 2014).

African sheep are morphologically categorised into two: thin-tailed (long-legged or tropical dwarf) and fat tailed (wool or hair) sheep (Epstein, 1971). The most widely dispersed sheep on the continent are fat-tailed sheep. They are distributed in the northern region (from Egypt to Algeria), eastern and the southern part of Africa (from Eritrea to South Africa) (Muigai and Hanotte, 2013). The tin-tailed sheep are distributed in the northern region (from Morocco to Egypt and Sudan) and in the western part of Africa (from Senegal to Nigeria) (Muigai and Hanotte, 2013).

Following domestication of sheep they became widely dispersed throughout the world, because of their ability to adapt to poor diets and their tolerance to extreme climatic conditions (Lancioni et al., 2013). Consequently, sheep are among the few animal species that have existing wild progenitors scattered over a wide geographical area (Hussain et al., 2017). With regard to autochthonous Zulu sheep, the ancestors of this breed were brought to the east coast of South Africa (KwaZulu-Natal) by Nguni people between 200 and 400 AD (Du Toit, 2008). The origins of many domestic livestock species have been investigated using mitochondrial sequencing (Othman et al., 2015). Mitochondrial DNA (mtDNA) is useful as a tool for phylogenetic enquiry, due to its characteristics. These characteristics include; maternal lineage heredity, low rate of recombination, and a greater rate of substitution than nuclear DNA (Wang et al., 2007; Meadows et al., 2011a; Yüncü et al., 2013; Liu et al., 2016). During phylogenetic studies the control region, also known as the displacement-loop region (D-Loop), is usually examined, because it is the hypervariable non-coding region for the transcription and replication of the mtDNA (Liu et al., 2016).

Phylogenetic analysis of domestic sheep mtDNA has revealed five maternal lineages (haplogroup A to haplogroup E), with the most frequently discovered haplogroups being haplogroup A and B. Haplogroup A is of Asian origin, while B lineage originated in Europe (Meadows, 2011). Haplogroup C has been identified in Portugal, Turkey, the Caucasus and

China (Othman et al., 2015). Haplogroup D has been discovered in Rumanian Karachai and Caucasian animals, and is probably related to haplogroup A. Haplogroup E has been identified in Syria, Israel and Turkey (Othman et al., 2015). However, due to the lack of mtDNA analysis the origin of African sheep and details of their domestication are still something of a mystery (Muigai and Hanotte, 2013; Brahi et al., 2015; Resende et al., 2016). In addition, the maternal origin of Zulu sheep is unknown. The aim of the present study was therefore to investigate the mtDNA diversity and origin of the Zulu sheep population using the mitochondrial DNA D-Loop.

4.2 Materials and Methods

4.2.1 Animal sampling

A total of 110 blood samples were randomly collected from 8 Zulu sheep populations as described in (section 3.2.2), and illustrated in (Fig 3.1). Blood sample collection and DNA extraction protocol was the same as described on section (3.2.2).

4.2.2 PCR amplification and Statistical analysis

According to the complete sheep mitochondrial sequence, with accession number NC001941 (Hiendleder et al., 1998) used as a reference, mtDNAs were amplified and sequenced from nucleotide position (np) 15,436 to np 157, which included the D-loop hypervariable region. PCR amplification was performed in 25 µl volume with 2 mM MgCl₂, 200 µM of each dNTP, 1 µM of each primer (OA_15346F: GGAGAACAACCAACCTCCCTA; OA 157R: TGATTCGAAGGGCGTTACTC) and 1 unit of tag DNA polymerase using ABI ProFlex PCR system thermocycler with the following protocol: initial denaturation step of 5 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at the 55°C, 120 secs at 72°C, and a final extension of 5 min at 72°C. PCR products were first purified using the ExoSAP-IT® enzymatic system ExoSAP-IT (USB Corporation, Cleveland, OH, USA). Purified PCR products along with an internal primer (OA_16032F, ATGCGTATCCTGTCCATTAG) were sent to Central Analytical Facilities. Stellenbosch University for standard dideoxysequencing. mtDNA profiles were determined by a fragment of 812 base pairs (from np 15,450 to np 16,260), which resulted from the standardisation and optimisation of the sequencing results. Electropherograms were aligned, assembled, and compared using Sequencher[™] 5.10 software (www.genecodes.com). Indices such as haplotype diversity (Hd), nucleotide diversity (π), and average number of nucleotide differences (k) were estimated by DnaSP 5.10 software (Librado and Rozas, 2009).

The evolutionary relationship of sequences was evaluated through a median-joining network of control-region haplotypes constructed using Network 4.6 software (www.fluxus-engineering.com). Nucleotide weighting (w) was adjusted to reflect the difference in mutational frequency among indels (w = 30), transversions (w = 20), and transitions (w = 10), where the least-common event received the highest value. The maximum parsimony (MP) calculation (Polzin and Daneschmand, 2003) was performed to identify unnecessary median vectors and links which could be switched off in the results display.

4.3 Results

4.3.1 Mitochondrial DNA haplotypes and diversity

A total of 233 polymorphic sites and 57 haplotypes were identified from 110 mtDNA control region sequences (Table 4.1). All analysed populations were found to be polymorphic. The highest polymorphic site value was found in JO (54), whereas the lowest value was observed in both ES and MS (16). The number of haplotypes found in Zulu sheep population ranged from 3 (MS) to 11 (NQ). The analysis with DnaSP software revealed that the highest haplotype diversity was observed in NQ (0.952), while the lowest was detected in ES (0.571). Furthermore, the mean value of haplotype diversity of all Zulu sheep sequences analysed was 0.8113. The highest nucleotide diversity was observed in UL (0.026), whereas the lowest showed in ES (0. 005). The average nucleotide diversity value was 0. 0115.

BREED/POPULATION	ID	Ν	П	nh	Hd	S
Jozini	JO	14	0.017	10	0.945	54
Eshowe	ES	14	0.005	4	0.571	16
Mtubatuba	MT	14	0.010	8	0.857	24
Nongoma	NG	15	0.009	9	0.924	26
Nquthu	NQ	15	0.010	11	0.952	31
Ulundi	UL	15	0.026	7	0.829	47
UNIZULU research station	UZ	15	0.007	5	0.733	19
Makhathini research station	MS	8	0.008	3	0.679	16

Table 4. 1Estimation of genetic diversity within sheep breeds

N= number of analysed samples; π = nucleotide diversity; nh= number of unique haplotypes; Hd= haplotype diversity; S= number of polymorphic sites

4.3.2 Mitochondrial DNA phylogeny

The mitochondrial sequences of 110 analysed Zulu sheep were aligned with the reference sequences of different haplogroups around Africa to identify the haplogroup lineages to which the analysed populations belong. Subsequently, the median-joining network constructed from 110 analysed samples revealed the presence of two haplogroup lineages (haplogroup A and haplogroup B) (Fig 4.1). The other three haplogroups lineages (C, D and E) mentioned in the literature were not identified. In particular, the predominant haplogroup was B lineage (93%), whereas haplogroup of lineage A was subordinate, representing only 7%. The UL and JO populations were found in both haplogroups (A and B), while the remaining six populations (ES, MS, MT, NO, NQ, UZ) showed only haplogroup B.



