MONITORING THE IMPACT OF TRANSLOCATION ON THE GENETIC DIVERSITY AND FITNESS OF THE CAPE MOUNTAIN ZEBRA (*EQUUS ZEBRA ZEBRA*)

by

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in Genetics at the University of the Free State

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DECLARATION

I, Rae Marvin Smith, hereby declare that this thesis, submitted to the University of the Free State as per the requirements for the degree, Doctor of Philosophy in Genetics is my own original work and has not been previously been submitted to any other University for a degree, in its entirety or in part.

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Rae Marvin Smith

DEDICATION

For Monique and Zoë

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“Discipline is the bridge between goals and accomplishment” – Jim Rohn

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SUMMARY

Historic efforts to restore the Cape Mountain zebra (*Equus zebra zebra*, CMZ) population from near extinction was implemented through initiatives by government conservation agencies. While the animals have recovered in number, concerns over the genetic diversity of this sub-species have been raised. Efforts to preserve genetic diversity and mitigate risks to population decline have been outlined in a Biodiversity Management Plan (BMP) gazetted in 2018. This study addressed various objectives of the BMP. Specifically, the aim of this study was to monitor the impact that translocations have on the genetic diversity, fitness and long-term survival of the Cape mountain zebra. The study was carried out in four parts: First, the temporal change in genetic diversity was investigated over the last decade using 14 microsatellite markers. This was done to determine how past translocations conservation management has affected the genetic diversity of key populations. Second, single nucleotide polymorphism (SNP) variation in Toll-like receptor 9 was investigated to evaluate genetic diversity at adaptive sites and how they relate to protein function. Thirdly, the prevalence of two protozoa attributed to equine piroplasmosis was surveyed using real-time PCR to determine the risks associated with translocation. Lastly, the Cation Sperm Associated (CATSPER) gene 1-3 was screened to identify SNPs that may be associated with reproductive fitness in CMZ. Temporal declines in measures of genetic diversity such as allelic richness, observed heterozygosity and well as a low estimated effective population size (N_e) were identified in MZNP. Notable shifts in genetic structure of DHNR were also observed. Loss of genetic variation in the TLR 9 gene was observed in DHNR and a private reserve in Grahamstown when compared to the source population, MZNP. When investigating the structural implications caused by the genetic mutations in the gene, mutations were determined to not affect active binding regions of the protein structure. All animals tested in three reserves were infected with the equine piroplasm, *Theileria equi*. Translocations and founding new populations pose a risk to domestic animals in adjacent farms and could potentially introduce new genotypes in these areas. Four SNPs were identified at intronic regions of CATSPER 1 while no SNPs were found in exons screened in CATSPER genes 2 and 3 for the CMZ. This study played a key role in informing conservation actions to be implemented through the BMP. The aim of future analyses will be to incrementally sample each generation for better estimations of effective population size as a measure of genetic health. Collaborative efforts with conservation agencies will enable monitoring and controlled mixing of relict populations to assess which populations would best enable the preservation of the maximum number of alleles.

Keywords: Cape mountain zebra, genetic diversity, translocation, equine piroplasms, Conservation, microsatellites, Single nucleotide polymorphisms

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LIST OF ABBREVIATIONS

Akap4	A-kinase anchoring protein 4
AMOVA	Analysis of Molecular Variance
ARC	The Agricultural Research Council
BMP	Biodiversity Management Plan
Ca^{2+}	Calcuim ion
CATSPER	Cation Sperm Associtated Genes
CI-ELISA	Competitive-inhibition ELISA
CITES	Convention of International Trade in Endagered Species
CMZ	Cape mountain zebra
CoP17	17th Conference of the Parties
CpG	Cytosine-phosphate-guanine
DAFF	Department of Agriculture, Forestry and Fisheries
DHNR	De Hoop Nature Reserve
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyzme-linked immunosorbant assay
EP	Equine Piroplasmosis
GHT	Private reserve in Grahamstown
GNR	Gamkaberg Nature Reserve
IFA	indirect fluorescent antibody assay
IUCN	International Union for the Conservation of Nature
KNP	Karoo National Park
KNR	Kammanassie Nature Reserve
LRR	Leucine-rich repeat
LRRCT	C-terminal LRR
LRRNT	N-terminal LRR
MZNP	Mountain Zebra National Park
NRF	National Research Foundation
NZG	National Zoological Garden of South Africa
OVR	Onderstepoort Veterinary Research
PBMCs	Peripheral blood mononuclear cells
PCA	Principle component analysis
PCR	Polymerase Chain Reaction

qPCR	Quantitative PCR also referred to as real-time PCR
QRICH2	Glutamine-rich protien 2
RAD	Restiction site-associated DNA
RADseq	RAD sequencing
RESC	Research Ethics and Scientific Committee
RNA	Ribonucleic acid
rt-PCR	see qPCR
SANBI	South African National Biodiversity institute
SanParks	South African National Parks
SMM	Step-wise mutation model
SNP(s)	Single Nucleotide Polymorphism
ssDNA	single stranded DNA
STR	Short tandem repeats (Microsatellites)
TIR	Toll/interleukin 1 recetor domain
TLR	Toll Like Receptor
TPM	Two-Phase mutation model

LIST OF SYMBOLS

a	alpha value
A	Adenine
A	Average Allele Number
A_r	Allelic richness
bp	Base pair (Genetics)
C	Cytosine
CI	Confidence interval
C_q	Quantification cycle
d^2	Difference in length of Mircrosatellite alelles
F	Fixation index
F_{BR}	Breeding females
F_c	Nei and Tajima (1981) model for mesuring N_e
F_{IS}	Inbreeding coeficient
F_{IT}	Overall inbreeding coeficient
F_s	Jorde and Ryman (2007) model for mesuring N_e
F_{st}	proporiton of ttal differentiation due to differences among populations
G	Guanine
GI	Generation interval
H_e	Expected Heterozygosity
H_o	Observed Heterozygosity
K	Mean for accounting for non-poisson distrubutions in Estimatinng population size
K	Number of populations
K_F	Annual number of young born to females
K_M	Annual number of young sired by males
L_F	Generation length for females
l_F	Probablity of female newborns surviving to reproductive age
L_M	Generation length for males
l_M	Probablity of male newborns surviving to reproductive age
$\ln(Pr(X\|K)$	estimated log-likelihood (Structure Harvester)
M_{BR}	Breeding Males

n	The Sample size
N	The total number of individuals in a population
N_e	Effective populations size
N_{eV}	Variance of effective populaiton size
p	Frequency of allele A
p	probability of an event
p	the proportion of population infected (In measuring CI)
P_{CRIT}	The criterion for excluding rare alleles
q	1-p (when measuing CI)
q	Frequency of allele a
qi	coeficient of membership
SE	Standard error
T	Thymine
V_K	Variance of the total number of offspring produced by an individual female
Z	The Z-index
ΔK	Posterior Propability of K
µl	Micro litre

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- Took a leading role in data and statistical analysis
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- Took a leading role in reviewing the final manuscript

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CHAPTER 1

Introduction

Within the last few decades, various components of overall biodiversity, from individual populations to ecosystems, have succumbed to massive reductions in genetic diversity (Hughes et al., 2008). The threat of climate change is projected to have significant effects on food webs, species distributions, disease distributions, biodiversity and ecosystems (Convey and Smith, 2006; Grosbois et al., 2008; Keith et al., 2008; Parmesan, 2006). Additional anthropogenic factors such as habitat fragmentation and isolation, poaching and the spread of disease is also affecting species' capabilities to survive, leading to the loss of genetic diversity, increase in inbreeding and reduced fertility (Arif et al., 2010; Hrabar et al., 2016; Moodley and Harley, 2005; Whiteman et al., 2006).

The Cape mountain zebra (*Equus zebra zebra*, CMZ) is presently listed as vulnerable by the IUCN (Novellie, 2008). The subspecies is however recommended to be downlisted to near threatened on condition that strict conservation actions be taken to ensure genetic diversity (Hrabar et al., 2016). The CMZ have also been down-listed from Appendix I to Appendix II as adopted through the 17th Conference of the Parties (CoP17) (CITES, 2016; Lombard, 2016) through the Convention of International Trade in Endangered Species (CITES). The CMZ is a sub-species of mountain zebra and has previously undergone a genetic bottleneck event involving the entire species. The sub-species was historically distributed in the south-western part of South Africa (Boshoff et al., 2015; Novellie et al., 2002). In the 1930s, CMZ numbers declined to less than 50 individuals (Hrabar and Kerley, 2013). The CMZ numbers have since recovered to over 4800 individuals, with 70% of the population now found in protected reserves (Hrabar and Kerley, 2015). The recovery of the CMZ is attributed to the work done through conservation agencies and government action. A biodiversity management plan that was recently gazetted contains clear objectives through which conservation agencies are to mitigate risks that impede on the long term survival of the sub-species. Moreover, these objectives aim to

further propagate the CMZ within its historical distribution range by founding new populations in unpopulated reserves (Birss et al., 2018).

The overall aim of this study is to determine the level of genetic diversity, risks of inbreeding, genetic structure, disease susceptibility and reproductive fitness of CMZ sub-populations. Recommendations will then be made based on the resulting data that will contribute to the implementation of a meta-population management strategy to ensure ecologically sustainable and genetically diverse meta-populations of CMZ.

1.1 The key objectives of this study were to:

- Investigate the current level of genetic diversity, possible inbreeding and the genetic structure of CMZ populations. The data obtained from recent samples will be compared to previous studies to determine if any temporal change in genetic diversity took place between the two periods.
- Investigate disease susceptibility as well as the prevalence and severity of key diseases in CMZ populations, as well as determining how translocations affect these factors.
- Assess the reproductive fitness of the CMZ by screening genes associated with normal sperm function for Single Nucleotide Polymorphisms. This will serve as a pilot study to enable improved management strategies.

1.2 The layout of the thesis:

Chapter 2 – This chapter provides an overview of the available literature surrounding Conservation as a tool for managing species that are at risk of extinction or loss of genetic diversity. Furthermore, a comprehensive history of the CMZ and the challenges that face the sub-species which could result in the loss of genetic diversity is presented. Also, concepts that relate to subsequent chapters are explained, highlighting current advancements and identifying aspects where knowledge is still lacking.

Chapter 3 - This chapter addresses point one in the outlined aims of this thesis. The Genetic diversity and structure of the CMZ are thus assessed for recent samples that were obtained from three reserves, two of which are the relict populations: Mountain Zebra National Park and Kammanassie Nature Reserve.

The Third Reserve is De Hoop Nature reserve, which was founded by animals from the two aforementioned relict populations. The chapter also uses molecular markers to estimate effective population size and makes use of appropriate statistical approaches to detect recent bottlenecks in populations.

Chapter 4 - Genetic diversity was historically deemed sufficient to base conservation management strategies on. However, in this chapter, we highlight that estimates of genetic diversity using markers influence CMZ survival through the resistance to disease. This will provide a tool for understanding the risks that CMZ will face as emerging diseases spread to regions where CMZ are located.

Chapter 5 - In this chapter, the prevalence of the two equine piroplasms - *Theileria equi* and *Babesia caballi* - in the blood of CMZ is assessed. The animals, from which samples were collected, were on-route to be translocated to various other reserves. Thus, inferences were made on how the presence of the piroplasms in the zebra blood will likely affect the spread of disease when founding new populations of CMZ, as part of future management strategies.

Chapter 6 - The CMZ has persisted in isolated populations for an extended period of time. This chapter investigates the possible ramifications of this on the genetic diversity found in genes associated with reproductive fitness. In this study, translocated males from the De Hoop Nature Reserve, as part a founding population, were investigated for genetic differences, such as single nucleotide polymorphisms.

Chapter 7 - In this chapter, all the research chapters are incorporated to determine how these various aspects of genetic diversity and fitness may influence the prospects for CMZs' long-term survivability as well as investigating how management strategies can alter the current survival trajectory for this species.

The remaining sections include appendices and supplementary material that support the findings reported in this thesis.

CHAPTER 2

Literature Study

2.1 Conservation Genetics

Conservation genetics is an interdisciplinary subfield of population genetics. The core objectives are that of understanding adaptation, genetic fitness and genetic characteristics of individuals as well as populations. Additionally, the field focuses on restoration of natural populations, determining population structure, measuring the effects of habitat loss, habitat fragmentation and isolation (Allendorf et al., 2013). For most species, adequate information on which to base decisions that lead to interventions and conservation actions are lacking, with data generally being collected opportunistically on the basis of competing interests and political climate (Pressey and Bottrill, 2008). However, increased effort are being made to ensure the best information is available to aid the conservation of nature (Ralls et al., 2018)

2.1.1 The role of conservation genetics in the wider field of conservation

The International Union for the Conservation of Nature (IUCN); which is the main international body for conservation, recognises the need for conservation at the levels of genetic, species and ecosystem diversity. The crucial role of genetic diversity in maintaining species-level diversity has been discussed by Frankham et al. (2005) and McNeely et al. (1990). Ralls et al. (2018) further highlighted the fact that species-specific data must be acquired for interventions using conservation genetics to be effective. Conservation efforts are prioritised for species that are listed as endangered, threatened and vulnerable, as set out by the IUCN. This allows for the concentration of resources and effort towards species that are most at risk of extinction.

Conservation of genetic variation has been shown to be of substantial importance in the conservation of biodiversity for mammals (Lacy, 1997), plants (Ajmone Marsan et al., 1998; Liu et al., 2003) and fish (Buchholz-Sørensen and Vella, 2016; Waples, 2005). Today, conservation strategies incorporating genetic management is regarded as necessary but is still a largely underused tool (Ralls et

al., 2018). In countries such as United States, Australia as well as in European countries, as few as 7% of conservation plans mention inbreeding as an important factor, with even fewer considering active genetic management strategies (Pierson et al., 2016). In terms of success stories, the genetic rescue of the Florida panther (*Puma concolor coryi*), where the near-extinct panthers were hybridized with a related species in order to reinforce the declining population, thereby leading to a sustainable population of panthers in the region (Pimm et al., 2006). Similarly, the immigration of a related species of the wolf (*Canis lupus*) into a small population in Scandinavia, lead to the rapid proliferation of the species, that was initially on the decline (Ingvarsson, 2002; Vila et al., 2003). The basis of success in conservation genetics is the ability to develop a model which accurately represents genetic systems in biology. Key concepts that play in role in conservation genetics will be highlighted in the next section.

2.2 Genetic Diversity

2.2.1 Hardy-Weinberg Principle

The Hardy-Weinberg principle is one of the fundamental models in population genetics. This principle describes a population in which individuals mate randomly and where there are no other factors that can cause genetic changes from generation to generation (Allendorf et al., 2013). Under Hardy Weinberg Equilibrium, it is expected that proportions of alleles are equivalent to the following formula:

$$p^2 + 2pq + q^2 = 1$$

Where: p and q are the frequencies of alleles in a population. Expected heterozygosity is described by the measure $2pq$ (Allendorf et al., 2013). This model is a fundamental component of genetic diversity measures and is incorporated into other measures of population genetics.

2.2.2 Genetic drift

Genetic drift is the natural tendency for an independent population to slowly succumb to a loss of genetic diversity over time, that results in a random subset of alleles being exclusively passed down to the later generations (Allendorf et al.,

2013). Genetic drift is generally the most significant force that acts within small populations and it can lead to the loss of allelic variants as well as the change in allele frequencies as seen in captive populations (Gautschi et al., 2003). Ohta (1982) showed that in cases where migration is limited between sub-populations, clear genetic differentiation is observed which can be attributed to genetic drift. Thus with populations that are isolated, fragmented and unable to disperse, the problem of genetic drift becomes a major concern.

2.2.3 Inbreeding

Inbreeding refers to the increased level of relatedness between individuals of a population, relative to that of other populations. The process occurs through the increase of homozygosity in individuals within a population, which results in the accumulation of deleterious, recessive alleles (Allendorf et al., 2013). Inbreeding in wildlife tend to reduce the fitness of individuals in populations, which in turn leads to higher mortality rates, whereas captive populations are sometimes better able to accommodate inbred individuals (Crnokrak and Roff, 1999). The risk of inbreeding is increased in wildlife reserves where founding populations were not established with an appropriate number of founding members (Newman and Pilson, 2006). Studies of inbreeding have been performed under controlled conditions with animals in captivity (Jensen et al., 2014) and plants in the lab (Carr and Dudash, 1996; Dudash et al., 1997). Excessive inbreeding results in depression, whereby the loss of genetic diversity is so severe that populations in this state display reduced adaptability to environmental stochasticity in the long term thereby increasing the risk of extinction (Carlson et al., 2014; Smith et al., 2014). One method that is employed to reduce the risk of inbreeding is translocation. With wildlife and captive animals, this method is a popular strategy used by conservation agencies, which can improve the genetic diversity and directly contribute to genetic management of species (Batson et al., 2015), and will be further discussed in Section 2.5.1.