Figure 4. 1 Median-joining network of the sheep control-region sequences

JO, Jozini; MT, Mtubatuba; NG, Nongoma; ES, Eshowe; UL, Ulundi; NQ, Nquthu; UZ, UNIZULU research station; MS, Makhathini research station; DO, Dorper; DA, Damara; ME, South African Merino.

4.4 Discussion

During migration humans would take along only a subset of domestic animals, leading to an assumption that as geographic distance increased there would follow a decline in, or loss of genetic line (Meadows et al., 2007). Dudu et al. (2016), reported that the diversity of animal breeds that appeared succeeding domestication raises difficulties of origin and classification.

The first sheep to migrate outside their centre of domestication were thin-tailed sheep (Epstein, 1971; Pereira et al., 2006). The thin-tailed sheep were the first to enter the African continent through the Isthmus of Suez and spreading overland and via maritime trade. The fat-tailed sheep migrated into Africa through the Isthmus of Suez and from the Arabian Peninsula through the horn of Africa, traveling southward. Data from historical linguistics, physical anthropology, genetic studies and archaeology suggest that the major gateway for

domesticated animals into Southern Africa was via Northern Botswana (Robbins et al., 2005).

The evolutionary history of 118 sequences from 110 Zulu sheep samples analysed through median-joining network revealed that haplogroup B lineage predominates in Zulu sheep. These results confirm studies conducted by Horsburgh and Rhines (2010), Gornas et al. (2011), Álvarez et al. (2013), (Brahi et al., 2015), Othman et al. (2015), Resende et al. (2016) and Ghernouti et al. (2017), confirming that haplogroup B lineage predominates in African sheep. These studies were conducted in four different cardinal points of Africa. In addition, the results are in with Hiendleder et al. (1998), where African sheep were classified as haplogroup B. The dominance of haplogroup B in Africa and Europe suggests that both African and European sheep share common ancestry in the Near East, consequently having a similar mtDNA profile (Álvarez et al., 2013; Brahi et al., 2015; Resende et al., 2016). Furthermore, the close genetic relationship between African and European sheep suggests subsequent introgression by European sheep via Mediterranean maritime trading, which has been suggested as another route for the sheep spreading along the northern African coastline (Pereira et al., 2006). The presence of slight haplogroup A in African sheep is probably due to the ancient migration of Arabian tribes into East Africa (Álvarez et al., 2013; Resende et al., 2016). Hence, 7% of Zulu sheep clustered with haplogroup A. The distribution of haplogroup B declines towards the east, whereas the frequency of lineage A increases (Dudu et al., 2016).

Two significant indices of mtDNA for evaluating genetic diversity are haplotype and nucleotide diversity (Pereira et al., 2006). The overall values of haplotype and nucleotide diversity were 0. 8113 and 0. 0115, respectively, thus showing a high level of genetic diversity within eight analysed Zulu sheep populations. More haplotype variation in mtDNA indicates that more ewes than rams have been used for breeding over time (Ghernouti et al., 2017). In comparison to other African indigenous sheep, the haplotype diversity detected in the current study is higher than that reported by Álvarez et al. (2013), while the nucleotide diversity is lower than that shown in the study by Othman et al. (2015).

4.5 Conclusion

The mitochondrial DNA D-Loop sequence analysis revealed a high level of genetic diversity in Zulu sheep. The phylogenetic analysis has shown that haplogroup B is the most

44

widespread haplogroup among the breed, which is the most important haplogroup in Europe. The predominance of haplogroup B lineage in the Zulu sheep is in agreement with the results from other African sheep breeds studies. However, future studies should be conducted by evaluating more populations of Zulu sheep mtDNA.

CHAPTER 5

5.1 General discussion

Animal genetic resources (AnGRs) contain traits that are potentially valuable or useful to mankind (Tapio, 2006). Generally, these traits are of economic, scientific and cultural value, particularly for food or agriculture either during the present or in the future (Alderson, 2010). However, DAFF (2016) reported that the comprehensive potential value of AnGRs is far from being recognised, since they are facing major erosion both in developing and developed countries. The major causes of this erosion are change in production systems, the outbreak of disease, uncontrolled breeding practices, and the unsuitable introduction of exotic breeds (FOA, 2015). In brief, the major motive of farmers to influence AnGRs is to meet their specific production goals, without considering the consequences of genetic erosion of indigenous AnGRs (Last, 2013). Indigenous breeds are essential in securing the necessities of life in rural areas (Tapio, 2006). This has also been confirmed by the study conducted by Mavule et al. (2013) where the production objective of keeping Zulu sheep for meat had the highest ranking.

In Africa, the majority of farmers own goats and sheep compared to other domestic animals, excluding poultry (Mukhongo et al., 2014). *Ovis aries* (domesticated sheep) are reared globally due to their diverse products (meat, milk and wool) and adaptive traits (Tapio, 2006). Nevertheless, the adaptive traits of indigenous breeds are threatened, since the sheep are crossbred with exotic breeds (FAO, 2015). This is because nowadays breeding systems are subjected to the economically essential traits of high market worth (DAFF, 2015). The replacement (introgression) of indigenous breeds by cosmopolitan breeds has caused the loss of unique gene combinations in local breeds (Groeneveld et al., 2010).

The phenomenon of cross breeding with exotic breeds in the current study was revealed by microsatellites (reported in chapter 3). In that chapter, the level of genetic dilution was determined by calculating F_{ST} values, where the Nquthu population was more genetically diluted by the Dorper breed (table 3.3). In addition, the Reynolds' genetic distance according to the neighbour-joining algorithm (Fig 3.2) showed the genetic introgression of the Dorper breed in the Eshowe, Nongoma, Ulundi and Nquthu populations. This was also confirmed by the structure assessment test at K=4 (Fig 3.3a). The genetic admixture of Zulu sheep with the Dorper breed is a threat, since their genetic ability to adapt to unfavourable conditions is probably will be diluted (genetic erosion). However, the

46

phenomenon of crossbreeding has been highlighted in many studies (Elbeltagy et al., 2015; Wei et al., 2015; Gaouar et al., 2016), where local sheep have been crossbred with commercial breeds. Therefore, this shows that crossbreeding is a huge challenge for autochthonous sheep globally. In addition, inbreeding contributes to the loss of genetic diversity, due to the effects from the recessive alleles leading to inbreeding depression (Ferenčaković et al., 2013). The highest level of inbreeding among the Zulu sheep studied was detected in Jozini, and Nongoma, followed by the Nquthu and Ulundi population (Table 3.2). However, the level of inbreeding in Zulu sheep was not remarkable. Last (2013), reported that the breeding system has a major role in causing continuous change in the genetic diversity within and among animal populations.

Conservation of genetic diversity is essential in animal adaptation or survival (Barker, 2001). This is because the potential of animals to respond to changing environments and local conditions, such as emerging diseases, depends on the quantity of genetic diversity (Furlan et al., 2012). Every individual animal has a unique set of gene combinations caused by mutation and the evolutionary process (Barker, 2001; Frankham et al., 2002). Therefore, to survive unpredictable future challenges it is essential to conserve a gene pool that can respond and adapt to changing environmental conditions and diseases (Qwabe et al., 2012). Conservation of adaptive gene pools acts as a warehouse for genetic diversity, which can help in addressing future challenges (FAO, 2010). Thus, the current study used two molecular markers (microsatellites and mtDNA) to gain insight into the genetic diversity of Zulu sheep and also to investigate their origin.

Since the development of molecular technology there has been an increased understanding of the genetic diversity, structure, processes and behaviour of the genome (Last, 2013). Fortunately, the use of both microstellites and mtDNA in this study helped to better understand the current genetic diversity of indigenous Zulu sheep. Microsatellites revealed a noticeable genetic diversity in Zulu sheep, where high genetic variation was found within individuals (Table 3.4). Mitochondrial DNA analysis also confirmed high genetic diversity within Zulu sheep populations (Table 4.1), where both haplotype and nucleotide diversity were high. These results indicate that the unique genetic diversity of Zulu sheep should be maintained. With the use of D-loop hypervariable region we were able to investigate the maternal lineage and origin of the Zulu sheep studied. mtDNA analysis reveals a maternal inheritance without recombination and with a rapid evolution rate (Liu et al., 2016). The dominant haplogroup in the Zulu sheep was found to be haplogroup B (97%), while haplogroup A had 7%.

The use of both microsatellites and mtDNA molecular markers is a complementary approach that merges the highly polymorphic microsatellites whose high mutation rates permit small scale resolution of more recent demographic events, with mtDNA whose D-loop region reveals insights into phylogeographic events that happened years ago (Feulner et al., 2004). In brief, this thesis merged microsatellites and mtDNA to investigate the genetic variation, genetic structure (admixture), genetic relationship (phylogeny) and the origin of the indigenous Zulu sheep found in KwaZulu Natal province (South Africa). These molecular data, or genetic information, obtained will help in improving and maintaining the gene pool of the Zulu sheep population, allowing the conservation of this breed. Genetic information is essential when it comes to animal breeding as it can be used to: (i) estimate breeding value of selected animals, (ii) manage inbreeding rates, and (ii) examine losses of genetic diversity through genetic drift (Hendry, 2013).

5.2 Conclusions

The use of microsatellites and mtDNA in the study showed that Zulu sheep are characterised by a unique genetic diversity. Microsatellites also revealed that the genetic diversity of Zulu sheep is threatened by crossbreeding (with the Dorper breed). This may be the reason for the declining number of Zulu. Microsatellites also detected inbreeding, though it was not significant. Genetic characterisation assists in understanding gene flow and the movement of alleles both within and among breeds. Thus, data obtained from molecular markers provide a better understanding for improving conservation techniques and methods. Moreover, the exchange of rams between villages or farms for breeding should be practiced, in order to avoid inbreeding depression. Genetic improvement needs new, distinctive and beneficial genes to be introduced to a new generation. Mitochondrial DNA (D-loop) analysis revealed two maternal lineages (haploid A and haploid B), with haplogroup B lineage predominating over haplogroup A lineage (97% and 7% respectively). None of Zulu sheep populations clustered with haplogroups C, B, D and E. D-loop analysis provided knowledge on the maternal lineage and origin of Zulu sheep.

In conclusion, the results obtained from this dissertation will help in developing an appropriate approach for the genetic improvement, utilisation and conservation of autochthonous Zulu sheep. Furthermore, it will give awareness to the rural based farmers of the importance of controlling breeding on their farms.

48

5.3 Recommendations

In the future there is a need for follow-up research using new generation molecular markers, such as high density single nucleotide polymorphisms (SNPs), to investigate the genetic structure of Zulu sheep, since they are now markers of choice. In addition, nematode resistance genes should also be investigated. This is due to the fact that it has been reported that Zulu sheep are resistant to gastrointestinal parasites, though no molecular approach has been done in this breed.

6. References

- Abdul-Muneer, P. 2014. Application of microsatellite markers in conservation genetics and fisheries management: recent advances in population structure analysis and conservation strategies. Genetics research international 2014.
- Agaviezor, B. O. et al. 2012. Morphological and microsatellite DNA diversity of Nigerian indigenous sheep. Journal of animal science and biotechnology 3: 38.
- Ahmed, Z. et al. 2014. GENETIC DIVERSITY ANALYSIS OF KAIL SHEEP BY USING MICROSATELLITE MARKERS. JAPS, Journal of Animal and Plant Sciences 24: 1329-1333.
- Al-Atiyat, R. M., N. M. Salameh, and M. J. Tabbaa. 2014. Analysis of genetic diversity and differentiation of sheep populations in Jordan. Electronic Journal of Biotechnology 17: 168-173.
- Alberts, B. et al. 2013. Essential cell biology. Garland Science.
- Alderson, L. 2010. Breeds at risk. In: Criteria and classification. Report from a seminar. 16-17 February 2010, London
- Álvarez, I. et al. 2013. Mitochondrial analysis sheds light on the origin of hair sheep. Animal genetics 44: 344-347.
- ARC-AII. 2001. The origin of the indigenous livestock of SouthAfrica: <u>http://www.arc.agric.za/institutes/aii/main/divisions/animalbreedgen/reproduction/ge</u> <u>nresdep.htm#top</u>.
- Arif, I., and H. Khan. 2009. Molecular markers for biodiversity analysis of wildlife animals: a brief review. Animal Biodiversity and Conservation 32: 9-17.