2.2.4 Bottlenecks

A bottleneck is described as a sudden decline in population size which results in the loss of genetic diversity. This can either be genetic, where there is a rapid loss

of alleles while the relative population remains the same or increasing, or demographic, where there is a marked reduction in population numbers (Allendorf et al., 2013). A large population could become fragmented due to either geographical barriers or habit loss as suggested for the speciation of the mountain zebra (Moodley and Harley, 2005). Founding new populations is also an example of a bottleneck, as seen in birds founding new island populations (Grueber et al., 2012). Additionally, effects in prairie chickens (*Tympanuchus cupido pinnatus*) were observed, where a severe bottleneck event resulted in reduced fitness, as demonstrated by infertility and the hatching rate of their eggs, which showed a long term decline. However, the action of introducing birds from larger breeding populations resulted in a sudden increase in fitness (Westemeier et al., 1998).

2.3 Genetic Markers

2.3.1 Neutral and adaptive selection

Genetic markers have been developed to describe diversity at various regions of the genomes of organisms. One key defining factor is whether the markers are positioned at neutral sites or adaptive sites of the genome. Genetic markers at adaptive sites aim to determine how the fitness of the individual is influenced by diversity. Mutations in these regions could lead to reduced fitness or death of the organism of interest (Misch and Hawn, 2008). Allelic variations at neutral sites do not carry through to the translation of proteins and are thereby prone to high levels of genetic variation (Allendorf et al., 2013; Holderegger et al., 2006). Previous studies show clear differences (resulting from selective pressure) between the two sites, in animals that have undergone a recent bottleneck event compared to those that have not. A rapid increase of heterozygosity at adaptive sites relative to the change at neutral sites was observed in water vole (*Arvicola terrestris*, Oliver and Piertney, 2012). In wild equids such as zebra species, selective pressures at adaptive sites, particularly against parasites and disease were observed (Kamath and Getz, 2011). In island populations such as *Anthus* sp., where selection against pathogens is expected, genetic drift is found to play a more important role particularly for small populations or new founding populations (Gonzalez-Quevedo et al., 2015).

2.3.2 Microsatellite DNA regions

Microsatellites, also known as short tandem repeats, are highly ubiquitous lengths of deoxyribonucleic acid (DNA) sequence consisting of 2-6 nucleotide base pair (bp) repeats (Jarne and Lagoda, 1996). Microsatellites can be genotyped using the polymerase chain reaction (PCR). These regions are common throughout the eukaryotic genome (Weber and May, 1989). Microsatellite repeats are hypervariable due to the process of DNA slippage during replication within DNA polymerase under normal conditions (Lai and Sun, 2003). The mutation rate is estimated to be between 1 x 10-4 and 1 x 10-2 mutations per generation in humans (Gaviria et al., 2017; Lai and Sun, 2003). The most common microsatellite repeats in mammals are the CA/GT repeats (Lagercrantz et al., 1993). In conservation genetics, the markers are used to estimate genetic distance, genetic diversity, allele frequencies and for parentage analysis among others (Kim and Sappington, 2013). Typically, 15-30 microsatellite markers are used to provide a measure of within as well as between species diversity (Gaviria et al., 2017; Veremeichyk et al., 2015; Zhao et al., 2015).

Microsatellite markers have historically been used at neutral sites due to their high level of genetic diversity. These Neutral sites are genetic alleles of loci at non-coding regions of an organism's genome and thus don't affect fitness (Allendorf et al., 2013). However, there are markers that have been identified as affecting fitness such as the repeat region that leads to incidence of fragile X syndrome in humans (Schlotterer, 2000; Sutherland and Richards, 1995). This has also been observed in the Androgen receptor gene in various zebra species (*Equus sp.*) which is associated with sexual behaviour and aggression (Ito et al., 2015). Microsatellite markers at non-coding DNA sites were found to undergo weak selective pressure, while selective pressures were found to be more prominent at coding sites, showing that there are limits to the number of possible repeats at the latter sites (Metzgar et al., 2000). When populations undergo bottlenecks, there are indications that genetic diversity of markers at neutral sites tend to recover very slowly (Oliver and Piertney, 2012). Neutral microsatellite markers have proven useful in genetic diversity studies in the past and have been used to make reasonably accurate predictions of diversity for selective traits (Keller and Waller, 2002; Reed and Frankham, 2003). A disadvantage to this method is that in order

to determine levels of inbreeding and estimate other parameters in population genetics, many markers are needed to accurately represent what is expected at adaptive loci; yet low correlation may be observed (Coltman and Slate, 2003; Reed and Frankham, 2001). While microsatellites have been widely used in the past, there are limitations to the power of using predominantly neutral markers to provide insights into the genetic diversity of a population (e.g. Candy et al., 2015). With recent advancements in genetic methods, there are clear distinctions in power between microsatellites when compared to more modern tools for detecting genetic diversity.

2.3.3 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) occur in DNA sequence variation between individuals within a population. Polymorphisms at the nucleotide level are described as positions where individuals have different nucleotides at a fixed position in the genome (Butler, 2012). In the human genome, it is estimated that SNPs occur, on average, once every 250-1000 bp and that they account for approximately 90% of DNA sequence variants (Collins et al., 1998; Kwok et al., 1996). When located within genes that transcribe proteins, SNPs can lead to synonymous changes in the subsequent proteins, or non-synonymous changes, which may lead to conformational changes that affect protein function (reviewed by Skevaki et al., 2015). There are a number of common SNPs that have been found to affect protein structure and functions. The algorithm, SIFT, which predicts the impact of amino acid substitutions on protein function, showed that 25% of 3084 non-synonymous SNPs lead to changes in protein function in humans (Ng and Henikoff, 2003, 2002). A number of non-synonymous SNPs have been identified that are attributed to infertility in mice (Singh and Schimenti, 2015). Moreover, some synonymous SNPs also affect protein function. This was found in the MDR1 (ABCB1) gene which encodes for membrane-bound transporter protein, had a synonymous mutation at 3435C>T, which led to potential ribosome stalling since a rare codon is being used which could have altered the kinetics during translation (Fung and Gottesman, 2009; Kimchi-Sarfaty et al., 2007).

2.3.4 Next-generation sequencing

With the advent of next-generation sequencing, new methods have been developed which make the field of conservation genetics more informative for the purpose of conservation of endangered species (Ekblom and Galindo, 2011; Leroy et al., 2018). Advancements in genomic techniques have greatly enhanced the power to assess SNPs throughout the genome in both natural and domesticated populations (Angeloni et al., 2012). Recent studies analyze heterozygosity difference correlations (Hoffman et al., 2014). This is carried out using a regression coefficient which is based on runs of homozygosity (Bjelland et al., 2013; Keller et al., 2011). Runs of homozygosity analysis can estimate the number of preceding generations in which inbreeding occurred as well as being capable of differentiating the regions involved in inbreeding depression for a given trait (Purfield et al., 2012). Applications in this field are still being developed, but one study (Leroy et al., 2018) emphasizes that more genomic studies need to be conducted to assess the extent to which selection may have purged deleterious alleles from the genome. An additional application of next-generation sequencing is restriction site-associated DNA sequencing (RADseq). This approach is a reduced-representation sequencing approach, whereby RADseq targets a subset of the whole genome. This approach has some advantage over the whole genome sequencing, such as greater depth of coverage per locus as well as the ability to sequence a higher number of samples for a limited amount of funding (Andrews et al., 2016). Using this approach, research questions pertaining to the genomics of adaptation, inbreeding and genomic diversity, effective population size, population structure, as well as others, can be addressed (Dann et al., 2013; Evans et al., 2014; Hoffman et al., 2014; Larson et al., 2014).

While modern techniques are enabling more informative outcomes in conservations, scientists who wish to carry out long-term studies often have to rely on older methods to evaluate studies. Studies that make use of temporal samples sometimes have to rely on previous data or highly degraded samples using microsatellite markers (Buchholz-Sørensen and Vella, 2016; Kamath et al., 2015). Although there is increasing work being done on making modern genetic tools accessible for non-model species, there are still issues in minimizing error rate obtaining reference genomes (Ekblom and Galindo, 2011). However, it is also

possible to work without a reference as was shown previously (Everett et al., 2011). Also, in museum samples, DNA may be extensively degraded to the point that alternative methods, such as the use of microsatellites or short stretches of SNPs must be used. This is particularly important in the RADseq method whereby small fragments of the starting genomic DNA may end up in the sequencing library, which results in wasted sequencing effort on non-RAD loci (Andrews et al., 2016).

2.4 Measures of Genetic diversity

2.4.1 Allelic frequency

Alleles are a specific arrangement of DNA at a particular region (locus) on a chromosome. The allele frequencies in a population are simply a measure of the proportions of alleles obtained for a given locus. When multiple loci are used for genetic analysis, the average across proportions is used. Populations with a low number of alleles at neutral markers are likely to have lower genetic diversity and reduced fertility compared with individuals from other populations (Charlesworth and Willis, 2009; Crnokrak and Roff, 1999). A more powerful measure is that of Allelic richness, which refers to the expected number of alleles should sample sizes differ across populations within a species (Kalinowski, 2004). The Program HP-Rare was developed to account for this difference and to correct for sets of populations that vary greatly in sample size (Kalinowski, 2005). Monitoring changes in allelic frequencies is one of the methods that is used to detect changes in genetic diversity between temporal samples of populations (Kamath et al., 2015). In addition to allelic frequency methods of monitoring genetic diversity include that of heterozygosity.

2.4.2 Heterozygosity

Populations whose heterozygosity deviate from Hardy Weinberg proportions signify that non-random mating is occurring which could be a result of various factors such as inbreeding, which leads to a reduction in heterozygosity (Allendorf et al., 2013) or a recent population bottleneck which is often determined through an excess of heterozygotes (Luikart et al., 1998). Numerous bioinformatic tools have been developed that calculates expected heterozygosity (H_e); which is the

probability that any given individual will be a heterozygote at a given locus or loci, and the observed Heterozygosity (H_o); which is the proportions for the given allelic data. Unbiased expected heterozygosity (uH_e) is preferred over H_e, since it corrects for multiple populations with unequal sample sizes (Nei, 2009). There are various computer programs available to calculate heterozygosity as well as other measures of population genetics when using microsatellites. These programs, such as STUCTURE, BOTTLENECK, Genalex and more, are explained and summarized by Kim and Sappington (2013). The versions used and program information will be discussed later in multiple chapters.

2.4.3 Inbreeding

F-statistics is a method commonly used with microsatellite markers, to test the nature of departures from Hardy Weinberg equilibrium (Allendorf et al., 2013). There are three coefficients namely, the inbreeding coefficient (F_{IS}), the measure of population structure (F_{ST}) as well as the overall inbreeding coefficient (F_{IT}), thought F_{IT} is not commonly used (Nei, 1977; Clark-Cockerham and Weir, 1993). An additional measure, namely, d^2 which is a measure of the difference in length of microsatellite alleles, is sometimes used. This approach has shown to be useful in measuring the levels of genetic diversity and inbreeding resulting from processes which have occurred thousands of generations back but is not applicable for populations that have recently undergone a bottleneck (Goudet and Keller, 2002).

2.4.4 Effective population size

Estimating effective population size (N_e) has become an important standard for estimating the long-term survivability of populations. This estimation helps in quantifying how populations are affected by drift and inbreeding (Braude and Harmon, 2010). The 50/500 rule that was established for the conservation of small and threatened populations (Frankham et al., 2014). This rule defines that a population with a N_e of less than 50 is highly susceptible to inbreeding but above that level, the risks of inbreeding will be decreased. The second component is that, when a population exceeds 500 individuals, they have increased adaptive potential and can better withstand environmental change (Jamieson and Allendorf,

2012). The magnitude of Ne and the total size (census size) of the population is generally not the same. A study by Nei and Tajima (1981) showed that a captive population of olive flies (*Dacus oleae*) had a N_e ten times smaller than the census size. In the past, N_e was measured using purely population demographics. Harris and Allendorf, (1989) presented the modified version of Reed et al., (1986):

$$\frac{1}{N_e} = \frac{1}{(4L_M M_{BR} k_M l_M)} + \frac{1}{(4L_F F_{BR} k_F l_F)}$$

Where L_M and L_F are mean generation lengths for all male and females that reproduce respectively. M_{BR} and F_{BR} represent the number of males and females that are breeding, K_M and K_F are the number of young that are sired by a male or born to a female for each year and L_M and L_F are the probabilities that a newborn male or female survives to the age of reproduction and then breeds. It was found that although this formula is highly accurate for overlapping generations, the accuracy of the formula may be increased if the product is applied using the following formula to account for non-Poisson reproductive success:

$$\frac{K}{(K + 1) + (V_K/K)}$$

Where K is the mean and V_k is the variance for the total number of offspring that is produced by an individual female over her entire lifetime which also survives to reproductive age (Harris and Allendorf, 1989). There are two other useful means of estimating N_e using genetic markers were developed and performs well. One such program, LDNE, measures the level of linkage disequilibrium in order to estimate N_e (Waples and Do, 2008). Another program, NeEsitmator, is based on genetic drift (Jorde and Ryman, 2007; Nei and Tajima, 1981). These computational methods perform well and can be used in combination with demographic methods (Kamath et al., 2015).

2.4.5 Adaptive potential

Adaptive potential refers to the ability of a population to respond to environmental change through natural selection, which acts through the collective genetic variation between individuals (Eizaguirre and Baltazar-Soares, 2014). In populations with low amounts of genetic variation, there is reduced likelihood of an

appropriate response to environmental changes and thereby can increase in the risk of extinction for the species (Bürger and Lynch, 1995; Thomas et al., 2004). The loss of adaptive capacity occur through the effects of inbreeding on phenotypic plasticity, the increased magnitude of inbreeding depression under stressful conditions and a reduced genetic variation for evolutionary adaptation (Bijlsma and Loeschcke, 2012; Frankham, 2005). For example, in Pacific salmon, low adaptive potential has limited their tolerance to increased ocean temperatures (Muñoz et al., 2015).

2.4.6 Genetic erosion

The term genetic erosion was coined in a conservation management context for the purpose of denoting a widespread or extreme loss of advantageous genes (alleles) and genotype combinations. The loss of advantageous genes is often onset through anthropogenic environmental change, which may result in population extinction even if a population seems to be increasing in size and their habitat appears favorable (Bijlsma and Loeschcke, 2012). The term genetic erosion tends to refer to the process of gradual curtailment of biodiversity for a particular location, over a certain period of time, which includes the loss of individual's genes and the loss of a combination thereof. Thus genetic erosion can be thought of as a function of changes of genetic diversity over time (Brown, 2008; Leroy et al., 2018). Indicators of genetic erosion have been hampered until recently, by a lack of sufficiently informative markers that can be analyzed efficiently, as well as economically. Technological advances in DNA sequencing and modern genomic approaches now offer new opportunities for monitoring genetic erosion.

2.5 Conservation genetics and Disease

2.5.1 Translocation and the spread of disease

Translocation is defined as the movement or relocation of living organisms from one area for release into another area (IUCN, 1987). Animals have been translocated into natural ranges (1) where they have become extinct, (2) where populations have become depleted and need to be restocked and reinforced, and (3) for the purpose of rehabilitating animals which have been illegally captured

(Woodford, 2000). Wildlife translocations have also been done for sporting purposes (hunting) (Woodford and Rossiter, 1994). Conservation managers often have limited information and resources when making decisions regarding the management of wildlife, which often leads to unclear outcomes at varied success rates (Frankham et al., 2014).

There are various implications of moving species between isolated reserves, where animals are unable to disperse naturally. Historically these concerns were left to the wildlife veterinarians, excluding stakeholders that fund translocations as well as plan and carry out the process (Cunningham, 1996). The risks when translocating animals are now increasingly being considered by conservation agencies prior to planned translocation activities (Cunningham, 1996). The act of translocating wild animals between reserves tends to be highly stressful to the animals and may lead to increased incidence of infectious disease (Woodford and Rossiter, 1994). Wildlife veterinarians regard animals that undergo translocation as a package, carrying a range of unseen viral, bacterial, protozoan as well higher-order parasites and disease agents (Griffith et al., 1989; Woodford, 2000). The resulting outbreaks of disease can lead to a significant reduction in fitness, mortality and become counterproductive in the augmentation of the target population (Kock et al., 2010). In species where there is already limited genetic variation, there is the added risk of introducing animals that have deleterious alleles in genes that may result in reduced fitness as demonstrated for endosymbiotic bacteria (Wernegreen and Moran, 1999). Translocations that are carried out without sufficient data may result in reduced fitness particularly in instances where the populations are small.

2.5.2 Disease susceptibility

There are two main mechanisms of immunity: adaptive immunity and innate immunity. Adaptive immunity develops over a period of days or weeks to establish protection when the host system encounters a specific pathogen for the first time. This process involves the synthesis of antibody-producing cells which responds to the presence of the pathogen in the host (Du et al., 2000). Alternately, a different pathway whereby immune cells are recruited to target the pathogen eliminate it from the host system occurs (reviewed by Barry and Bleackley, 2002). Adaptive

immune genetic markers such as the multi-histocompatibility complex (MHC) type I and type II has been particularly well studied. MHC molecules are highly polymorphic and contribute to a long-lasting immunity against pathogens that individuals encounter in their lifetime (reviewed by Abbas and Janeway Jr., 2000). MHC diversity has been studied and compared to neutral markers with regard to an isolated population of water vole showing that adaptive markers recover heterozygosity more rapidly than neutral markers (Oliver and Piertney, 2012). MHC diversity selection in relation to parasites in zebra species (Kamath et al., 2014).