- Arora, R., H. Yadav, and B. Mishra. 2013. Mitochondrial DNA diversity in Indian sheep. Livestock Science 153: 50-55.
- Bailey, J. et al. 2002. Genetic variation of mitochondrial DNA within domestic yak populations. In: Proceedings of the third international congress on yak, in Lhasa, China, 4-9 September 2000. p 181-189.
- Barker, J. S. 2001. Conservation and management of genetic diversity: a domestic animal perspective. Canadian Journal of Forest Research 31: 588-595.
- Benjamini, Y., P. Borsa, L. Chikhi, N. Raufaste, and F. Bonhomme. 2000. GENETIX 4.02,
 logiciel sous Windows TM pour la génétique des populations. La-boratoire Génome,
 Populations, Interactions, CNRS UPR 9060, Université de Mont-pellier II, Montpellier.
 France.
- Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the royal statistical society. Series B (Methodological): 289-300.
- Bhatia, S., and R. Arora. 2005. Biodiversity and conservation of Indian sheep genetic resources-an overview. ASIAN AUSTRALASIAN JOURNAL OF ANIMAL SCIENCES 18: 1387.
- Boettcher, P. et al. 2010. Objectives, criteria and methods for using molecular genetic data in priority setting for conservation of animal genetic resources. Animal Genetics 41: 64-77.
- Brahi, O., H. Xiang, X. Chen, S. Farougou, and X. Zhao. 2015. Mitogenome revealed multiple postdomestication genetic mixtures of West African sheep. Journal of Animal Breeding and Genetics 132: 399-405.
- Bruford, M. W., D. G. Bradley, and G. Luikart. 2003. DNA markers reveal the complexity of livestock domestication. Nature Reviews Genetics 4: 900-910.
- Buduram, P. 2004. Genetic characterization of Southern African sheep breeds using DNA markers. MSc (Agric) thesis, University of the Free State, Bloemfontein, South Africa.
- Cardellino, R. A., and J. Boyazoglu. 2009. Research opportunities in the field of animal genetic resources. Livestock Science 120: 166-173.
- Ceccobelli, S., P. Di Lorenzo, F. Panella, E. Lasagna, and F. M. Sarti. 2016. Morphological and genetic characterisation of Pagliarola breed and its genetic relationships with other three indigenous Italian sheep breeds. Italian Journal of Animal Science 15: 47-54.

- Ceccobelli, S. et al. 2015. Genetic diversity of Cornigliese sheep breed using STR markers. Small Ruminant Research 123: 62-69.
- Chistiakov, D. A., B. Hellemans, and F. A. Volckaert. 2006. Microsatellites and their genomic distribution, evolution, function and applications: a review with special reference to fish genetics. Aquaculture 255: 1-29.
- Cloete, S., M. Snyman, and M. Herselman. 2000. Productive performance of Dorper sheep. Small Ruminant Research 36: 119-135.
- Clutton-Brock, J. 1999. A natural history of domesticated mammals. Cambridge University Press.
- DAFF. 2015. Department of Agriculture Forest and Fisheries-South Africa. A profile of the South African wool market value chain. <u>http://www.nda.agric.za/doaDev/sideMenu/Marketing/Annual%20Publications/Com</u> <u>modity%20Profiles/INDUSTRIAL%20PRODUCTS/WOOL%20MARKET%20VALUE</u> <u>%20CHAIN%20PROFILE%202015.pdf</u>.
- DAFF. 2016. National plan for conservation and sustainable use of farm animals genetic resources. Report submitted for the 2015/16 Strategenic Output of the Department of Agriculture, Forestry and Fisheries. South Africa. http://www.nda.agric.za/doaDev/sideMenu/geneticResources/docs/National%20Pla n.pdf.
- Dancause, K. N., M. G. Vilar, R. Steffy, and J. K. Lum. 2011. Characterizing genetic diversity of contemporary pacific chickens using mitochondrial DNA analyses. PloS one 6: e16843.
- Degen, A., and M. Kam. 1992. Body mass loss and body fluid shifts during dehydration in Dorper sheep. The Journal of Agricultural Science 119: 419-422.
- Devendra, C., and G. McLeroy. 1982. Goat and sheep production in the tropics. Intermediate tropical agriculture series. First Publ. Longman. London. New York. Singapore.
- Di Lorenzo, P., S. Ceccobelli, F. Panella, G. Attard, and E. Lasagna. 2015. The role of mitochondrial DNA to determine the origin of domestic chicken. World's Poultry Science Journal 71: 311-318.
- Diamond, J. 2002. Evolution, consequences and future of plant and animal domestication. Nature 418: 700.
- do Amaral Crispim, B., L. de Oliveira Seno, A. A. do Egito, F. M. de Vargas Junior, and A. B. Grisolia. 2014. Application of microsatellite markers for breeding and genetic

conservation of herds of Pantaneiro sheep. Electronic Journal of Biotechnology 17: 317-321.

- Dobney, K., and G. Larson. 2006. Genetics and animal domestication: new windows on an elusive process. Journal of Zoology 269: 261-271.
- Du Toit, D. 2008. The indigenous livestock of Southern Africa. http://www.damaras.com/newsletters/du-toit.pdf.
- Dudu, A., E. Ghiţă, M. Costache, and S. E. Georgescu. 2016. Origin and genetic diversity of Romanian Racka sheep using mitochondrial markers. Small Ruminant Research 144: 276-282.
- Earl, D. A. 2011. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation genetics resources 4: 359-361.
- El Nahas, S. M. et al. 2008. Analysis of genetic variation in different sheep breeds using microsatellites. African Journal of Biotechnology 7.
- Elbeltagy, A., A. Aboul-Naga, H. Hassen, B. Rischkowsky, and J. Mwacharo. 2015. Genetic diversity and structure in Egyptian indigenous sheep populations mirror patterns of anthropological interactions. Small Ruminant Research 132: 137-142.
- Ellegren, H. 2004. Microsatellites: simple sequences with complex evolution. Nature reviews genetics 5: 435-445.
- Epstein, H. 1971. The origin of the domestic animals of Africa. Africana Publishing Corporation.
- Erwee, S., and W. Young. 2008. The Zulu. E-print. <u>www.south-africa-homeschool-</u> <u>curriculum.com/zulu-people.html</u>.
- Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular ecology 14: 2611-2620.
- Excoffier, L., and H. E. Lischer. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular ecology resources 10: 564-567.
- Fan, B., Z.-Q. Du, D. M. Gorbach, and M. F. Rothschild. 2010. Development and application of high-density SNP arrays in genomic studies of domestic animals. Asian-Australasian Journal of Animal Sciences 23: 833-847.
- FAO. 2000. World Watch List for domestic animal diversity. Information Division, FAO, Rome.

- FAO. 2004. Secondary Guidelines for Development of National Farm Animal Genetic Resources Management Plans. Food and Agricultural Organization of United Nations (FAO), Roma, Italy.
- FAO. 2004b. Secondary Guidelines for Development of National Farm Animal Resources Management Plas. Measurement of Domestic Animal Diversity (MoDAD) Recommended Microsatellites Markers.
- FAO. 2005. Genetic characterization of livestock populations and its use in conservation decision making, by O. Hannotte & H. Jianlin. In J. Ruane & A. Sonnino, eds. The role of biotechnology in exploring and protecting agricultural genetic resources, pp. 89–96. Rome.
- FAO. 2006. Molecular markers, a tool for exploring genetic diversity The State of the World's Animal Genetic Resources for Food and Agriculture, first draft. Edited by Marsjan, PA & Oldenbroek, JK.Rome. p 319-337.
- FAO. 2007a. Global Plan of Action for Animal Genetic Resources and the Intertaken Declaration. Rome.
- FAO. 2007b. The state of the world's animal genetic resources for food and agriculture. edited by B. Rischkowsky. & D. Pilling. Rome
- FAO. 2009. The State of Food and Agriculture, Livestock in the Balance. Rome.
- FAO. 2010. Progress report of Food and Agriculture Organization of the United Nations (FAO) on selected activities related to agriculture biodiversity. Rome, Italy.
- FAO. 2011a. Molecular genetic characterization of animal genetic resources. FAO Animal Production and Health Guidelines. No. 9. Rome.
- FAO. 2011b. Status and trends of animal genetic resources 2010. Commission on Genetic Resources for Food and Agriculture, Thirteenth Regular Session. Rome.
- FAO. 2012a. Phenotypic characterization of animal genetic resources. Animal Production and Health Guidelines No. 11. Rome.
- FAO. 2012b. Cryoconservation of animal genetic resources. FAO Animal Production and Health Guidelines No. 12. Rome.
- FAO. 2013. In vivo conservation of animal genetic resources. FAO Animal Production and Health Guidelines. No. 14. Rome.
- FAO. 2015. The Second Report on the State of the World's Animal Genetic Resources for Food and Agriculture edited by Scherf, BD and Pilling, D. Rome.

- Felsenstein, J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington. Seattle. http://www.evolution.genetics.washington.edu/phylip/getme.html.
- Ferenčaković, M. et al. 2013. Estimates of autozygosity derived from runs of homozygosity: empirical evidence from selected cattle populations. Journal of Animal Breeding and Genetics 130: 286-293.
- Feulner, P. et al. 2004. Mitochondrial DNA and microsatellite analyses of the genetic status of the presumed subspecies Cervus elaphus montanus (Carpathian red deer). Heredity 93: 299.
- Fimland, E. 2007. Genetic diversity and sustainable management of animal genetic resources, globally. Animal Genetic Resources/Resources génétiques animales/Recursos genéticos animales 41: 45-52.
- Frankham, R., D. A. Briscoe, and J. D. Ballou. 2002. Introduction to conservation genetics. Cambridge university press.
- Furlan, E. et al. 2012. Small population size and extremely low levels of genetic diversity in island populations of the platypus, Ornithorhynchus anatinus. Ecology and evolution 2: 844-857.
- Galtier, N., B. Nabholz, S. Glémin, and G. Hurst. 2009. Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Molecular ecology 18: 4541-4550.
- Gandini, G. et al. 2004. Criteria to assess the degree of endangerment of livestock breeds in Europe. Livestock Production Science 91: 173-182.
- Gaouar, S. B. S. et al. 2015. Admixture and local breed marginalization threaten Algerian sheep diversity. PLoS One 10: e0122667.
- Gaouar, S. B. S., S. Kdidi, and L. Ouragh. 2016. Estimating population structure and genetic diversity of five Moroccan sheep breeds by microsatellite markers. Small Ruminant Research 144: 23-27.
- Ghafouri-Kesbi, F., M. Eskandarinasab, and A. Hassanabadi. 2008. Investigation of genetic variability and inbreeding characteristics in a population of Zandi sheep. Canadian journal of animal science 88: 409-417.
- Ghazy, A. et al. 2013. Genetic diversity and distances of three Egyptian local sheep breeds using microsatellite markers. Research in Zoology 3: 1-9.
- Ghernouti, N. et al. 2017. Control Region of mtDNA identifies three migration events of sheep breeds in Algeria. Small Ruminant Research 155: 66-71.

- Gissi, C., F. Iannelli, and G. Pesole. 2008. Evolution of the mitochondrial genome of Metazoa as exemplified by comparison of congeneric species. Heredity 101: 301-320.
- Gornas, N., C. Weimann, A. El Hussien, and G. Erhardt. 2011. Genetic characterization of local Sudanese sheep breeds using DNA markers. Small Ruminant Research 95: 27-33.
- Groeneveld, L. et al. 2010. Genetic diversity in farm animals–a review. Animal Genetics 41: 6-31.
- Hanotte, O., and H. Jianlin. 2005. Genetic characterization of livestock populations and its use in conservation decision-making. The Role of Biotechnology in Exploring and Protecting Agricultural Genetic Resources. Food and Agriculture Organization of the United Nations, Rome: 89-96.
- Hayashi, K., N. Hashimoto, M. Daigen, and I. Ashikawa. 2004. Development of PCR-based SNP markers for rice blast resistance genes at the Piz locus. Theoretical and Applied Genetics 108: 1212-1220.
- Hendry, A. 2013. Key questions in the genetics and genomics of eco-evolutionary dynamics. Heredity 111: 456.
- Hiendleder, S., K. Mainz, Y. Plante, and H. Lewalski. 1998. Analysis of mitochondrial DNA indicates that domestic sheep are derived from two different ancestral maternal sources: no evidence for contributions from urial and argali sheep. Journal of Heredity 89: 113-120.
- Hlophe, S. 2011. Genetic variation between and within six selected South African sheeop breeds using radom amplified polymorphism DNA and protein markers. MSc. Thesis, University of Zululand. http://uzspace.ac.za/handle/10530/611
- Horsburgh, K. A. 2008. Wild or domesticated? An ancient DNA approach to canid species identification in South Africa's Western Cape Province. Journal of Archaeological Science 35: 1474-1480.
- Horsburgh, K. A., and A. Rhines. 2010. Genetic characterization of an archaeological sheep assemblage from South Africa's Western Cape. Journal of Archaeological Science 37: 2906-2910.
- Hussain, T. et al. 2017. Mitochondrial DNA D-Loop diversity and evolutionary relationship of wild Punjab Urial sheep (Ovis vignei punjabiensis) with closely related taxa. Small Ruminant Research 148: 22-32.