Though these studies have provided valuable insights, it has been recommended that innate immunity be studied in addition to Adaptive immunity (Acevedo-Whitehouse and Cunningham, 2006)

2.5.3 Toll-like receptors and innate immunity

Innate immunity is an ancient mechanism that is common to a multitude of organisms. This mechanism makes use of membrane-bound receptors, called toll-like receptors (TLR), which can recognize and bind to pathogen-associated molecular patterns (PAMPs). These PAMPs bind to leucine-rich repeat regions of the TLR receptors. At least ten different TLRs have been identified in humans as well as other organisms (reviewed by Jin & Lee, 2008). The TLRs are found on either the cell membrane or internally embedded into the membrane of endosomal vesicles. Each TLR serves to identify a particular kind of molecule. For TLR 2, associations with either TLR 1 or TLR 6 were able to recognize lipoproteins as well as lipopeptides from more than 400 proteins in a wide variety of bacterial species. The TLR 3 protein was able to recognize dsRNA produced in viral replication, while TLR 4 in conjunction with co-receptor MD-2 recognizes Lipopolysaccharides, specifically outer-membrane glycolipids of Gram-negative bacteria (reviewed by Jin and Lee, 2008). TLR 5 is embedded in the plasma membrane which was able to recognize an extracellular ligand in humans (Zarember and Godowski, 2002).

There are SNPs in the TLRs that are associated with increased susceptibility to infectious diseases. For example, in TLR 1, a SNP change of 1805G>T, caused a Serine to Isoleucine change at position 602 which increased the probability of

females contracting *Chlamydia trachomatis* (Taylor et al., 2012). Moreover, the study showed that TLR4 rs1927911 CC genotype also increases the odds of Chlaymidia infections identified in its expression in the female reproductive system (Taylor et al., 2012). A significantly increased susceptibility to Tuberculosis in Tunisian patients was observed that were found to have the non-synonymous mutation, 2029C>T which led to a change from Arginine to Tryptophan at position 677 (Ben-ali et al., 2004). The association of increased risk of infection by the human papillomavirus and TLR 9 was investigated, but there was no significant association with clearing or causing the infection to persist (Oliveira et al., 2013).

2.6 Reproductive fitness

Loss in reproductive fitness is generally attributed to changes in genetic diversity within a population, particularly as a result of inbreeding depression that occurs (Frankham, 1999). Reproductive fitness is the measure of an individual animal's ability to produce offspring. Recent studies were able to associate low reproductive fitness to particular genes, such as the Cation channel Associated Sperm (CATSPER) 1 gene in humans (Avenarius et al., 2009).

2.7 Cape Mountain zebra

2.7.1 Conservation history of the Cape mountain zebra

Conservation efforts initially consisted of declaring the CMZ a protected species and establishing reserves whereby the animals were able to replenish their numbers. The Mountain Zebra National Park (MZNP) was established in 1937, located in the Eastern Cape province, South Africa for this purpose (Young et al., 1973). In addition, four other reserves including the Kammanassie Nature Reserve (KNR) (1923) and the Gamkaberg Nature Reserve (GNR) (1974) were set out as protected reserves for the CMZ (Novellie et al., 2002). GNR was established to protect the 13 CMZ on that land. However, five were shot by a local farmer prior to the establishment of the reserve (Barry, 1997; Watson et al., 2005). Additional populations were protected at the Outeniqua and Baviaanskloof Nature Reserve but succumbed to local extinction. This was thought to be due to excessive translocations to other reserves and unregulated poaching. Baviaanskloof was later restocked with CMZ originating from MZNP (Novellie et al., 2002).

The population of CMZ was slow to recover from the bottleneck event with the rate increasing only gradually. The initial population within the MZNP died out by 1950. A total of 11 CMZ were then donated to the park by a neighbouring farmer, with the first new foal being born in 1953 (Penzhorn, 1985). By 1965, through the purchase of an additional 30 CMZ, the population in MZNP increased to 55 animals (Penzhorn, 1985). Between 1960 and 1970, the first translocations occurred from MZNP, one of the first being translocated to the De Hoop Nature Reserve (DHNR) around 1963. Notably, CMZ were translocated to DHNR from two different relict populations, with four originating from KNR and six from MZNP (Novellie et al., 2002; Smith et al., 2008). In 1973, MZNP had approximately 130 zebra (Young et al., 1973). This number grew to 230 (Penzhorn, 1985) with a total of 474 zebra in 1985 (Kriek et al., 1994; Smithers, 1986). In 1998, there was an estimated total population of 1200 zebra with the largest population being 250 at the Karoo National Park (Novellie, 2008; Novellie et al., 2002). By the turn of the century, the CMZ population increased further to 1600 zebra (Figure 2.1) (Moodley and Harley, 2005). There are currently, approximately 4872 zebra in 76 sub-populations (Hrabar and Kerley, 2015).

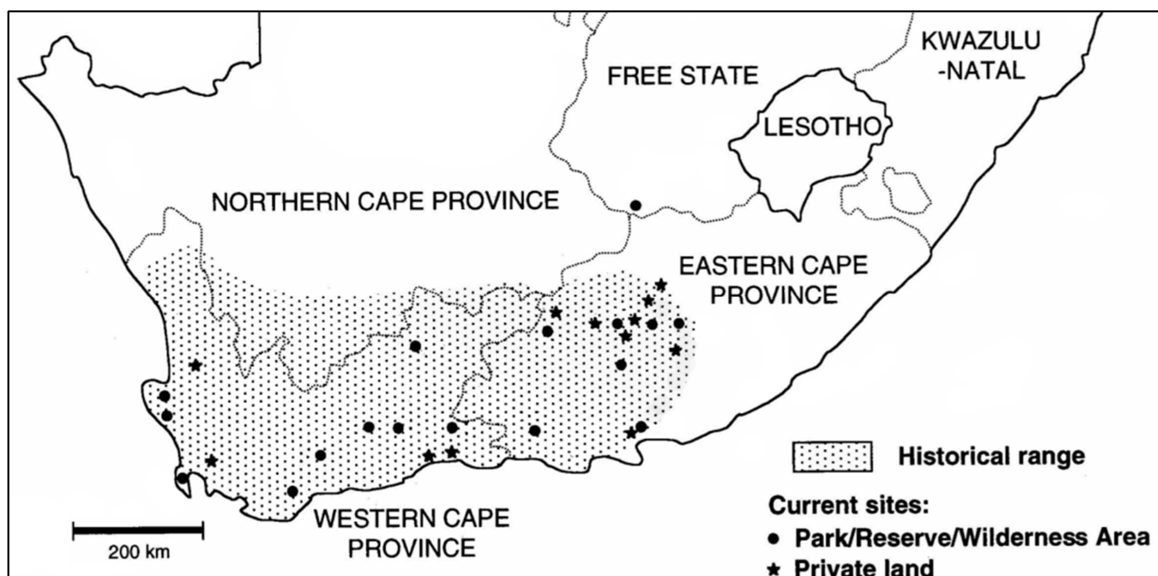

Figure 2.1: The historical range of CMZ population, also showing some of the current populations in either publicly owned or private conservations sites in the South-Western regions of South Africa (Novellie et al., 2002).

Notwithstanding the growth in number, the majority of CMZ populations are derived from a single relict population. Approximately 91% of extant CMZ were

sourced from the MZNP (Moodley and Harley, 2005). The other two relict populations have not been able to supply the foundation of new CMZ populations (Hrabar and Kerley, 2015; Novellie, 2008; Novellie et al., 2002). Translocations were severely limited since policies held by reserve management only permitted sourcing animals from either the GNR or the KNR when population levels exceed the designated maximum capacity of 90 animals (Watson and Chadwick, 2007). Current (2018) population estimates for Kammanassie are approximately 60 individuals and for Gamkaberg approximately 27 individuals (Briss, 2018 pers. comm.) and thus have not been used for translocations. The lack of population growth has been primarily attributed to insufficient availability of palatable grasses (Watson et al., 2005), further discussed in Section 2.7.4. Furthermore, there was a concern that translocating the animals from these key-reserves may lead to local extinctions. In a previous translocation attempt from KNR, seven of the nine animals died. The KNR reserve management has since refrained from translocations out of their reserve. Watson et al., (2005) criticized the KNR management’s reluctance and noted that to reach a population of more than 90 CMZ without drastic action is near unachievable. Watson & Chadwick (2007) later reiterated the advancements made in translocating animals and recommended that translocating CMZ from these key reserves KNR and GNR should be carried out to increase gene-flow. It is, however, a sizeable undertaking to ensure that genetic diversity is preserved and such actions require intensive monitoring and management (Novellie et al., 2002).

2.7.2 Mountain zebra Phenotype

Cape mountain zebra are medium-sized and have stripe patterns which vary from that of the plains zebra (*Equus quagga*) and Grevy’s zebra (*Equus grevyi*). There are also slight differences to that of the Hartmann’s Mountain zebra (*Equus zebra hartmannae*) (HMZ). Both CMZ and HMZ typically have a white underside with a black line running along the center of their chest and belly (Figure 2.2). The stripes on the body also differ between sub-species, while CMZ has broader stripes that are closer together, HMZ have narrower stripes spaced slightly further apart (Figure 2.3). The pattern on the heads of both sub-species consists of narrow stripes leading up to the muzzle, and a fade to an orange colour that ends in a black muzzle tip (Figure 2.4). There are no shadow stripes on the rump of CMZ

whereas these shadow stripes are typical of the Plains zebra. The hind legs of the HMZ have broader stripes which are typically two or three times the width of stripes on the rump, whereas, CMZ have stripes of similar width on their rumps and legs (Figure 2.5). Mountain zebra, in general, have a distinct dewlap, which is more conspicuous in the CMZ (Novellie et al., 2002). The mains of the HMZ also extend further forward between the ears, than is typically observed in CMZ (CITES, 2016).

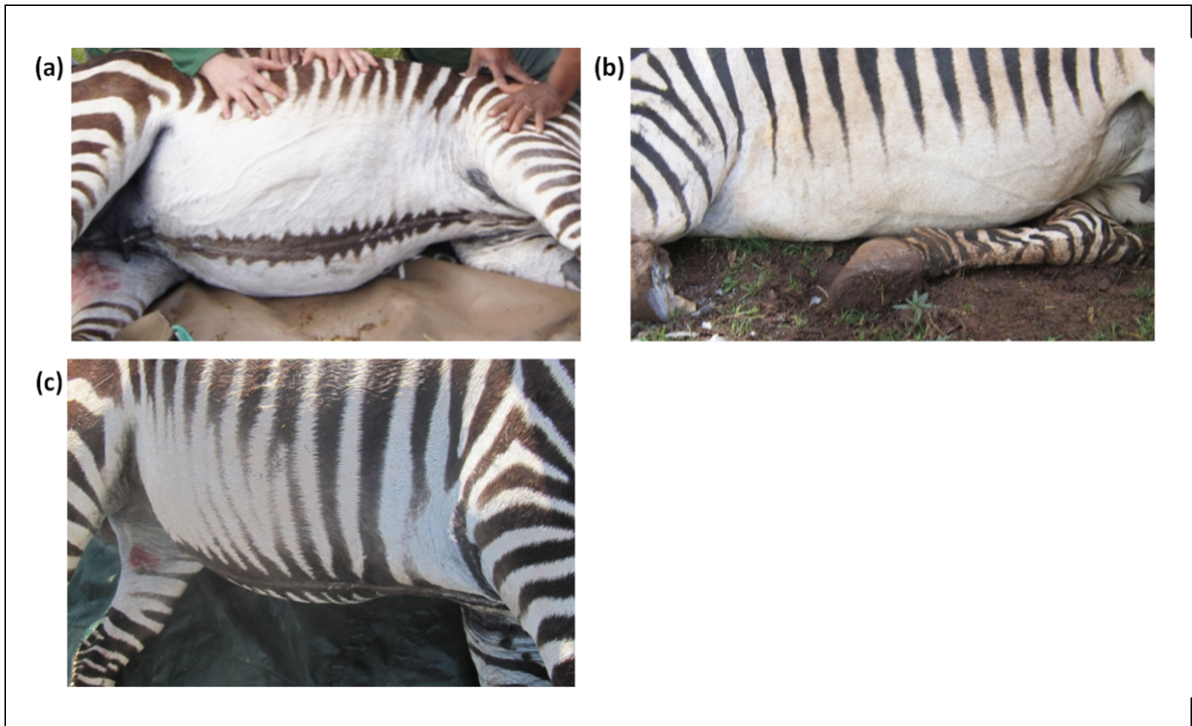

Figure 2.2: Images showing the undersides of (a) a sedated CMZ, (b) HMZ as well as an atypical (c) CMZ striping pattern. The stripes on the CMZ body do not typically continue all the way to the belly-line of the animal. (a) Shows the stripe that runs along the center of the animal's chest and belly, though this view is obscured in (b).

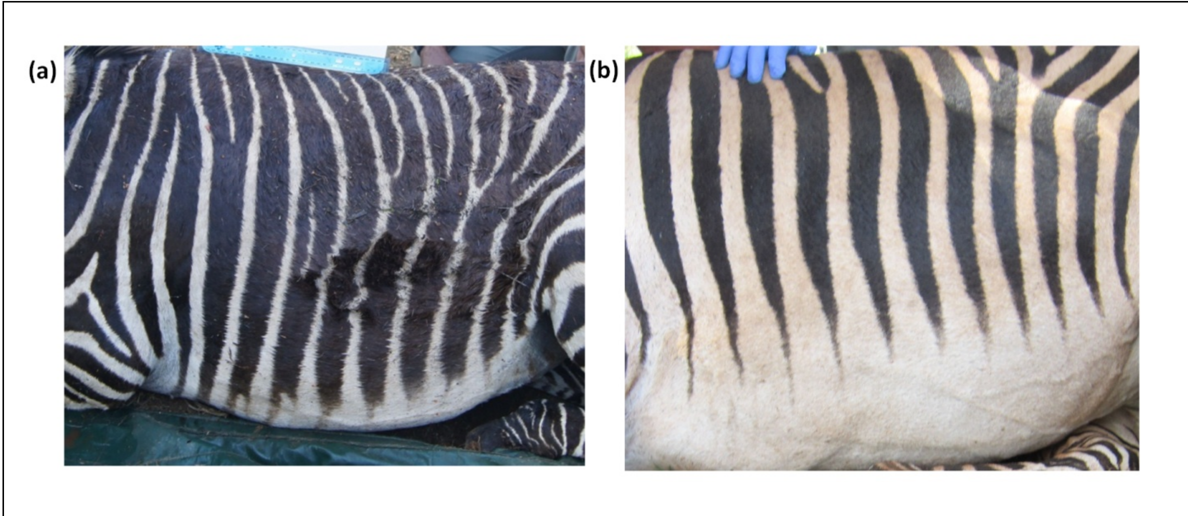

Figure 2.3: Images showing the stripes on the body from (a) a sedated CMZ and (b) HMZ. These images are representative of the difference between the width of the stripe and the distance between stripes on the body of these sub-species.

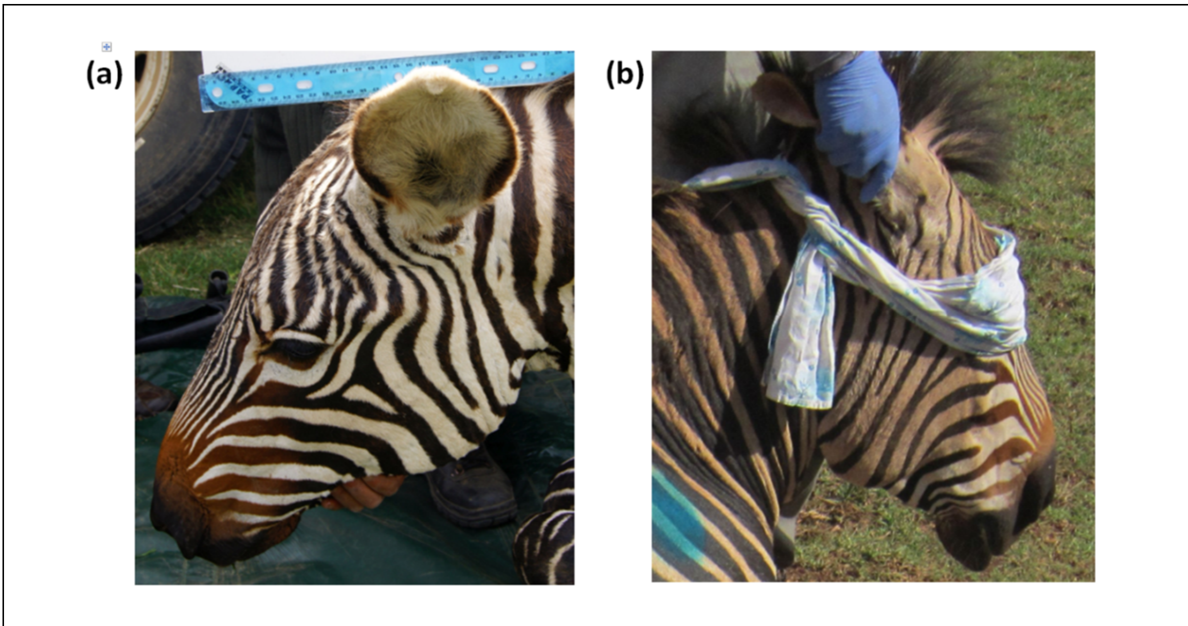

Figure 2.4: Images showing the side profile of the head of (a) a sedated CMZ and (b) HMZ with the characteristic narrow stripes that fade to orange with a black muzzle common to both sub-species.

2.7.3 Social structure and reproduction

The CMZ move in family groups, consisting of a dominant male and a harem of one or more females with their foals (Nel et al., 2006; Rubenstein and Nuñez, 2009). Zebra fillies leave their maternal herd between the age of 21-85 months and are able to produce foals at around 38 months old. Mares that are

approximately 21 months old have been able to produce foals with intervals typically being between 1 and 2.5 years (Penzhorn, 1985). Juvenile males tend to form temporary groups until they successfully take over a harem of their own. As females reach maturity, they immediately join a harem, particularly if there are few females. In larger groups, it has been found that females work in unison to exclude new female additions. (Rubenstein, 1994). This practice is thought to decrease the resulting competition for grazing, which would affect the reproductive success of the group (Rubenstein, 1994). A complex hierarchy exists among females which are also observed in the plains zebra, where higher ranked females tend to have more reproductive success (Pluháček et al., 2006; Rubenstein and Nuñez, 2009).

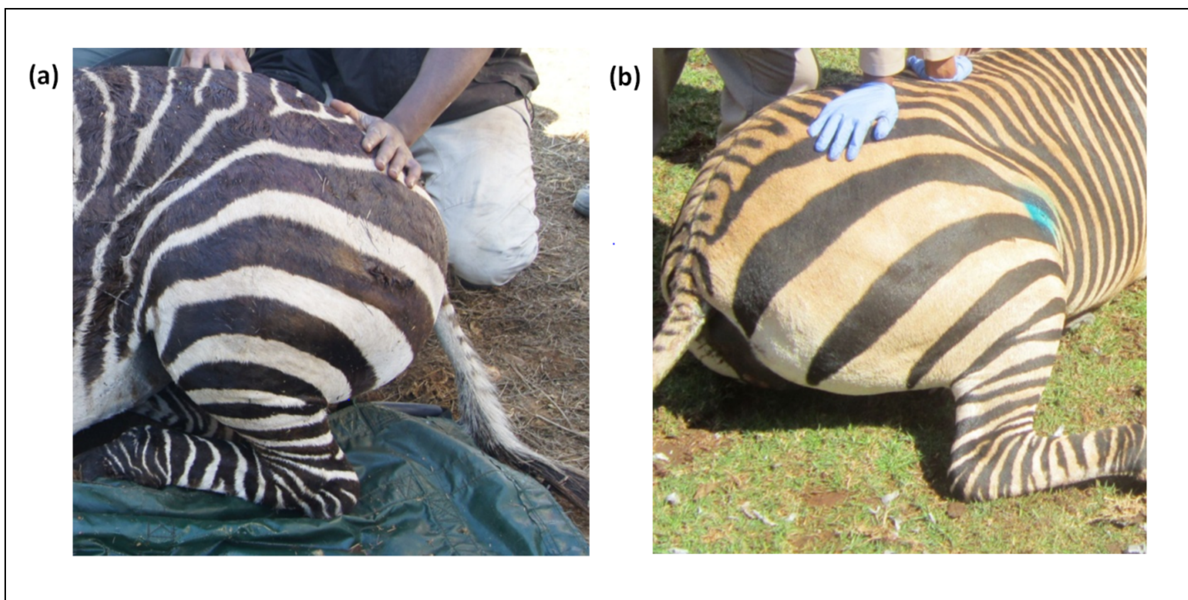

Figure 2.5: Images showing the hind legs of a sedated (a) CMZ and (b) HMZ. The images show the difference between their stripe patterns, where CMZ stripes are similarly sized to that of the body stripes. The HMZ hind leg stripes are larger than that of their body stripes.

2.7.4 Feeding habits and habitat use

CMZ are highly selective feeders (Grobler, 1983). Their preferred diet consists of palatable grasses, predominantly *Themeda traindra* and *Eragrostis curvula* found mainly in the Renosterveld habitat located in MZNP as well as in GNR (Watson et al., 2005). The CMZ population in KNR prefer to occupy the Arid Restiod fynbos, Waboomveld and Renosterveld, while the population in GNR shows a preference for Renosterveld (Watson et al., 2005). The terrain in KNR is significantly more

rugged and has a smaller proportion of low-lying lands where nutrient-rich soils and higher quality grasses are able to grow. The surrounding properties are privately owned. Negatively, the KNR and GNR are not permitted to carry out controlled burns, which affect the quality of the grasses in the reserve (Watson et al., 2005; Watson and Chadwick, 2007). In recent years, more populations are having to inhabit areas that are on the fringes of geographical distributions which are associated to conditions that are not favorable for CMZ and are thus then considered a refugee species (Lea et al., 2016; reviewed by Willi et al., 2006). Based on fossil evidence it is more likely that CMZ readily had access to grassland areas in the Fynbos regions where they are not able to support a dense population of the animals (Faith, 2012).

2.7.5 Threats to the Cape mountain zebra

Fragmented populations of CMZ which remain small and isolated become increasingly susceptible to the incidence of genetic drift, loss of alleles and reduced heterozygosity (Hrabar and Kerley, 2015, 2013; Moodley and Harley, 2005; Novellie et al., 2002). Moreover, inbreeding has become increasingly prevalent. Hybridization between zebra species poses a risk, particularly with the relict populations. Hrabar and Kerley, (2015) reported that 62% of sub-populations of CMZ have been exposed to plains zebra, which are known to hybridize with CMZ (Dalton et al., 2017). Due to the geographical distance between sub-populations of CMZ gene-flow is reliant on human intervention. Currently, there are challenges with the translocation process due to the Western Cape being a quarantine zone for African Horse sickness. A recent attempt to translocate animals between Anysberg Nature Reserve and Camdeboo National Park has been postponed due to a local outbreak of the disease in the Western Cape (K. Labuschagne 2018 per. comm.).

2.7.6 Genetic status of the Cape mountain zebra

The first study of genetic diversity in CMZ commenced in 2002, using neutral markers to measure the genetic diversity of the sub-species (Moodley, 2002; Moodley and Harley, 2005). Studies of the genetic status of the CMZ revealed several useful findings: Due to the lack of dispersal in the three relict populations,

risks of genetic drift and reduced genetic heterozygosity are prevalent. Since the three relict populations retain the majority their allelic diversity, it was suggested that mixing of these populations would mitigate the loss of genetic diversity (Moodley and Harley, 2005; Watson and Chadwick, 2007). Recent reports have shown that little has been done in that regard (Birss et al., 2018; Hrabar and Kerley, 2015). It was reported that there is low genetic variability in most of the sub-populations of CMZ distributed in South Africa, but that relict populations, as well as the DHNR, showed genetic diversity that was higher than average (Moodley and Harley, 2005).

2.7.7 Disease and parasites of Cape mountain zebra

The CMZ are known hosts to several parasites and diseases. Species of horse bot flies, namely, *Gasterophilus pecorum*, *G. intestinalis*; a tapeworm, *Anplocephala magna* and a nematode, *Trichonema* were isolated from CMZ in MZNP. Additionally, four tick species are associated with CMZ, namely: *Rhipicephalus evertsi evertsi*, *R. glabroscutatum*, *Hyalomma rufipes* and *Margaropus winthemi* (Young et al., 1973). The *H. truncatum* tick was also observed in Bontebok National Park (Guo et al., 2014). These ticks often travel along with animals being translocated (Figure 2.6). A total of 18 species of nematodes were identified in CMZ from MZNP in 1994 (Kriek et al., 1994). The disease, Babesiosis, also referred to as, equine piroplasmosis (from *Theileria equi* and *Babesia caballi*), has been confirmed in CMZ found at Bontebok National Park as well as Karoo National Park, transmitted by the tick vectors *Rickettisia evertsi evertsi* and *Hyalomma truncatum* (Bhoora et al., 2010a; Lampen et al., 2009; Zweygarth et al., 2002). Within the last two decades, sarcoidosis (Bovine papillomavirus) was reported to be prevalent in a number of CMZ found at Bontebok National Park and Gariep Dam Nature Reserve, while no incidence was identified in Karoo National Park or Karoo Nature Reserve (Sasidharan et al., 2011). Encephalitis induced by equine herpes virus has also been isolated from other zebra species (*Equus gravyi* and *Equus burchelli*) (Guo et al., 2014).

When translocating CMZ between reserves it is highly likely that these animals are carrying diseases, or vectors that are able to transmit disease (Gaughan, 2001) (Figure 2.6). The goal of a wildlife manager in this regard is executing

translocations in a manner that simultaneously reduces risks to natural populations and the translocated animals (Aiello et al., 2014). The first step of monitoring the spread of disease in the CMZ meta-population is by identifying diseases that may be most detrimental as a result of translocation (Miller, 2007). Two diseases of interest that have been identified in CMZ, namely, babesiosis and sarcoidosis.

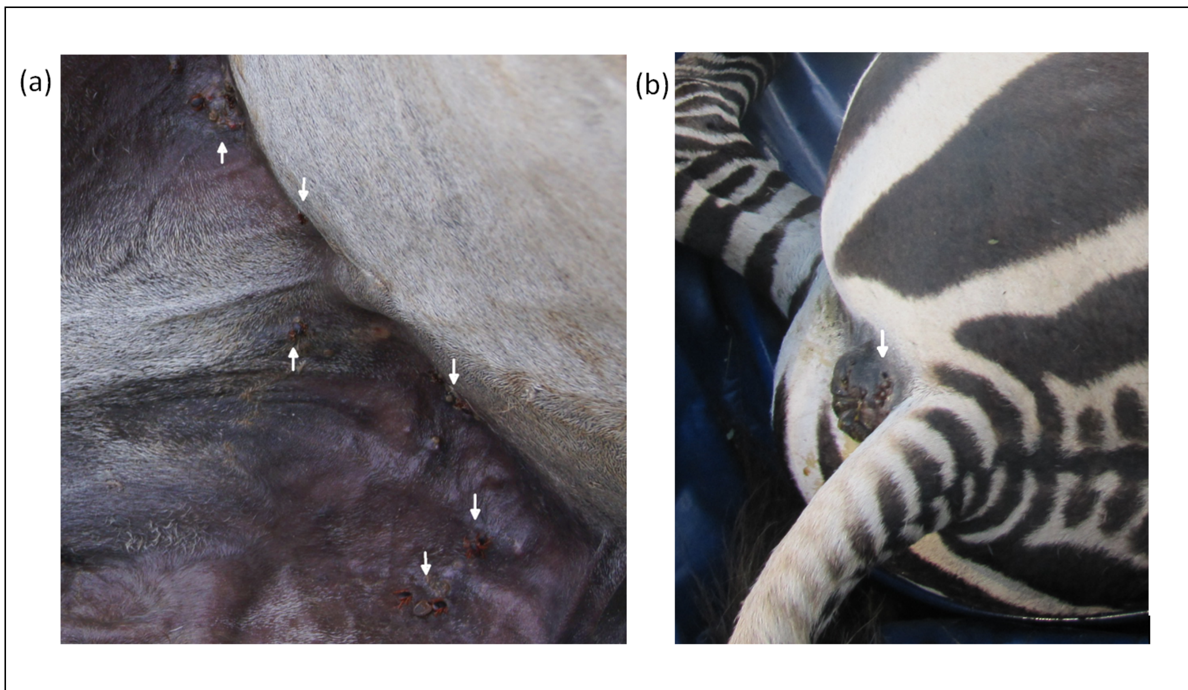

Figure 2.6: (a) Male and female ticks attached to the groin area of a male Cape mountain zebra; and (b) Ticks attached at the anal region of a male Cape mountain zebra. The White arrows show locations where ticks were attached at the time of translocation.

2.8 Disease susceptibility in the Cape mountain zebra

2.8.1 Equine piroplasmosis in Cape mountain zebra

Equine piroplasmosis can be attributed to the two agents, *Babesia equi* and *Theileria equi* which can infect the CMZ independently or in tandem (Wise et al., 2013). The disease is transmitted through ticks from the genera *Boophilus*, *Hyalomma*, *Rhipicephalus* and *Dermacentor*. In South Africa, only two tick species known to transmit the disease occur in the distribution range of CMZ, namely *R. everstsi evertsi* and *H. truncatum* (Bhoora et al., 2010a). In piroplasmosis-endemic areas, horses tend to become infected within the first year of life, though in native populations mortality may range between 5-10%. The effect is dependent on the

strain which is present, the availability of treatment and the general health of the animals. When previously unexposed, mature animals are introduced into endemic areas, deaths exceed 50%. Severely infected horses experience oedema, haemorrhages, anoxia as well as organ dysfunction. It was found that horses can acquire life-long immunity as long as the parasites are present, but there is no cross-immunity between *T. equi* and *B. caballi* (reviewed by Rothschild, 2013). Since, the growth of international equine sport and trading in horses, the risk to introduce the pathogen into previously unaffected areas has increased (Brüning, 1996). While most control measures take place on an international level, local translocations tend to be overlooked, which may pose a risk to animals that are intended to move across international borders.

There has been extensive research into equine piroplasmosis and many screening tools that have been developed in order to detect an infection in Zebra (Alhassan et al., 2005; Bhoora et al., 2010b; Brüning, 1996; Kubelová et al., 2013; Malekifard et al., 2014; Rothschild, 2013). The simplest screening method is through microscopic analysis of a blood smear, stained with Giemsa 10%. However, this method has been found to be insufficient due to the low levels of parasitaemia for both organisms in blood during an infection (Rothschild, 2013). A newer screening tool is through a PCR-based process which amplifies DNA exclusive to the parasites of interest (Bhoora et al., 2010b). It was recommended that when screening for this disease, both the genetic screening method and classical microscopy techniques are employed (Bhoora et al., 2010a; Lampen et al., 2009; Malekifard et al., 2014). It would be useful to apply these methods to monitoring how piroplasms move between populations. This may be crucial in preventing the loss of genetic diversity in the individuals translocated from relict populations.

2.8.2 Reproductive fitness

Little work has been done to understand the reproductive traits of the CMZ. While the CMZ population is able to reproduce and have generally been increasing in number, there are concerns that their low genetic diversity may affect reproductive fitness (Birss et al., 2018). In two of the relict populations, KNR and GNR population numbers are not increasing (Birss per. Comm. 2018), and in DHNR, the

population is under strain with very low birth rates and a skewed sex ratio (Smith et al., 2008).

2.8.3 Cation Channel of Sperm (CATSPER) genes

Isolation and inbreeding may have increased the prevalence of deleterious alleles into the genes that are linked to reproductive fitness. Identifying mutations in genes that impact reproductive health may serve to improve the growth of the population. Moreover, increased translocation may unknowingly introduce animals with reduced fitness into populations and cause the proliferation of the associated alleles throughout the population. Sperm or spermatozoa acquire motility in the epididymis (Qi et al., 2007). Spermatozoa are then capacitated in the female reproductive tract and this is where hyperactivation takes place, which is necessary for the penetration of the zona pellucida which encapsulates the mature ovum (Bedford, 1970; Brackett, 1975; Ren et al., 2001; Stauss et al., 1995). The Cation Channels of Sperm (CATSPER) has been attributed to the normal functioning of the tail during the process of capacitation as well as during hyperactivation (Qi et al., 2007). Hyperactivation refers to the tail motion changing from sinusoidal, which is symmetric fast and low in amplitude, to whip-like, which is asymmetric slow and large amplitude (Carlson et al., 2003). There are four CATSPER genes that are all expressed in the sperm tail, all of which were determined to facilitate retained sperm motility (Qi et al., 2007). The CATSPER genes have been extensively studied in humans (Avenarius et al., 2009; Hildebrand et al., 2010; Singh and Rajender, 2015; Strünker et al., 2011), as well as in horse (Loux et al., 2013) and mice (Jin et al., 2007; Qi et al., 2007). In equines, the CATSPER channel is also needed to induce hyperactivity of sperm which resulted from the Ca^{+} gradient *in vitro* (Loux et al., 2013). When the expression of this gene is impaired by mutation, there tends to be infertility and this pathway was previously suggested as a target for male contraceptive in humans (Avenarius et al., 2009). CATSPER ion channels subunits form a heteromer complex which is expressed around a Ca^{2+} pore where they regulate the flow of the ions through the channel, which is thought to be analogous to other six-transmembrane spanning ion α-channel subunits (Lishko and Mannowetz, 2018). In addition, there are three auxiliary subunits, namely the CATSPER β, CATSPER δ and CATSPER γ, which all play a role in the hyperactivity and motility

as seen in males (Chung et al., 2011; Liu et al., 2007; Wang et al., 2009). Management practices in CMZ that effects diversity of the CATSPER genes can thus impact significantly on the fitness of the species.