- Jombart, T. 2008. Adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24: 1403-1405.
- Kalinowski, S. T. 2004. Counting alleles with rarefaction: private alleles and hierarchical sampling designs. Conservation genetics 5: 539-543.
- Kalinowski, S. T. 2005. hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. Molecular Ecology Resources 5: 187-189.
- Kdidi, S. et al. 2015. Genetic relationship and admixture in four Tunisian sheep breeds revealed by microsatellite markers. Small Ruminant Research 131: 64-69.
- Kemp, S., Y. Mamo, B. Asrat, and T. Dessie. 2007. Domestic Animal Genetic Resources Information System. Addis Ababa, Ethiopia. International Livestock Research Institute.
- Kevorkian, S. E. M. et al. 2010. Genetic diversity using microsatellite markers in four Romanian autochthonous sheep breeds. Rom Biotech Lett 15: 5059-5065.
- Kijas, J. W. et al. 2012. Genome-wide analysis of the world's sheep breeds reveals high levels of historic mixture and strong recent selection. PLoS Biol 10: e1001258.
- Kijas, J. W. et al. 2009. A genome wide survey of SNP variation reveals the genetic structure of sheep breeds. PloS one 4: e4668.
- Köhler-Rollefson, I. 2003. Community-based management of animal genetic resources–with special reference to pastoralists. Community-based management of animal genetic resources: 13-26.
- Kumar, P., V. K. Gupta, A. K. Misra, D. R. Modi, and B. K. Pandey. 2009. Potential of Molecular Markers in Plant Biotechnology. Plant Omics Journal 2: 141-162.
- Kunene, N., C. Bezuidenhout, and I. Nsahlai. 2009. Genetic and phenotypic diversity in Zulu sheep populations: Implications for exploitation and conservation. Small Ruminant Research 84: 100-107.
- Kunene, N., and A. Fossey. 2006. A survey on livestock production in some traditional areas of Northern Kwazulu Natal in South Africa. Livestock Research for Rural Development 18.
- Kunene, N., E. Nesamvuni, and A. Fossey. 2007. Charaterisation of Zulu (Nguni) sheep using linear body measurements and some environmental factors affecting these measurements. South African Journal of Animal Science 37: 11-20.
- Kunene, N. W., C. C. Bezuidenhout, I. V. Nsahlai, and E. A. Nesamvuni. 2011. A review of some characteristics, socio-economic aspects and utilization of Zulu sheep: implications for conservation. Tropical animal health and production 43: 1075-1079.

- Kunene, N. W. et al. 2014. Genetic diversity in four populations of Nguni (Zulu) sheep assessed by microsatellite analysis. Italian Journal of Animal Science 13.
- Lancioni, H. et al. 2013. Phylogenetic relationships of three Italian Merino-derived sheep breeds evaluated through a complete mitogenome analysis. PloS one 8: e73712.
- Last, L. 2013. The assessment of genetic diversity in agricultural production systems. Doctoral thesis. ETH Zurich. https://www.research-<u>collection.ethz.ch/handle/20.500.11850/76416</u>.
- Lewis, P., and D. Zaykin. 1999. Genetic data analysis. Computer program for the analysis of allelic data. Version 1.0. Lewis Labs, Univ. of Connecticut, Storrs, CT.
- Librado, P., and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452.
- Ligda, C., and M. Zjalic. 2011. Conservation of animal genetic resources in Europe: overview of the policies, activities, funding and expected benefits of conservation activities. Animal Genetic Resources/Ressources génétiques animales/Recursos genéticos animales 49: 75-86.
- Ling, Y. et al. 2010. Identification of Y chromosome genetic variations in Chinese indigenous horse breeds. Journal of Heredity 101: 639-643.
- Liu, J. et al. 2016. Genetic Diversity and Phylogenetic Evolution of Tibetan Sheep Based on mtDNA D-Loop Sequences. PloS one 11: e0159308.
- Liu, J. et al. 2013. Analysis of copy number variations in the sheep genome using 50K SNP BeadChip array. BMC genomics 14: 1.
- Liu, Z., and J. Cordes. 2004. DNA marker technologies and their applications in aquaculture genetics. Aquaculture 238: 1-37.
- Loukovitis, D. et al. 2016. Genetic diversity of Greek sheep breeds and transhumant populations utilizing microsatellite markers. Small Ruminant Research 136: 238-242.
- Mäki-Tanila, A., and S. Hiemstra. 2010. Regional issues on animal genetic resources: trends, policies and networking in Europe. Animal Genetic Resources/Ressources génétiques animales/Recursos genéticos animales 47: 125-136.
- Marsjan, P., and J. Oldenbroek. 2006. Molecular markers, a tool for exploring genetic diversity. The State of the World's Animal Genetic Resources for Food and Agriculture, first draft, Rome. p 319-337. FAO.
- Mavule, B., V. Muchenje, C. Bezuidenhout, and N. Kunene. 2013a. Morphological structure of Zulu sheep based on principal component analysis of body measurements. Small Ruminant Research 111: 23-30.

- Mavule, B., V. Muchenje, and N. Kunene. 2013. Characterization of Zulu sheep production system: Implications for conservation and improvement. Scientific research and essays 8: 1226-1238.
- Mavule, B. S. 2012. Phenotypic characterization of Zulu sheep: implications for conservation and improvement, University of Zululand.
- Mavule, B. S., F. M. Sarti, E. Lasagna, and N. W. Kunene. 2016. Morphological differentiation amongst Zulu sheep populations in KwaZulu-Natal, South Africa, as revealed by multivariate analysis. Small Ruminant Research.
- Mburu, D., and O. Hanotte. 2005. A practical approach to microsatellite genotyping with special reference to livestock population genetics. ILRI, Nairobi, Kenya.
- McFarlane, K., G. A. Wilson, J. S. Nishi, and N. Territories. 2006. Management Strategies for Conservation of Genetic Diversity in Wood Bison (Bison Bison Athabascae). Northwest Territories, Resources, Wildlife & Economic Development.
- Meadows, J. et al. 2006. Globally dispersed Y chromosomal haplotypes in wild and domestic sheep. Animal genetics 37: 444-453.
- Meadows, J., S. Hiendleder, and J. Kijas. 2011a. Haplogroup relationships between domestic and wild sheep resolved using a mitogenome panel. Heredity 106: 700-706.
- Meadows, J. R., I. Cemal, O. Karaca, E. Gootwine, and J. W. Kijas. 2007. Five ovine mitochondrial lineages identified from sheep breeds of the near East. Genetics 175: 1371-1379.
- Meadows, J. R., S. Hiendleder, and J. W. Kijas. 2011b. Haplogroup relationships between domestic and wild sheep resolved using a mitogenome panel. Heredity 106: 700-706.
- Miah, G. et al. 2013. A review of microsatellite markers and their applications in rice breeding programs to improve blast disease resistance. International journal of molecular sciences 14: 22499-22528.
- Miller, J. M., J. Poissant, J. W. Kijas, and D. Coltman. 2011. A genome-wide set of SNPs detects population substructure and long range linkage disequilibrium in wild sheep. Molecular Ecology Resources 11: 314-322.
- Muigai, A., A. Okeyo, A. Kwallah, D. Mburu, and O. Hanotte. 2009. Characterization of sheep populations of Kenya using microsatellite markers: implications for conservation and management of indigenous sheep populations. South African Journal of Animal Science 39: 93-96.
- Muigai, A. W., and O. Hanotte. 2013. The origin of African sheep: archaeological and genetic perspectives. African Archaeological Review 30: 39-50.