CHAPTER 3

Lessons for conservation management: monitoring temporal changes in genetic diversity of Cape mountain zebra (*Equus zebra zebra*)

Submitted:

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3.1 Abstract

The Cape mountain zebra (*Equus zebra zebra*) is a subspecies of mountain zebra endemic to South Africa. The Cape mountain zebra experienced near extinction in the early 1900's and their numbers have since recovered to more than 4,800 individuals. However, there are still threats to their long-term persistence. A previous study reported that Cape mountain zebra had low genetic diversity in three relict populations and that urgent conservation management actions were needed to mitigate the risk of further loss. As these suggestions went largely unheeded, we undertook the present study, fifteen years later to determine the impact of management on genetic diversity in three key populations. Our results show a substantial loss of heterozygosity across the Cape mountain zebra populations studied. The most severe losses occurred at De Hoop Nature Reserve where expected heterozygosity reduced by 22.85% from 0.385 to 0.297. This is alarming, as the De Hoop Nature Reserve was previously identified as the most genetically diverse population owing to its founders originating from two of the three remaining relict stocks. Furthermore, we observed a complete loss of multiple private alleles from all populations, and a related reduction in genetic structure across the subspecies. These losses could lead to inbreeding depression and reduce the evolutionary potential of the Cape mountain zebra. We recommend immediate implementation of evidence-based genetic management and monitoring to prevent further losses, which could jeopardise the long term

survival of Cape mountain zebra, especially in the face of habitat and climate change and emerging diseases.

3.2 Introduction

The Cape mountain zebra (CMZ, *Equus zebra zebra*) provides a useful case study to help understand and advance the usefulness of genetic tools and monitoring for biodiversity conservation. The CMZ is a subspecies of mountain zebra that is endemic to South Africa. The subspecies is listed as Vulnerable on the International Union for Conservation of Nature (IUCN) Red List (Novellie 2008) and on Appendix II by the Convention on International Trade in Endangered Species (CITES; CITES, 2016; Lombard, 2016). Historically, CMZ had a widespread distribution in the mountainous Fynbos, Karoo, and grassland regions of the Western Cape, the Eastern Cape and portions of the Northern Cape Provinces of South Africa (Millar 1970a; Millar 1970b; Hrabar et al. 2016). However, by the 1950's, as a direct result of hunting pressure and habitat loss, CMZ experienced a 90% reduction in its geographic distribution and were reduced to less than 50 individuals in a few relict populations, inhabiting the most inaccessible parts of the subspecies' range (Novellie et al., 2002; Moodley and Harley 2005; Hrabar and Kerley 2015). Only three relict populations survived to the present day, and these are found in the Mountain Zebra National Park (MZNP) located near the town of Cradock in the Eastern Cape, and Kammanassie National Reserve (KNR) and Gamkaberg National Reserve (GNR) in the Klein Karoo region of the Western Cape.

Following historical decline, CMZ numbers have since recovered to an estimated 2,650 individuals within the historical range (Birss et al., 2018; Hrabar et al., 2016), due mainly to dedicated conservation efforts by the South African National Parks (SANParks), who are the custodians of the MZNP. The low proportion of habitat that contains palatable grasses such as *Themeda trinadra* is one of the main limiting factors to CMZ growth in many of the current populations (Penzhorn, 1982; Winkler and Owen-Smith, 1995; Lea et al., 2016). Therefore, SANParks employed a range expansion strategy, where "excess" MZNP stock was used to seed several other populations within the former CMZ range including: Baviaanskloof Nature Reserve, Karoo National Park, Camdeboo National Park, Tankwa Karoo

National Park, Bontebok National Park, Oorlogskloof Nature Reserve, De Hoop Nature Reserve (DHNR); and outside its natural distribution range including: Addo Elephant National Park, Table Mountain National Park, West Coast National Park, Commando Drift Nature Reserve, Tsolwana Nature Reserve and Gariep Dam Nature Reserve. In addition, approximately 1,500 CMZ are reported to occur on private reserves (Hrabar and Kerley 2015). The populations of MZNP-derived CMZ in South Africa have steadily increased and the estimated number of mature individuals in protected areas has exceeded the threshold of 1,000 for more than five years, resulting in a regional IUCN Red Listing of Least Concern (Hrabar et al. 2016).

In contrast, to the SANParks approach, CapeNature, the authority managing the remaining relict populations at KNR and GNR, has historically opted for a more conservative approach, citing low population numbers and lower growth rates as reasons not to remove animals from their reserves to establish populations elsewhere. The only exception to this rule was the population at DHNR, founded from a mix of MZNP and KNR individuals in the 1960s and 1970s. Thus, despite KNR and GNR containing a large proportion of the CMZ's historic genetic variation (Moodley and Harley 2005), they remain isolated and at critically low numbers.

Genetic factors likely influencing the persistence of CMZ have been previously reported. Moodley and Harley (2005) indicated that individual CMZ populations exhibited low genetic variation. The three relic CMZ sub-populations (MZNP, KNR and GNR) were inbred with the lowest microsatellite heterozygosity being identified in KNR (He = 0.239). In sharp contrast, the only known mixed population at DHNR had the highest genetic diversity (He = 0.380, Moodley and Harley 2005). However, the overall genetic variation in the metapopulation (populations from National South African Reserves) was considered moderate because substantial remnant allelic variation existed in the subspecies (Moodley and Harley, 2005). Inbred populations of CMZ with low genetic diversity show an increased incidence of sarcoidosis (Marais et al., 2007; Sasidharan, 2006; Sasidharan et al., 2011). In addition, the two relict CMZ populations (GNR and KNR) further exhibited inflated genetic differentiation due to genetic drift and inbreeding effects resulting from lack of dispersal (Moodley and Harley, 2005).

To manage and monitor the evolutionary potential of the CMZ metapopulation, a Biodiversity Management Plan (BMP) for this species in South Africa was developed by various stakeholders. The purpose of the BMP is to ensure the long term survival of CMZ in nature, using a strategy underpinned by specific goals and objectives aimed at addressing the threats faced by this subspecies, such as population fragmentation, disease, inbreeding, hybridization with plains zebra and habitat loss (Hrabar et al., 2016; Birss et al., 2018). It was suggested by several authors (Moodley and Harley, 2005; Hill, 2009; Sasidharan et al., 2011; Hrabar and Kerley, 2013; Hrabar et al., 2016; Hrabar and Kerley, 2015) that mixing of aboriginal populations is required to further reduce genetic diversity losses, especially considering that only populations descending from MZNP stock are experiencing high population growth whereas KNR and GNR are not.

However, it was recommended that introductions into either KNR and/or GNR populations be avoided due to the observed population structure, and mixing should only be considered at alternative locations which would be monitored. These plans were hindered, however, by ecological studies that suggested CMZ numbers in the GNR were too low to risk removal of individuals to seed mixed populations (Watson et al., 2005, Watson and Chadwick 2007), despite the high diversity reported for the mixed DHNR population. Currently, an estimated 40 mountain zebra are being removed from the MZNP for the purpose of re-establishing a breeding herd within the historical range as well as stocking private reserves with animals, both within and outside the natural distribution range. To date, CMZ occur in more than 75 localities, including over 30 national- and regional parks. Population sizes are estimated to vary between 4 and 1,191 individuals, with the largest population found in the MZNP. The average annual population increase for the subspecies over the period 2009-2015 was 11% (Hrabar and Kerley, 2015).

Although the CMZ metapopulation continues to grow, it is yet to be determined whether management strategies have affected genetic diversity over time. Thus, this study aims to investigate temporal changes in genetic diversity in three key CMZ sub-populations by comparing present day (2015-2016) levels with those of samples collected from 1999 to 2001 (Moodley and Harley, 2005). This represents a time span of approximately 15 years or approximately 1.5 generations (Moodley

2002, C. Birss unpublished data). Genetic diversity within populations can only be expected to increase through gene flow between relict stocks (e.g., as in DHNR) or new mutations, with the latter considered inconsequential in the timescale involved. Natural selection is unlikely to increase diversity except at individual loci under balancing selection. Given that our survey is only across a single generation, and there has been no gene flow between relict stocks during this time, we do not expect diversity to have increased over the study period.

However, diversity may be maintained in larger populations experiencing rapid growth. Therefore, we expect that within one generation, populations with larger size and high growth rates (e.g., MZNP) should maintain diversity, whereas populations with lower growth (e.g., DHNR), and smaller size (KNR), would be expected to show a loss in genetic diversity. Furthermore, changes in genetic diversity such as loss of heterozygosity and rare alleles may also have consequences for how populations are structured relative to each other. The loss of shared alleles from populations would be expected to inflate genetic structure (differentiation), as seen in Moodley and Harley (2005), whereas a loss of private alleles would reduce genetic differentiation, making populations appear more similar. The results of this study will be used to inform management strategies employed by the CMZ BMP, by providing additional data on population diversity and differentiation.

3.3 Methods

3.3.1 Ethical approval and sample collection

Ethical approval was obtained from the Research Ethics and Scientific Committee (RESC) of the National Zoological Garden, South African National Biodiversity Institute (NZG SANBI, NZG/RES/P17/19), as well as the Animal Ethics Committee of University of the Free State (UFS-AED2017/0011). Permission was also obtained from the Department Agriculture Forestry and Fisheries of South Africa under Section 20 of the Animal Disease Act 1984 with (Ref: 12/11/1/1/8). Whole blood samples were collected from the MZNP (n=75) and DHNR (n=27) in 2016 and four samples were obtained from KNR in 2015. In addition, this study included samples from MZNP (n=12), DHNR (n=15) and KNR (n=9) collected between

1999 and 2001 (Moodley and Harley, 2005). Thus, a total of 142 samples were analysed. Samples were stored at -20°C in the Biobank of the NZG, SANBI until used.

3.3.2 Molecular Methods

We extracted DNA using the Quick-DNA™ Universal kit (Zymo Research) following the manufacturer's protocol for blood. We selected 14 cross-species microsatellite markers (AHT21, UCDEQ505, HTG3, HTG7, HTG9, HTG11, HTG14, HTG15, LEX20, LEX52, TKY273, VHL47, HMB1 and COR014) used in the study conducted by Moodley and Harley, (2005). Polymerase Chain Reaction (PCR) amplification was conducted in a 12.5 µl reaction volume consisting of Ampliqon Taq DNA Polymerase Master Mix RED, forward and reverse primers (0.5 µM each), and 50 ng genomic DNA template. The conditions for PCR amplification were as follows: 5 min at 95°C denaturation, 30 cycles for 30 sec at 95°C, 30 sec at 55-60°C (depending on the marker amplified, Supplementary Table 1), and 30 sec at 72°C, followed by extension at 72°C for 40 min in a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.). PCR products were run against a Genescan™ 500 LIZ™ internal size standard on an ABI 3130 Genetic Analyzer (Applied Biosystems Inc.). Samples were genotyped using GeneMapper v. 4.0 software (Applied Biosystems Inc.).

3.3.3 Genetic variation

MICRO-CHECKER software (van Oosterhout et al. 2004) was used to detect possible genotyping errors, allele dropout, and null alleles. Allelic richness (A_r), was estimated correcting for sample size through rarefaction using HP-RARE v. June-6-2006 (Kallinowski, 2005). Allele Frequencies, observed Heterozygosity (H_o) and expected heterozygosity (H_e) and number of private alleles per population were calculated using GenAlEx 6.5 (Peakall and Smouse 2005; Peakall and Smouse 2012). In order to determine the significance of changes (H_e or A_r) between the two time periods, a one-tailed pairwise T-test (α = 0.05 and α = 0.1) was performed with a null hypothesis that no loss in diversity has occurred. Deviations from expected Hardy-Weinberg (HW) proportions were tested (Markov Chain length of 105 and 100,000 dememorization steps). We also tested for

gametic disequilibrium between all pairs of loci using the exact test described by Guo and Thompson (1992) in GenAlEx 6.5.

3.3.4 Genetic structure

Since changes in genetic diversity can also affect genetic structure, we determined the relative structure (differentiation) among our three sampled populations at the time periods 1999-2001 and 2015-2016 using three methods. First, we used the Principal Component Analysis (PCA), which is a multivariate method using K-means clustering, and implemented in the R package Adegenet version 2.1.1 (Jombart, 2008). We then used the model-based Bayesian clustering algorithm in STRUCTURE version 2.3.4 (Pritchard et al., 2000), which determines the most probable number of populations and assigns individuals to their most likely population of origin.

We ran STRUCTURE with the following models: admixture model with both correlated and independent allele frequencies and no admixture model with correlated and independent allele frequencies. Each of the models were run without prior population information for ten replicates each with K = 1 – 10, with a run-length of 700,000 Markov Chain Monte Carlo repetitions, following a burn-in period of 200,000 iterations. The ten values for the estimated log-likelihood (ln(*Pr*(X|K)) were averaged across runs and posterior probabilities were calculated (Supplementary Figure 1). The K with the greatest increase in posterior probability (ΔK, Evanno et al., 2005) was identified as the optimum number of sub-populations using STRUCTURE HARVESTER (Earl & von Holdt 2012). The membership coefficient matrices (Q-matrices) of replicate runs for the optimum number of sub-populations was combined using CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007) with the FullSearch algorithm and G′ pairwise matrix similarity statistics. The results were visualized using DISTRUCT version 1.1 (Rosenberg 2003). Lastly, we used an F_{st}-based hierarchical analysis of molecular variance (AMOVA, Excoffier et al. 1992) to estimate how genetic diversity was partitioned between and within the MZNP and KNR populations for both time periods (Arlequin 3.5; Excoffier and Lischer 2010). We excluded the DHNR population for this particular analysis as it is descended from a mix of MZNP and KNR.

3.3.5 Bottleneck simulations

The program Bottleneck version 1.2.02 (Piry et al., 1999) was used to detect evidence of recent population bottlenecks. This program measures significant differences between the measured expected heterozygosity (H_e; i.e., gene diversity) and the theoretical expected heterozygosity assuming mutation-drift equilibrium (H_{eq}) given the sample size and observed number of alleles (A). This test identifies populations that have recently undergone a decline in effective population size (N_e), resulting in a heterozygosity excess and deficit of rare alleles (Cornuet and Luikart, 1996). The test is able to detect a population size reduction of ~50 N_e that may have occurred approximately 25-250 generations ago. Previously recommended parameters for microsatellite data, using the two-phase mutation model, were used (Di Rienzo et al., 1994; Piry et al. 1999). This model accommodates for a small proportion of multiple-step mutations, with most mutations being a single step change in allele length. We used two different mutation models including 95% and 80% single-step mutations (SMM) with a two-phase model variance of 12% for a total of 10,000 iterations (Luikart et al., 1998a). Heterozygosity excess was tested for using the Wilcoxon sign-rank test (Significance at $\alpha = 0.05$) (Luikart et al., 1998c). Allelic frequency, mode-shift deviation from the L-shaped distribution was examined, which was to corroborate detection of a recent bottleneck event (Luikart et al., 1998b).

3.3.6 Effective population size (N_e) estimation

The two-sample temporal method (Krimbas & Tsakas 1971; Nei & Tajima 1981; Waples 1989) was applied to estimate the variance effective population size (N_{eV}). This method assumes temporal changes in allele frequencies are caused solely by genetic drift, based on the Wright-Fisher model (Fisher 1930; Wright 1931). The standardized variance in allele frequency was calculated using two moment-based *F*-statistic estimators, namely F_s (Jorde & Ryman, 2007) and F_c (Nei & Tajima 1981) under the model that assumes animals sampled will contribute to future generations. This was implemented using the program NeEstimator v2.1 (Do et al. 2014). This analysis was performed using population samples from MZNP which consisted of adults and foals. The KNR and DHNR were omitted from this analysis due to the small sample size and the recent admixture.

3.4 Results

3.4.1 Genetic variation in different populations and time periods:

Deviations from HW proportions, following Bonferroni correction (Rice, 1989), were only observed in two loci from two populations namely: COR014 (MZNP, DHNR, 2015-2016) and UCDEQ505 (DHNR, 2015-2016). One locus, COR014, showed evidence of null alleles in the MZNP population at both time periods. The locus, UCDEQ505 showed evidence of null alleles in one population at one time period (2015-2016) according to the MICRO-CHECKER results (Supplementary Table 2). Thus, all further analysis was performed with and without the marker COR14. The KNR 2015-2016 population was not tested for null-alleles due to insufficient sample size. Significant gametic disequilibrium was not observed between loci in any population. The markers, HTG15, LEX52 and HTG03 were monomorphic in all CMZ samples for both time periods and were omitted from further analysis. Analysis of microsatellite data identified low to moderate genetic diversity in populations from both time periods (Supplementary Table 3). In the 1999-2001 population, the A_r was 2.069, the average H_o was 0.327 (range of 0.115 to 0.531) and the average H_e was 0.503 (range of 0.356 to 0.720). In the 2015-2016 population, the A_r was 1.86, the average H_o was 0.274 (range of 0.061 to 0.621) and the average H_e was 0.337 (range of 0.119 to 0.627).

3.4.2 Genetic diversity and effective size (N_e): temporal changes within populations

Analysis per population indicated that the highest heterozygosity and A_r was observed for the DHNR population at both temporal periods compared to the MZNP and KNR populations (Table 3.1). Heterozygosity within each reserve (KNR, DHNR and MZNP) declined between temporal sampling periods (Table 3.1). A decline in genetic diversity was observed for DHNR in A_r, which decreased from 2.35 to 2.1 between 2015-2016 and 1999-2001. H_e declined from 0.385 to 0.297 during this same ~16 year time period. In the MZNP population, the mean A_r declined from 1.65 to 1.53 and H_e was reduced from 0.264 to 0.230. In the KNR population, A_r decreased from 1.59 to 1.53 and H_e declined from 0.258 to 0.230 (Table 3.1). However, only the decline in H_e in DHNR was observed to be

statistically significant ($p > 0.05$). The number of private alleles for KNR, MZNP and DHNR in 1999-2001 was 0.143, 0.357 and 0.071 respectively, whereas the frequency of private alleles in these populations in 2015-2016 was 0.0 (Figure 3.1). The temporal variance estimates of N_e ranged from 1.7 to 6 when performing F_c analysis and varied from 1.7 to 18.2 when performing F_s analysis (Table 3.2A). Analysis where the locus COR014 was removed, was similar for F_c analysis but differed for F_s (1.6 to 31.2, Table 3.2B).

Table 3.1: Genetic diversity at the two time-points for the Cape mountain zebra in the three reserves, Mountain zebra National Park (MZNP), De Hoop Nature Reserve (DHNR) and Kammanassie Nature Reserve (KNR). Population genetic diversity over for two periods spanning around 16 years (1999-2001 to 2015-2016) was evaluated based on allelic richness (A_r), observed heterozygosity (H_o) as well as unbiased expected Heterozygosity (H_e) and fixation (F_{IS}) across 11 polymorphic microsatellite loci. Detection of recent bottlenecks were tested by identifying whether Mountain Zebra National Park had significant levels of heterozygous excess using the Two-Phase Mutation model (Wilcoxon P_{TPM} and P_{SMM}) and then confirmed by checking allelic mode shift at mutation-drift equilibrium (A_{DIST}). * = significant temporal change, $p < 0.05$. [1]indicates estimates of A_r are corrected for sample size through rarefaction in HP-RARE using the smallest number of gene copies per population (MZNP = 14, DHNR = 24, KNR = 4) and [2]indicates were analysis was not applicable, since sample size was too small for KNR and in DHNR, the population is an admixture of MZNP and KNR.

Reserve	**Period**	**Sample size**	**A_r[1] (SE)**	**H_o(SE)**	**H_e(SE)**	**F_{IS}(SE)**	**Wilcoxon P_{TPM}**	**Wilcoxon P_{SMM}**	**A_{DIST}**
MZNP	1999-2001	12	2.27(0.33)	0.167(0.07)	0.264(0.09)	0.383(0.09)	0.922	0.922	NA[2]
	2015-2016	75	1.95(0.33)	0.221(0.07)	0.230(0.07)	0.076(0.05)	0.615	0.688	Normal
DHNR	1999-2001	15	2.35(0.21)	0.394(0.07)	**0.385(0.05)**	-0.011(0.10)	NA[2]	NA[2]	NA[2]
	2015-2016	27	2.1(0.21)	0.266(0.07)	**0.297(0.07)***	0.042(0.10)	NA[2]	NA[2]	NA[2]
KNR	1999-2001	9	1.59(0.12)	0.205(0.07)	0.258(0.07)	0.126(0.13)	NA[2]	NA[2]	NA[2]
	2015-2016	4	1.53(0.12)	0.218(0.10)	0.230(0.09)	-0.080(0.12)	NA[2]	NA[2]	NA[2]

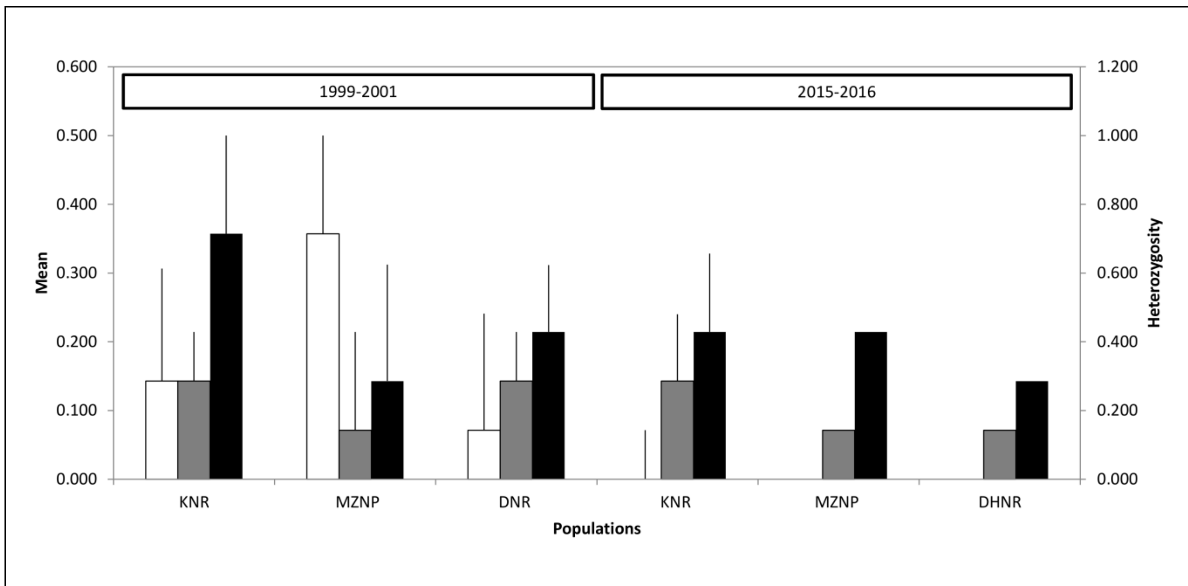

Figure 3.1: Loss of private alleles (red) in a single generation from 1999/2001 to 2015/2016 in each of three CMZ populations. White indicates the frequency of private alleles unique to a population, grey indicates the frequency of locally common alleles (frequency of <= 5%), black indicates the frequency of locally common alleles found in 50% or fewer populations (frequency of >= 5%). Mountain zebra National Park (MZNP), De Hoop Nature Reserve (DHNR) and Kammanassie Nature Reserve (KNR) for the temporal periods 1999-2001 and 2015-2016.

Table 3.2: Estimated effective population sizes for the Mountain zebra National Park (MZNP) using the temporal method. The two analyses used were the *F*c method (Nei and Tajima, 1981) and the *F*s method (Jorde and Ryman, 2007) using the Program NeEstmator ver. 2.1 (Do et al., 2014). P_{crit} = the criterion for excluding rare alleles if the frequency of rare alleles and less than the P_{crit} value they are excluded, GI = the Generation interval, N_e = the estimated effective population size and Min and Max = confidence interval values. A) Indicates analysis with marker COR14 and (B) indicates analysis without marker COR14.

A)

MZNP	F_c		F_s	
	P_{crit} = 0,05	P_{crit} = 0,02	P_{crit} = 0,05	P_{crit} = 0,02
GI	1	1	1	1
N_e	3.2	3.4	3.2	3.2
CI 95% (Min - Max)	1.7 - 5.8	1.9 - 6	1.7 - 18.2	1.8 - 15.1

B)

MZNP	F_c		F_s	
	P_{crit} = 0,05	P_{crit} = 0,02	P_{crit} = 0,05	P_{crit} = 0,02
GI	1	1	1	1
N_e	3.1	3.3	3.1	3.2
CI 95% (Min-Max)	1.5 – 6.2	1.7 – 6.2	1.6 – 31.2	1.7 – 24.1

3.4.3 Bottleneck tests

The bottleneck tests were only carried out on the MZNP, for the two temporal periods. Similar results were obtained for both 80% and 95% SMM (Table 3.1). The DHNR population was not tested as the heterozygote excess method assumes that no recent admixture has taken place in KNR, and the sample size was too low. No significant heterozygote excess was detected for MZNP ($p > 0.10$).

3.4.4 Genetic structure

Principal component analysis (PCA) revealed a clear separation between MZNP and KNR for both the 1999 – 2001 and 2015 – 2016 time periods (Figure 3.2). The position of DHNR was intermediate in the multivariate space between the two relict populations. However, in the 1999-2001 dataset, DHNR appeared closer to the MZNP population, whereas in 2015-2016 it was closer to KNR. In general, populations in 2015-2016 appeared more closely related to each other than in 1999-2001 (Figure 3.2). Similar results were obtained for STRUCTURE analysis

with and without admixture, supporting the occurrence of two distinct genetic clusters (K = 2, Figure 3.3 and Supplementary Figure 2)

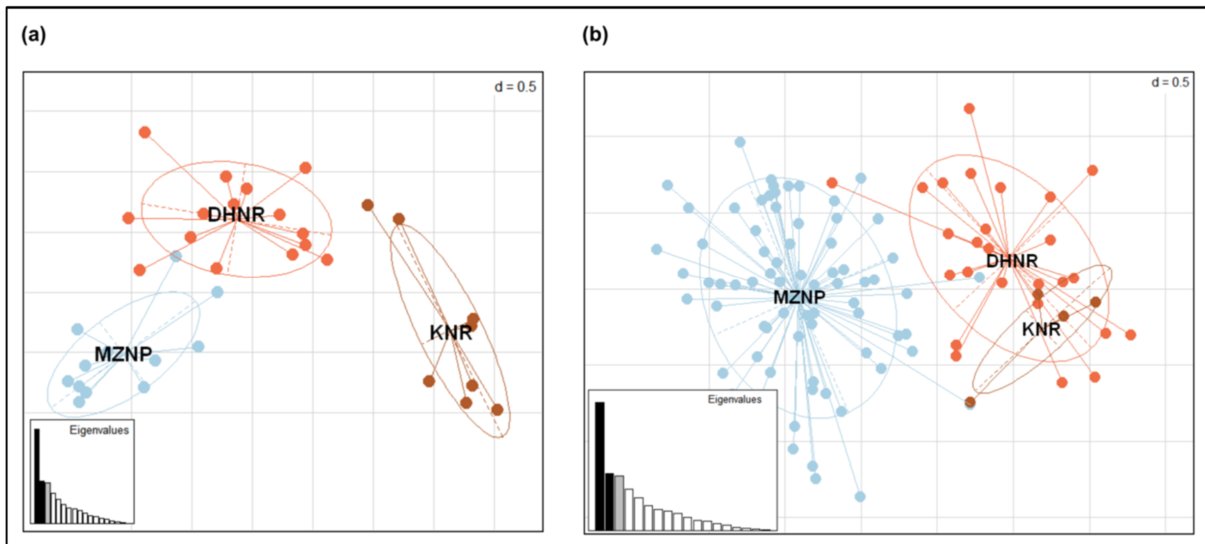

Figure 3.2: Principal Component analysis for three Cape mountain zebra populations at two temporal periods. (a) 1999-2001 and (b) 2015-2016, for the populations Mountain Zebra National Park (MZNP), De Hoop Nature Reserve (DHNR) and Kammanassie Nature Reserve (KNR).

In both time periods, MZNP and KNR were assigned to two distinct clusters with high individual coefficient of membership (*qi*) for 1999-2001 MZNP *qi* = 0.9639; 2015–2016 MZNP *qi* = 0.8465 and for the 1999–2001 KNR *qi* = 0.9715; 2015 – 2016 KNR *qi* = 0.9783. The 1999-2001 DHNR population indicated an approximately 50:50 mixed ancestry with qi_{MZNP} = 0.5313 and qi_{KNR} = 0.4687 (Figure 3.3a), while the 2015–2016 DHNR populations were clustered mainly with KNR (*qi* = 0.9048, Figure 3.3b). Analysis of Molecular Variance (Table 3.3) also provided support for variation between the MZNP and KNR populations. For the 1999-2001 time period (MZNP and KNR populations) F_{ST} comparisons indicated that variation between the populations was 54.4% ($p < 0.001$) and variation within populations was 45.58%. In the 2015-2016 time period variation was between MZNP and KNR populations was lower (F_{ST} = 35.35%) with much higher variation within populations (64.65%).

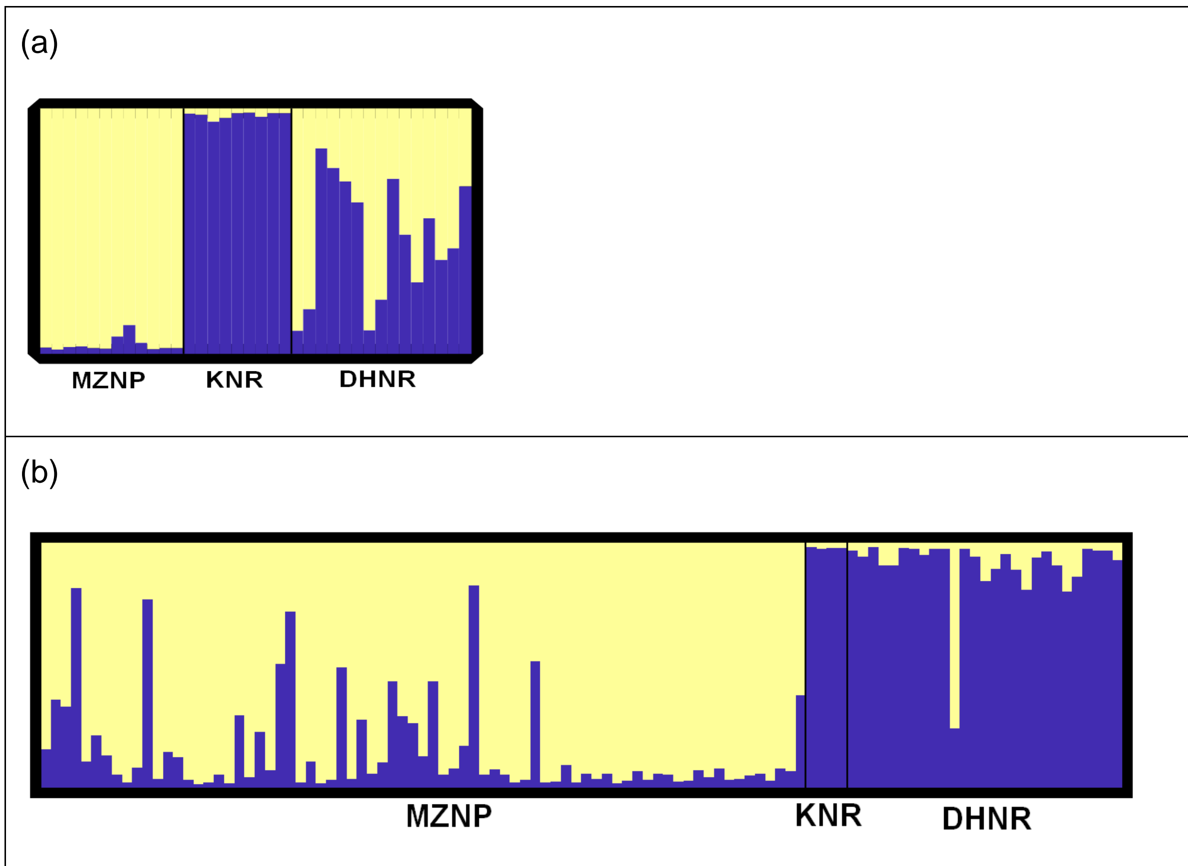

Figure 3.3: Histogram of multilocus population assignment for three Cape mountain zebra populations. The optimal number of clusters for the dataset was K=2. Several models were run with very similar results; the admixture model is displayed here. The Mountain zebra National Park (MZNP), De Hoop Nature Reserve (DHNR) and Kammanassie Nature Reserve (KNR) for the two temporal periods, (a) 1999-2001 and (b) 2015-2016.

Table 3.3: Results from Fst-based hierarchical analysis of molecular variance (AMOVA).

Source of variation	**Percentage Variation**	**F_{st}**	***P*-value**
Between 1999 – 2001 MZNP and KNR populations	54.42	0.544	0.00
Within 1999 – 2001 MZNP and KNR populations	45.58		
Between 2015 – 2016 MZNP and KNR	35.35	0.354	0.00
Within 2015 – 2016 MZNP and KNR populations	64.65		

3.5 Discussion

In this study, we provide a rare test of the genetic consequences of different conservation management strategies among populations of a large mammal. We compare genetic diversity and population structure among three key CMZ populations sampled at two time periods (1999-2001 and 2015-2016). The study was carried out to assess the value of the actions previously proposed to address the reported low genetic diversity in the CMZ (Moodley and Harley, 2005). We also evaluate the importance of management decisions for the long-term persistence of the CMZ, which is the primary objective for the BMP gazetted through the South African government (Birss et al., 2018). We predicted genetic diversity to be more stable in fast-growing populations, and to decline in smaller or slower growing populations. Our data provides strong support for a general reduction in all measures of genetic diversity (A_r, H_o and H_e) across all loci in the generations elapsed between 1999-2001 and 2015-2016. These results suggest that despite a high metapopulation growth rate, the CMZ has lost a significant proportion of its genetic diversity within a single generation.