- Mukhongo, D., O. Mwai, M. Tapio, and A. Muigai. 2014. Genetic diversity and population structure of the indigenous sheep in Kenya based on microsatellite analysis: implications for their conservation. Journal of Livestock Science 5: 65-78.
- Naderi, S. et al. 2007. Large-scale mitochondrial DNA analysis of the domestic goat reveals six haplogroups with high diversity. PLoS One 2: e1012.
- Nanekarani, S., C. Amirinia, and N. Amirmozafari. 2011. Genetic analysis of Karakul sheep breed using microsatellite markers. African Journal of Microbiology Research 5: 703-707.
- Naqvi, A. et al. 2017. Assessment of genetic diversity and structure of major sheep breeds from Pakistan. Small Ruminant Research 148: 72-79.
- NDA. 2004. National Livestock industry strategy and implementation frame work 2004. National Department of Agriculture. South Africa. http:/<u>www.nda.agric.za/docs/Trends2003/Animal_production.pdf</u>.
- Niemi, M. et al. 2013. Mitochondrial DNA and Y-chromosomal diversity in ancient populations of domestic sheep (Ovis aries) in Finland: comparison with contemporary sheep breeds. Genetics Selection Evolution 45: 1.
- Notter, D. R. 1999. The importance of genetic diversity in livestock populations of the future. Journal of animal science 77: 61-69.
- Othman, O. E., L. Pariset, E. A. Balabel, and M. Marioti. 2015. Genetic characterization of Egyptian and Italian sheep breeds using mitochondrial DNA. Journal of Genetic Engineering and Biotechnology 13: 79-86.
- Othman, O. E. M. et al. 2016. Sheep diversity of five Egyptian breeds: Genetic proximity revealed between desert breeds: Local sheep breeds diversity in Egypt. Small Ruminant Research 144: 346-352.
- Page, R. 1996. TreeView: An application to display phylogenetic trees on personal computers. Comput Appl Biosci. 12: 357–358.
- Parés Casanova, P.-M., E. S. Mwaanga, M. Caballero, J. Sabaté, and S. Valenzuela. 2013.
 Biometrical multivariate study of the Zambian indigenous Fat-tailed sheep.
 International Journal of Livestock Production, 2013, vol. 4, num. 9, p. 148-154.
- Pariset, L. et al. 2006. Characterization of 37 breed-specific single-nucleotide polymorphisms in sheep. Journal of Heredity 97: 531-534.
- Pariset, L. et al. 2011. Genetic diversity of sheep breeds from Albania, Greece, and Italy assessed by mitochondrial DNA and nuclear polymorphisms (SNPs). The Scientific World Journal 11: 1641-1659.
- Park, S. D. E. 2001. Trypanotolerance in West African cattle and the population genetic effects of selection. Ph. D. thesis, University of Dublin.
- Peery, M. Z. et al. 2012. Reliability of genetic bottleneck tests for detecting recent population declines. Molecular Ecology 21: 3403-3418.
- Pereira, F. et al. 2006. Genetic signatures of a Mediterranean influence in Iberian Peninsula sheep husbandry. Molecular Biology and Evolution 23: 1420-1426.
- Pérez, T., S. E. Hammer, J. Albornoz, and A. Domínguez. 2011. Y-chromosome phylogeny in the evolutionary net of chamois (genus Rupicapra). BMC evolutionary biology 11:
 1.
- Pilling, D., and I. Hoffmann. 2011. Climate change and animal genetic resources for food and agriculture: state of knowledge, risks and opportunities. Background study paper.
- Pleurdeau, D. et al. 2012. "Of sheep and men": earliest direct evidence of caprine domestication in southern Africa at Leopard Cave (Erongo, Namibia). PloS one 7: e40340.
- Plug, I., and S. Badenhorst. 2001. The distribution of macromammals in southern Africa over the past 30 000 years. Transvaal Museum Monograph 12: 1-234.
- Pollott, G., and R. T. Wilson. 2009. Sheep and goats for diverse products and profits. FAO Diversification Booklet.
- Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945-959.
- Qwabe, S. O., E. van Marle-Köster, and C. Visser. 2012. Genetic diversity and population structure of the endangered Namaqua Afrikaner sheep. Tropical animal health and production 45: 511-516.
- Ramsay, K., L. Harris, and A. Kotzé. 2000. Landrace breeds: South Africa's indigenous and locally developed farm animals. Farm Animal Conservation Trust, Pretoria, pp. 38-39.
- Rao, K. A., K. Bhat, and S. Totey. 1996. Detection of species-specific genetic markers in farm animals through random amplified polymorphic DNA (RAPD). Genetic analysis: biomolecular engineering 13: 135-138.
- Raymond, M., and F. Rousset. 1995. An exact test for population differentiation. Evolution 49: 1280-1283.
- Resende, A., J. Gonçalves, A. W. Muigai, and F. Pereira. 2016. Mitochondrial DNA variation of domestic sheep (Ovis aries) in Kenya. Animal genetics.

- Reynolds, J., B. S. Weir, and C. C. Cockerham. 1983. Estimation of the coancestry coefficient: basis for a short-term genetic distance. Genetics 105: 767-779.
- Robbins, L. et al. 2005. The Advent of Herding in Southern Africa: Early AMS Dates on Domestic Livestock from the Kalahari Desert 1. Current Anthropology 46: 671-677.
- Rosenberg, N. A. 2004. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Resources 4: 137-138.
- Rust, J., and T. Rust. 2013. Climate change and livestock production: A review with emphasis on Africa. South African Journal of Animal Science 43: 256-267.
- Ruto, E., G. Garrod, and R. Scarpa. 2008. Valuing animal genetic resources: a choice modeling application to indigenous cattle in Kenya. Agricultural Economics 38: 89-98.
- Sadr, K. 2015. Livestock first reached southern Africa in two separate events. PloS one 10: e0134215.
- Sassi-Zaidy, Y. B. et al. 2014. Genetic structure and variability within and among populations of the fat-tailed Barbarine sheep breed using microsatellites markers. African Journal of Biotechnology 13: 44-54.
- Sharma, P. C., A. Grover, and G. Kahl. 2007. Mining microsatellites in eukaryotic genomes. Trends in biotechnology 25: 490-498.
- Sharma, R. et al. 2016. Genetic diversity estimates point to immediate efforts for conserving the endangered Tibetan sheep of India. Meta gene 8: 14-20.
- Sheikhlou, M., and M. Abbasi. 2016. Genetic diversity of Iranian Lori-Bakhtiari sheep assessed by pedigree analysis. Small Ruminant Research 141: 99-105.

Simpson, M. C. 2012. Microsatellite analysis of Ceratocystis fimbriata, University of Pretoria.

- Snyman, M. A., E. van MarleKöster, S. O. Qwabe, and C. Visser. 2013. GENETIC AND PHENOTYPIC PROFILE OF THREE SOUTH AFRICAN NAMAQUA AFRIKANER SHEEP FLOCKS. 13.
- Soma, P., A. Kotze, J. Grobler, and J. Van Wyk. 2012. South African sheep breeds: Population genetic structure and conservation implications. Small Ruminant Research 103: 112-119.
- Steinfeld, H. et al. 2006. Livestock's long shadow: environmental issues and options. Food and Agriculture Organization of the United Nations (FAO).
- Taberlet, P., E. Coissac, J. Pansu, and F. Pompanon. 2011. Conservation genetics of cattle, sheep, and goats. Comptes rendus biologies 334: 247-254.