At the population level, all three reserves lost genetic diversity (A_r and H_e), however only in DHNR was this loss statistically significant. The loss of diversity in DHNR was compounded by decreases in H_o and an increase in the inbreeding coefficient (F_{IS}). In contrast, non-significant increases in H_o and a reduction in F_{IS} were found in the relict populations MZNP and KNR. This slight increase in observed heterozygosity may reflect a sampling effect in KNR, since only four samples were genotyped from this reserve in the 2015-2016 time periods. It is more likely due to several loci within these two populations reaching HWE, where H_o is not significantly different from H_e. Both KNR and MZNP, therefore, did not display significant heterozygous excess.

The N_e for the MZNP was very low suggesting that continued isolation of this population will result in further loss of genetic variation through drift. A small effective population size could assist in explaining how genetic diversity was lost even though the population demographic census size increased. The effective size can be far smaller than the census size due to high variance in reproductive success such as few males fathering most or all of the offspring. However, bias in

this case may be introduced by small sample size, a limited time period between collection of samples and number of microsatellite markers (Nei and Tajima, 1981; Waples and Yokota, 2007). Bias is unlikely to explain entirely the low N_e estimates which are well below $N_e = 50$ and can lead to excessive inbreeding and inbreeding depression (Jamieson and Allendorf 2012). Similar low N_e values have been reported in other species including red deer (*Cervus elaphus*) from Sardinia and Mesola ($N_e = 2$ to 8) as a consequence of bottlenecks and near-extinction (Zachos et al., 2016). Detected differences between as little as 1 generation in small isolated populations may be valuable in monitoring the shift in N_e as new conservation activities are implemented.

Of additional concern is the complete loss of private alleles from all three sampled populations. This suggests rare and low-frequency alleles have likely been lost genome-wide. While a loss in such population-specific alleles may not have an effect on overall heterozygosity (Norris et al., 1999), it represents a loss of alleles potentially important for future adaptation and loss the uniqueness of the KNR and MZNP stocks. Though, the true consequences of this loss in heterozygosity will need to be confirmed using markers, known to be adaptive. This means that a proportion of the historical diversity of the CMZ, conserved through different conservation strategies and present in 1999-2001 has already been lost in the present generation. The loss of unique alleles in MZNP and KNR could also affect an individual or populations ability to adapt and cope with future environmental change. Equally worrying is that the third stock population, inhabiting the Gamkaberg Nature Reserve (GNR), with a growth rate even lower than that of KNR, could also be similarly affected.

The erosion of private alleles may have affected population genetic structure (differentiation) of these populations. Model-based and model-free algorithms all document a decrease in genetic differentiation between the KNR and MZNP stocks, which are now more similar to each other than they were a generation ago. Furthermore, the allele frequencies for DHNR, which were intermediate between its MZNP and KNR founding stocks in 1999-2001, are now clearly more Kammanassie-like, with 90% of genotyped individuals assigned to the KNR cluster (Figure 3.3). In addition, these results are supported by a reduction in F_{ST} (0.544 to 0.354). Such a substantial shift or homogenization in allele frequencies within a

single generation underscores the erosive effect of random genetic drift, even in populations that are expanding demographically, and threatens to undo much of the population benefits of a strategy to restore gene flow.

Major changes in the number of private alleles could also be brought about by non-random mating, where a handful of males dominate most of the breeding opportunities. This idea is corroborated by empirical data showing that in 2005, the population was already male-biased, with a deficit of females resulting in an excess of non-breeding males with limited reproductive potential. Population growth at DHNR has thus declined from 6.6% in 1995–1999 to 4.5% in 1999–2005 (Smith et al., 2007). Thus, DHNR, a population that previously benefitted from admixture, now requires urgent intervention to mitigate this loss. These striking results stress the necessity of establishing studbooks with more hands-on management for all newly founded mixed-stock populations.

The lower growth rates of KNR and GNR has been attributed to lower abundance of palatable grass species in those reserves (Vlok et al. 2005, Lea et al. 2016), and given the evidence of genetic declines reported in this study, the erosive effects of genetic drift and non-random mating can only be rectified through new introductions. The conservative practice of managing these populations separate to protect their uniqueness has had the opposite effect of loss of alleles that made them unique in the first place. We therefore advocate a paradigm shift among the conservation authorities managing these important populations, to try to arrest these worrying population trends, likely to cause further loss of diversity, evolutionary potential and the onset of fitness related problems.

Results and the approach from this study could help design and implement management and conservation strategies in other species with only a few small populations remaining. Genetic monitoring of multiple metrics (e.g., heterozygosity, allelic uniqueness, and effective size) can provide early detection of loss of diversity even when a population is large or growing.

CHAPTER 4

Molecular characterization in the Toll-like receptor 9 gene of Cape Mountain Zebra (*Equus zebra zebra*) from three populations

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Submitted:

Smith, R.M., Kotze, A., Grobler, J.P. and Dalton, D.L. Molecular characterization in the Toll-like receptor 9 gene of Cape Mountain Zebra (*Equus zebra zebra*) from three populations. *Molecular Immunology*, Submitted May 2019. (Reference: MIMM_2019_279)

4.1 Abstract

Toll-like receptors (TLR) are a family of proteins that signal activation of the innate immune response through the recognition of a variety of pathogen molecular compounds. Toll-like receptor 9 forms part of a subfamily that recognizes unmethylated nucleic acids of pathogens such as *Trypanosoma evansi* and Equine herpesvirus 1. Here, we characterized the complete TLR9 gene in Cape mountain zebra (*Equus zebra zebra*) from three populations in South Africa and compared sequences to a variety of horse and donkey breeds. The possible implications of amino acid substitutions on function were also investigated. Overall, we identified six single nucleotide polymorphisms (SNPs). A single SNP (G586S) was non-synonymous, resulting in a substitution in the amino acid sequence, whereas the remaining SNPs were synonymous. The G586S alteration was detected in Cape mountain zebra populations from Mountain Zebra National Park, De Hoop Nature Reserve and from a private game reserve with varying frequency. In addition, adaptive diversity was found to be discordant with variation based on neutral markers. The mutation is unique to the Cape mountain zebra when compared to other equid species. However, the resulting amino acid substitution was found to have minimal interaction with active sites in the protein. Lastly, as with other equid species, the structure of TLR9 is relatively conserved. Future studies can explore the effects of this potentially functional mutation which is highly prevalent in two CMZ reserves and contributes to our understanding of the genetic diversity within adaptive sites of the Cape mountain zebra genome.

4.2 Introduction

To produce an accurate representation of genetic diversity within a species, both neutral and adaptive markers should be used. Previous studies have shown that the assumption that neutral makers, primarily subject to genetic drift, does not correlate to adaptive traits that are subject to selection (Reed and Frankham 2001, Bekessy et al 2003). There are clear patterns that distinguish the neutral markers from adaptive markers. In water vole (*Arvicola terrestris),* adaptive markers from the major-histocompatibility complex (MHC), demonstrated a rapid recovery in diversity after a known bottleneck, whereas neutral markers preserved the signature of the bottleneck (Oliver and Piertney, 2012). The (MHC) has a known role in adaptive immunity (Abbas and Janeway Jr., 2000, Kamath and Getz, 2012). Moreover, Additional adaptive markers are being evaluated to offer a more complete picture of genetic diversity as well to assess adaptive potential (Acevedo-Whitehouse and Cunningham, 2006).

The toll-like receptors (TLRs) are a family of proteins that function by recognizing pathogen-associated molecular patterns (PAMPs) (reviewed by Medzhitov, 2001). Toll-like receptors are type I transmembrane glycoproteins which consist of a N-terminal signal peptide, an ectodomain containing tandem Leucine Rich Repeats (LRR) consensus motifs defined by hydrophobic residues, a single transmembrane and cytoplasmic region including a linker region, toll/interleukin 1 receptor (TIR) domain and a C-terminal tail region (Botos et al., 2011). Toll-like receptor 9 (TLR9) recognizes single-stranded DNA (ssDNA) and cytosine-phosphate-guanine (CpG) dideoxynucleotide motifs present in bacteria and viruses which activates the mammalian immune system (Hemmi et al., 2001). Toll-like receptor 9 is predominantly expressed in the endosomes of peripheral blood mononuclear cells (PBMCs), lymphoid tissues (Hashimoto et al., 2005, Ohto et al., 2015) and neutrophils (Julia et al., 2007). The structure and function of TLR9 has been elucidated by Ohto et al., (2015). These authors identified the CpG binding site and the manner in which TLR9 becomes dimerized. More recently, a second binding site was discovered which augments dimerization and activation of the TLR9 complex (Ohto et al., 2018). A total of 13 TLRs have been identified in mammals (Li et al., 2009). Single nucleotide polymorphisms (SNPs), which are the most abundant type of genetic variation in species (Collins et al., 1998), have been

identified in the TLRs of birds (Grueber et al., 2008), horses and donkey breeds (Manuja et al., 2019); and are associated with increased susceptibility to disease (Skevaki et al., 2015).

Toll-like receptor 9 is well studied in humans (Ashkar and Rosenthal, 2005) and domestic equids (Manuja et al., 2019; Zhang et al., 2008) as well as bovines (Griebel et al., 2005). In mice, type I interferon production is stimulated upon exposure to CpG DNA motifs belonging to Herpes simplex virus 1 and 2 (Hochrein et al., 2004; Krug et al., 2004; Lund et al., 2003), as well as exposure to cytomeglalovirus (Wagner, 2004). Moreover, activation of immune cells against *Trypanosomma cruzi* was observed through signaling of TLR9 and TLR2 (Bafica et al., 2006). Similarly, TLR9 and TLR7 work together in signaling for the increase in type I interferon to distinguish between pathogenic and non-pathogenic acquired immunodeficiency syndrome infections in the cases of Human and Simian Immunodeficiency virus as determined in Sooty mangabey (*Cercocebus atys*) plasmacytoid dendritic cells (Mandl et al., 2008). Horse lungs were demonstrated to have high TLR9 expression as an immune response against unmethylated bacterial DNA (Schneberger et al., 2009). Increased expression of TLR9 and TLR3 and related cytokines have been observed when horses were infected with Equine herpesvirus-1 (Soboll Hussey et al., 2014). Immunostimulation using CpG-DNA was shown to protect horses against *Rhodococcus equi* and *Trypanosomma evansi* through the TLR9 immune pathway (Liu et al., 2009; Manuja et al., 2014). Little is known as to how SNPs in the TLR9 gene affect signaling functionality and the clinical impact these identified SNPs play in immune system (Skevaki et al., 2015), though some SNPs in the TLR9 gene such as the T1237C in the promoter region leads to allergic bronchopulmonary apergillosis in humans (Carvalho et al., 2008). Mutations in close proximity to the TLR9 are correlated to the development of sepsis and multiple organ dysfunction in humans (Zhao et al., 2012). Little research has however been done TLR9 function in wild equids.

The Cape mountain zebra (*Equus zebra zebra*, CMZ) from South Africa have undergone a severe bottleneck event in the early 1930s (Novellie et al., 2002). The CMZ is listed as vulnerable by the International Union for the Conservation of Nature (IUCN) (Novellie, 2008). Additionally, the species is listed on Appendix II by the Convention on International Trade of Endangered Species (CITES) (CITES,

2016), which regulates permission for hunting and trade of animals and their products. In recent years, CMZ have recovered in number to more than 4800 individuals, though genetic diversity within the metapopulation was reported to be low and in need of intervention (Moodley and Harley, 2005). A Biodiversity Management Plan (BMP) was established by conservation agencies to mitigate risks that threaten the long-term survival of the species as well as to facilitate population growth through the development of conservation strategies (Birss et al., 2018). Disease and disease risk management have been highlighted as an objective needed to achieve these goals. Genetic data are an important tool that informs conservation strategies as included in the BMP (Ralls et al., 2018). The aim of the current study was to characterize polymorphisms in the TLR9 gene of CMZ from selected reserves, as well as to investigate the structural implications of the amino acid substitutions.

4.3 Method and Materials

4.3.1 Sample collection

Ethical approval was obtained from the Research Ethics and Scientific Committee (RESC) of the National Zoological Garden, South African National Biodiversity Institute (NZG, SANBI, Ref: NZG/RES/P17/19) and the Animal Ethics Committee of the University of the Free State (Ref: UFS-AED2017/0011). Additionally, this project was approved by the Department of Agriculture, Forestry and Fisheries of South Africa (DAFF) under Section 20 of the Animal Disease Act 1984 (Ref: 12/11/1/1/8). A total of 60 Ethylenediaminetetraacetic acid (EDTA) whole blood samples collected in 2016 from three South African reserves; Mountain Zebra National Park (MZNP), De Hoop Nature Reserve (DHNR), and a private reserve in Grahamstown (GTR), were included in this study. Samples of animals from MZNP and DHNR were obtained while the animals were in the process of being translocated to other reserves. The blood samples from GTR were obtained through the NZG SANBI Biobank.

4.3.2 DNA extraction, amplification and purification

DNA was extracted from the CMZ samples using the Quick-DNA™ Universal Kit (Zymo Research) according to the manufacturer’s instructions. The eluted DNA

concentration of eluted DNA was determined using a Nanodrop™ (Thermo Scientific) Spectrophetometer and the DNA was used either used immediately used or stored at -20°C. Polymerase chain reaction of TLR9 was conducted using primers (Table 4.1), developed for horse (*Equus caballus*) and donkey (*Equus asinus*) (Menzies and Ingham, 2006). Briefly, DNA was amplified using 10 µl Taq DNA Polymerase Master Mix RED (Ampliqon©), 10 pmol of each primer, 10 ng of DNA, made up to 20 µl with nuclease-free water. The samples were run at 95°C for 5 min, followed by 35 cycles of 95 for 30 sec, 60°C for 30 sec and 72°C for 1 min and a final extension at 72°C for 10 min. Amplicons were run on a 2% agarose electrophoresis gel to confirm fragment size using the following conditions: 2% agarose gel, 145 volts for 40 min. PCR products were purified using Exonuclease I (10 U) (Thermo Scientific inc.) and FastAP Thermosensitive Alkaline Phosphatase (1U) (Thermo Scientific inc.). The PCR products were sequencing using the BigDye™ Terminator 3.1 Cycle sequencing kit (Applied Biosystems) as per the manufacturer's instructions, with an annealing temperature of 53°C. Briefly, a master mix was prepared using 4 µl BigDye™ Terminator 3.1 Ready Reaction Mix, 1 X BigDye™ Terminator v1.1 and v3.1 sequencing buffer, 3.2 pmol of the forward or reverse primer, 5 µl PCR product as a template and the reaction was made up to 10 µl with deionized water. Samples were run on a thermocycler under the following conditions: 94°C for 2 min; 40 cycles at 85°C for 10 sec, 53°C for 10 sec, and 60°C for 2 min 30 sec, then samples were stored at 4°C until used. Samples were purified using the BigDye® XTerminator™ purification kit as per manufacturer's instructions. In a 96-well plate, 45 µl SAM™ solution was added to wells corresponding to the number of samples. Thereafter, 10 µl of Big Dye® XTerminator solution was added, followed by the entire sequencing product. The mixture was vortexed at room temperature for 30 min at the plate was centrifuged at 2000 rpm. Once complete, the plate was run on a 3130 Genetic Analyser (Applied Biosystems).

Table 4.1: List of Polymerase chain reaction primers used in this study for amplification Toll-like receptor 9 gene in Cape mountain zebra, developed previously (Fisher, 2013).

Name	Forward	Reverse
TLR9_04	CACTCTCACCCAATCTCCAC	CGCAGGGGTTCTTGTAGTAG
TLR9_05	CTCGTGTCCCTGATCCTGA	GAATGCCTTGGTTTTGGTG
TLR9_06	GGCCTCGTGTTGAAGGATAG	AGGTCCAAGGTGAAGCTGAG
TLR9_07	CATCAGTGGAGCTGTGGAG	CACATCTGGCTCAGGGAAT
TLR9_08	TGGACCTCAGCTACAACAGC	CTGCTAGGAAACCAAACCAG
TLR9_09	CTAGACCTGTCCCAGAATCG	ACCAGCAATGAGAGACCAAA
TLR9_10	CCTTTGTGGACTTCCTGCT	CTCAGGATTACCAGCACCAC
TLR9_11	CCTGAGCTATGATGCCTTTG	ATAGGCAGAGAGGCAAGGTC

4.3.3 Data preparation and genetic statistical analysis

Sequences obtained were inspected and edited using Geneious v10.2.6 (Biomatters inc., https://www.geneious.com). Sequences were trimmed and edited manually using geneious software. A multiple alignment was created for each of the TLR9 gene fragments. We then examined the alignment to identify positions with two overlapping peaks in the chromatogram that had reduced height, or differences between individual samples. We aligned all gene fragments spanning the TLR9 gene. Two consensus sequences namely, CMZ set 1 and 2, of the TLR9 gene were generated from the consensus sequence. Bases where SNPs were present were edited to represent all possible variations within the 3 CMZ populations. Haplotypes were not considered since the non-synonymous SNPs are expected to independently affect protein function.