- Taberlet, P. et al. 2008. Are cattle, sheep, and goats endangered species? Molecular Ecology 17: 275-284.
- Tapio, M. 2006. Origin and maintenance of genetic diversity in Northern European sheep. University of Oulu Oulu.
- Tapio, M. et al. 2010. Microsatellite-based genetic diversity and population structure of domestic sheep in northern Eurasia. BMC genetics 11: 76.
- Team, R. C. 2013. R: A language and environment for statistical computing.
- Tibbo, M. 2006. Productivity and health of indigenous sheep breeds and crossbreds in the central Ethiopian highlands.
- Tolone, M., S. Mastrangelo, A. Rosa, and B. Portolano. 2012. Genetic diversity and population structure of Sicilian sheep breeds using microsatellite markers. Small Ruminant Research 102: 18-25.
- Toro, M., J. Fernández, I. Shaat, and A. Mäki-Tanila. 2011. Assessing the genetic diversity in small farm animal populations. animal 5: 1669-1683.
- Toro, M. A., J. Fernández, and A. Caballero. 2009. Molecular characterization of breeds and its use in conservation. Livestock Science 120: 174-195.
- Wang, X., Y. Ma, H. Chen, and W. Guan. 2007. Genetic and phylogenetic studies of Chinese native sheep breeds (Ovis aries) based on mtDNA D-loop sequences. Small Ruminant Research 72: 232-236.
- Wang, Y. et al. 2015. Y chromosomal haplotype characteristics of domestic sheep (Ovis aries) in China. Gene 565: 242-245.
- Wei, C. et al. 2015. Genome-wide analysis reveals population structure and selection in Chinese indigenous sheep breeds. Bmc Genomics 16: 194.
- Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. evolution 38: 1358-1370.
- Wood, N., and S. Phua. 1996. Variation in the control region sequence of the sheep mitochondrial genome. Animal Genetics 27: 25-33.
- Wright, D. K. 2014. East and Southern African Neolithic: geography and overview. Encyclopedia of global archaeology: 2281-2298.
- Yüncü, E. et al. 2013. Comparative study of three simple molecular approaches in search of mtDNA haplogroup identification of domestic sheep. Small Ruminant Research 114: 64-71.

- Zeder, M. A. 2008. Domestication and early agriculture in the Mediterranean Basin: Origins, diffusion, and impact. Proceedings of the national Academy of Sciences 105: 11597-11604.
- Zhang, M. et al. 2014. Y chromosome haplotype diversity of domestic sheep (Ovis aries) in northern Eurasia. Animal genetics 45: 903-907.

Appendices

Appendix 1: Images of Zulu sheep



Population /breed ‡	Phenotypic description	Geographic localization	Adaptive traits	Management practices
JO	Fat-tailed hair sheep. Coat colour: dark-brown and white, dark-brown, black and white. Horn absent.	latitude: 27° 42' 94"S longitude: 32° 06' 57"E	Hot and wind (adapted to long walks)	Extensive sheep production system
MT	Long fat-tailed Hair sheep. Coat colour: dark-brown, brown, fawn, dark-brown and white, brown and white, fawn and white, black and white. Horn absent.	latitude: 28° 40' 59''S longitude: 32° 21' 43''E	Hot and cold (adapted to long walks)	Extensive sheep production system
NG	Long fat-tailed hair sheep. Coat colour: dark-brown, brown, fawn, dark-brown and white, brown and white, fawn and white, black and white. Horn absent.	latitude: 27° 89' 43''S longitude: 31° 64' 54''E	Hot and cold (adapted to long walks)	Extensive and semi-intensive sheep production system
ES	Long fat-tailed hair sheep. Coat colour: dark-brown, brown, fawn. Horn absent.	latitude: 28° 89' 47''S longitude: 31° 46' 28'' E	Hot and cold (adapted to long walks)	Extensive sheep production system
UL	Long fat-tailed hair sheep. Coat colour: dark-brown, fawn, dark-brown and white, brown and white, black and white. Horn absent.	latitude: 28° 29' 97''S longitude: 31° 43' 42''E	Hot and cold (adapted to long walks)	Extensive sheep production system
NQ	Long fat-tailed hair sheep. Coat colour: dark-brown, fawn, dark-brown and white, brown and white, black, black and white. Horn absent.	latitude: 28° 30' 08''S longitude: 30° 80' 39''E	Hot and cold (adapted to long walks)	Extensive sheep production system
UZ	Long fat-tailed and thin tailed hair sheep. Coat colour: dark-brown, brown, fawn, dark-brown and white, brown and white, black, black and white. Horn absent.	Pilot farm latitude: 28° 85' 24''S longitude: 31° 84' 91''E	Hot and cold	Semi-intensive sheep production system

Appendix 2: Details of 11 studied sheep populations

MS	Long fat-tailed and thin- tailed hair sheep Coat colour: dark-brown, brown, fawn, dark-brown and white, brown and white, black, black and white. Horn absent.	Pilot farm latitude: 27° 39' 53''S longitude: 32° 17' 64''E	Hot and cold	Semi-intensive sheep production farming
DO	Short tailed sheep. Coat colour: black and white Horn absent.	latitude: 29° 80' 0''S longitude: 30° 65' 0''E	Arid to semi- arid conditions	Semi-intensive sheep production system
DA	Long fat tailed hair sheep. Coat colour: black and white, brown and white. Horn present.	latitude: 25° 16' 74''S longitude: 29° 39' 87''E	Arid, semi- desert areas	Semi-intensive sheep production system
ME	Wool sheep. Coat colour: white Horn absent.	Site 1: Pietermaritzburg, latitude: 29° 60' 06"S longitude: 30° 37' 94"E Site 2: Isipingo, latitude: 29° 98' 25" S longitude:30° 92' 17"E	Semi-arid areas up to high rainfall regions	Semi-intensive sheep production system

* Data on the census of the Zulu sheep is unavailable.

JO, Jozini; MT, Mtubatuba; NG, Nongoma; ES, Eshowe; UL, Ulundi; NQ, Nquthu; UZ, UniZulu research station; MS, Makhathini research station; DO, Dorper; DA, Damara; ME, South African Merino.

Appendix 3: Microsatellites PCR conditions according to Kunene et al. (2014)

An initial denaturation step of 5 min at 94°C, 35 cycles of 30 secs at 95°C, 45 secs at the annealing temperature of each multiplex PCR, 30 secs at 72°C and a final extension of 15 min at 72°C. The reaction volume of 11.95 μ l contained 25 ng of genomic DNA, 2.5mM MgCl2, 1 μ l of 10X PCR buffer, 0.5U Hot start Taq (Sigma–Aldrich, St. Louis, MO, USA), 200 μ M dNTPs

and 0.2 pmol of each primer.