4.3.4 Functional changes associated with amino acid substitutions

Toll-like receptor 9 amino acid and nucleotide sequences were obtained from Genbank. The nucleotide sequences obtained consisted of horse breeds: (*E. Caballus*, Accession No.: DQ390541, ALE31224.1 AMA97029.1) and donkey breeds (*E. asinus*, Accession No.: ALE31225.1, NW_014638427), while the amino acid sequences were obtained from *E. caballus* in UniProt (Accession No. Q2EEY0) (https://www.uniprot.org/). The annotations of the horse TLR9 were added using the *E. caballus* (Q2EEY0) protein and functional information found on

UniProt (https://www.uniprot.org/) and corroborated with annotations and 3D structures determined previously (Ohto et al., 2015). The CMZ sequences were further analyzed by comparing the amino acid domains as per the predictions obtained from the online utility expasy prosite (http://prosite.expasy.org) with the horse annotated sequence. To assess the effect of amino acid substitutions on the function of the TLR9 amino acid sequences, we used the Sorting Intolerant from Tolerant (SIFT) algorithm (https://sift.bii.a-star.edu.sg/) which was used to sort amino acid substitutions between tolerable and intolerable substitutions. Sites of Asparagine (N)-linked Glycosylation in the CMZ amino acid sequence was predicted using the online tool, NetNGlyc, available from the Centre for Biological Sequence Analysis prediction service (http://www.cbs.dtu.dk/services/). The results were compared to sites previously identified in horses and other orthologs (Ohto et al., 2015). The properties of the amino acid substitutions found were compared to the sequence and functional variability that was observed in related equids (Manuja et al., 2019). Lastly, substitutions were evaluated for their influence in TLR9 dimerization as described recently (Ohto et al., 2018).

4.4 Results

4.4.1 Identification of single nucleotide polymorphisms and diversity

A total of six SNP variants were identified in the nucleotide sequences of the CMZ (Table 4.2) in exon 2 of TLR9. The MZNP and DHNR populations harbored all six SNPs, whereas five SNPs were observed in the GHT population. The frequency of the SNPs varied in the three populations, with a higher prevalence being observed in MZNP, followed by DHNR and GHT (Table 4.2). Of the six identified SNPs, five are synonymous and one was non-synonymous (G1756A) which resulted in a G586S substitution in the amino acid sequence. The non-synonymous SNP had a lower prevalence in MZNP (GA: 35%, AA: 25%, GG: 25%) compared to DHNR (GA: 25%, AA: 65%) and GHT (GA: 10%, AA: 70%).

Table 4.2: Single nucleotide polymorphisms (SNPs) detected in the Toll-like receptor 9 gene of Cape mountain zebra from three reserves namely; Mountain Zebra National Park (MZNP), De Hoop Nature Reserve (DHNR) and Grahamstown (GHT). The amino acid (AA) residue, the position and the respective DNA position are shown. Only one amino acid substitution was identified as non-synonymous, represented by (*) and the remaining SNPs are synonymous. nd indicates no data.

AA position	S205		V312		P415		G586S*		E908		L991	
DNA position	G615A		G936C		C1245T		G1756A		G2724A		C2973A	
Total	20%	GA	23%	GC	30%	CT	23.3%	GA	28%	GA	30%	CA
	50%	AA	53%	CC	52%	TT	53.3%	AA	58%	AA	52%	AA
	5%	GG	8%	GG	7%	CC	8.3%	GG	5%	GG	8%	CC
	25%	nd	15%	nd	12%	nd	15%	nd	8%	nd	10%	nd
MZNP	15%	GA	35%	GC	50%	CT	35%	GA	50%	GA	50%	CA
	65%	AA	25%	CC	20%	TT	25%	AA	20%	AA	25%	AA
	0%	GG	25%	GG	20%	CC	25%	GG	15%	GG	25%	CC
	20%	nd	15%	nd	10%	nd	15%	nd	15%	nd	0%	nd
DHNR	45%	GA	25%	GC	25%	CT	25%	GA	30%	GA	35%	CA
	25%	AA	65%	CC	65%	TT	65%	AA	65%	AA	60%	AA
	0%	GG	0%	GG	0%	CC	0%	GG	0%	GG	0%	CC
	30%	nd	10%	nd	10%	nd	10%	nd	5%	nd	5%	nd
GHT	0%	GA	10%	GC	10%	CT	10%	GA	5%	GA	5%	CA
	60%	AA	70%	CC	70%	TT	70%	AA	90%	AA	70%	AA
	0%	GG	0%	GG	0%	CC	0%	GG	0%	GG	0%	CC
	40%	nd	20%	nd	20%	nd	20%	nd	5%	nd	25%	nd

4.4.2 Function and structure

A comparison of amino acid sequence alignments between CMZ and other Equine species (Figure 4.1) indicated that the amino acid substitution, G586S was unique to the CMZ, with the Gly residue being conserved for other Equine species. Analysis of amino acid substitutions between species also revealed the amino acid substitution, Q860R, is exclusive to CMZ. An additional two amino acid substitutions, I420V and S970R were common to CMZ and donkey breeds but was not present in horse breeds. Amino acids substitutions I420V and S970R that were found in both CMZ and donkey species, as well as the G586S substitution were assessed using the structural and functional algorithms. The SNP, Q860R is located five residues upstream of the TIR domain. Structural information for TLR9

is currently only known up until residue 810, thus we could only use SIFT to determine that the substitution was tolerable within the protein sequence. The I420V and S970R SNPs were predicted to be tolerated substitutions as reported previously (Manuja et al. 2019) (Table 4.3). G586S was also determined to be tolerated.

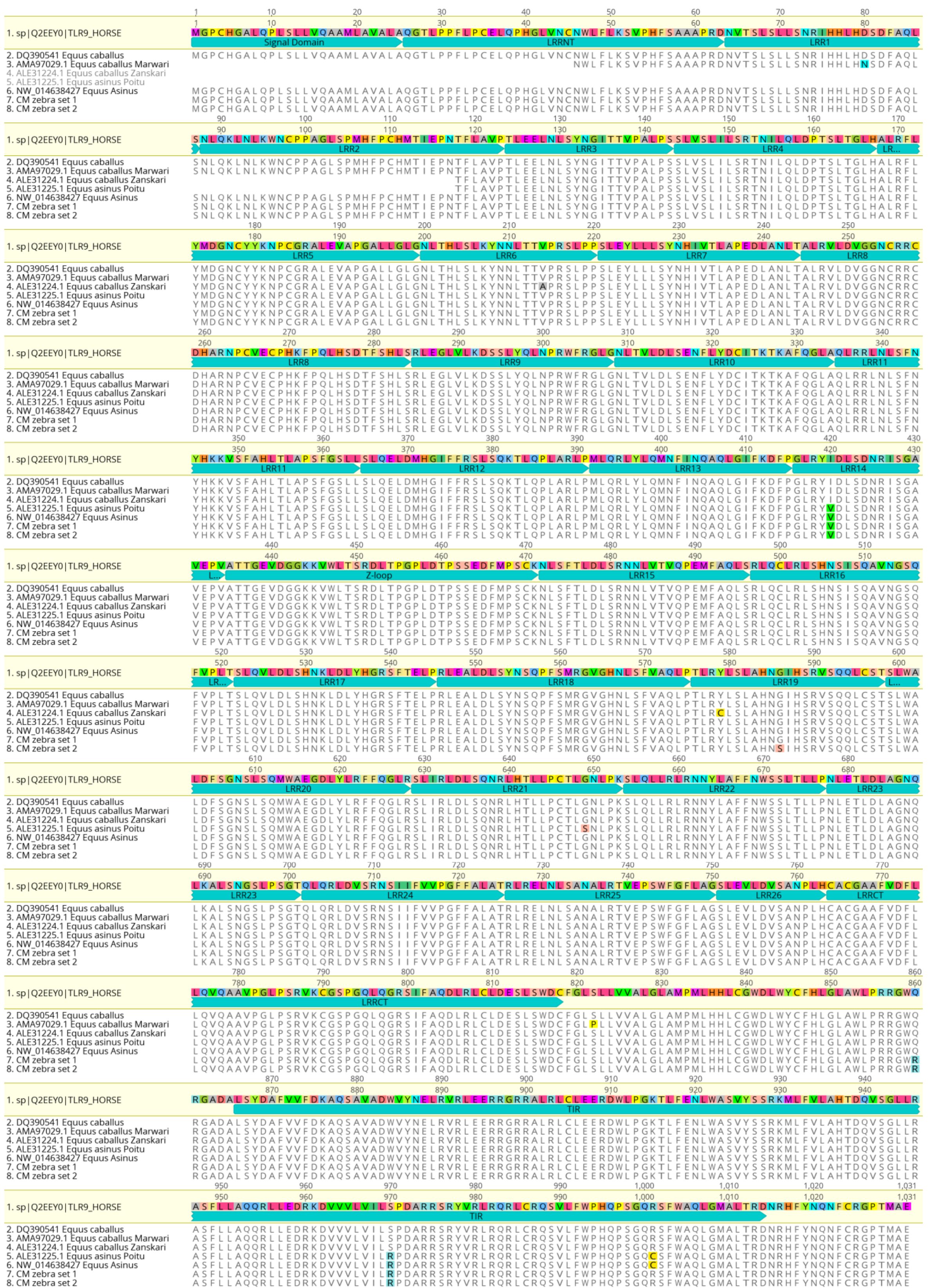

Figure 4.1: Comparison of the Two Cape mountain zebra amino acid sequence sets to previously deduced sequences of TLR9 for various horse (*Equus caballus*) and (*Equus asinus*) donkey breeds. Aligned domains are annotated in blue, and

amino acid variants from the horse reference sequence are highlighted. The domains shown are the leucine-rich repeat (LRR) domains 1-26, the N-terminal (LRRNT) and C-terminal (LRRCT) leucine-rich repeat domains, the Z-loop and the toll-interleukin 1 receptor (TIR) domain.

Table 4.3: The amino acid substitutions and characteristics, previously determined in various equines, compared to the Cape mountain zebra (CMZ) set 1 and set 2 containing representative amino acid substitutions.

Amino Acid Position	Substitutions	Domain	Functional Characteristics	Polarity	Class	Charge	Species	Ref.
D80N	Aspartic acid / Asparagine	LRR	Tolerated	A P to P	Acid to amide	Negative to Neutral	Horse (Marwari)	Manuja et al. 2019
V214A	Valine / Alanine	LRR	Affect protein function	Both NP	Both aliphatic	Both Neutral	Horse (Zanskari)	Manuja et al. 2019
I420V	Isoleucine / Valine	LRR	Tolerated	Both NP	Both aliphatic	Both Neutral	Donkey, CMZ both	Manuja et al. 2019
Y579C	Tyrosine / Cysteine	LRR	Affect protein function	P to NP	Aromatic to sulfur-containing	Both Neutral	Horse (Zanskari)	Manuja et al. 2019
G586S	Glycine /Serine	LRR	Tolerated	NP to P	Aliphatic to hydroxyl-containing	Both Neutral	CMZ Set 2	Current Study
S822P	Serine / Proline	none	Tolerated	P to NP	Hydroxyl-containing to Cyclic	Both Neutral	Donkey , Horse (Marwari)	Manuja et al. 2019
Q860R	Glutamine / Arginine	none	Tolerated	P to basic	Amide to basic	Neutral to Positive	CMZ both	Current Study
S970R	Serine / Arginine	TIR	Tolerated	P to basic P	Hydroxyl-containing to basic	Postive (pH) to Neutral	CMZ both, Donkey	Manuja et al. 2019

Comparison of sites of amino acid modification, namely, the 13 sites of N-linked Glycosylation, the 8 disulfide bond sites as determined previously for the horse TLR9 (Ohto et al, 2015) were determined to be identical in CMZ. The active binding site where the ligand, CpG DNA and inhibitory DNA, was identified in horse was also conserved in the CMZ. All equine amino acid sequences in the multiple alignments were highly similar for all 26 LRR domains and the 1 TIR domain. Moreover, the site of the post-translational modification, the z-loop, was highly conserved in all equid species. The SNP, G586S was located within LRR19 domain. This residue does not form part of the alpha-helix or Beta sheet secondary structure within LRR19 but is located five residues downstream from the beta-sheet.

4.5 Discussion

This study is the first to report characterization of the TLR9 gene in CMZ via direct sequencing. Single nucleotide polymorphisms present in functional genes such as TLR genes are considered an important component to evaluate an organism's adaptive potential (Grueber et al., 2012). Here, we identified a single non-synonymous SNP in three populations of CMZ. TLR9 gene is thus highly conserved, as has been reported for other Equus species. Manuja et al. (2019) identified two non-synonymous alterations in Marwari horses, two in Zanskari horses and four non-synonymous SNPs in Poitu donkeys. The observed variation of frequency is consistent with the reported population size on reserves. Smaller populations are reported to have reduced adaptive potential compared to larger populations (Willi et al., 2006). The MZNP includes 1191 individuals (Hrabar and Kerley, 2015), DHNR consists of approximately 115 individuals (Hrabar and Kerley, 2015) and GHT is a private reserve and most likely consists of less than 100 individuals (population size is not known). However, genetic diversity at neutral markers is slightly lower in the MZNP (H_o = 0.221) compared to DHNR (H_o = 0.266, Kotze et al., unpublished). Discordance between microsatellite heterozygosity and functional diversity has been previously reported. Reed and Frankham (2001) showed through a meta-analysis, that direct evidence of selection in alleles, is needed in order to estimate local adaptive potential. In South Africa, hunting and habitat destruction over the last 300 years decimated

populations of CMZ with only three relic populations surviving to the present day (Moodley and Harley, 2005). The higher genetic diversity observed in the DHNR may be attributed to this population being founded from two of the relict populations, namely, MZNP and Kammanassie Nature Reserve (Smith et al., 2007). This data, however, cannot infer adaptive potential. Thus, functional genomic variation in addition to heterozygosity at neutral markers should be monitored to ensure that CMZ populations in National and private reserves are able to cope with future environmental change.

In this study, all equine species compared had a highly conserved amino acid sequence which is consistent with the domains annotated previously which was identified by the xLxxLxLxxN motif (Ohto et al., 2015), showing that CMZ, as well as other equids, have 26 LRR along with an LRRNT and LRRCT. Thus, we inferred that structure should also be relatively conserved. The CpG binding regions were determined to be at LRRNT to LRR 10 while the other regions form a dimer with another TLR9 whose LRR 20-22 domains interact with the CpG DNA (Ohto et al., 2015). Moreover, during dimerization LRR18 and LRR19 of the two protomers formed a tight interaction (Ohto et al., 2018). In CMZ, the G586S substitution was not in close proximity to the CpG binding sites but was situated close to a secondary binding D534 site that augmented the activation and dimerization of the TLR9 protein. It is unclear if the Ser residue has an effect on this interaction and thus further studies should be conducted to determine this. Two populations, DHNR and GHT, were predominantly monomorphic for the G586S substitution, based on the predictive algorithms in this study, the Ser residue was identified as a tolerated substitution. Since homozygous and heterozygous individuals were present in MZNP at proportions expected for population that mates randomly, it is likely that these mutations don't directly affect protein function.

Mutations that lead to structural changes in functional genes in small populations may be the most direct indication of a population's survivability (Lynch et al., 1995; Willi et al., 2006). Thus, future studies should screen additional toll-like receptor genes as well as other adaptive genes in order to identify alleles that are under selection, which can be used to offer a better understanding of the adaptive potential of the species. This work shows that it may not sufficient to identify SNPs

within exonic regions for the purpose of conservation, rather, that SNPs, directly linked to functional activity should be used when measuring adaptive potential. Identifying genes that have known effects on function may better dictate which populations to prioritize when carrying out translocations or when reinforcing existing populations. This study enables future work, exploring the effects of this potentially functional mutation in the LRR motif of TLR9 which is highly prevalent in two CMZ reserves and contributes to our understanding of the genetic diversity within adaptive sites in the CMZ genome.

CHAPTER 5

Translocation a potential corridor for equine piroplasms in Cape mountain zebra (*Equus zebra zebra*)

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5.1 Abstract

Translocation of animals in fragmented habitats is an important means of dispersal and gene flow, however, the movement of animals has led to the spread of various diseases globally and wildlife are often the reservoirs of these diseases. Currently, Cape mountain zebra are translocated within South Africa as a management method for augmentation of isolated and fragmented populations. The movement of pathogens due to translocations in local regions have gone largely unchecked, particularly where there may still be isolated regions that can be negatively affected. Equine piroplasmosis is a tick-borne disease caused by *Theilaria equi* and/or *Babesia caballi* reported to occur in equids (Bhoora et al., 2010; Zweygarth et al., 2002). Here, the presence of *T. equi* and *B. caballi* was detected in 137 clinically healthy Cape mountain zebra from three South African reserves, Mountain Zebra National Park, De Hoop Nature Reserve and Karoo National Park using the multiplex EP real-time PCR assay. We observed 100% prevalence for *T. equi* and identified only one animal from MZNP with *B. caballi*. These results affirm that precautions should be taken prior to founding new populations of Cape mountain zebra and that potential farms and properties adjacent to prospective reserves should be screened for the presence of the organisms in order to mitigate risks of infection to domestic animals.

5.2 Introduction

The Cape mountain zebra (CMZ) is endemic to South Africa and is a subspecies of mountain zebra (*Equus zebra*). Due to various threats including hunting and habitat loss, the population declined in the early twentieth century to less than 100 animals (Moehlman, 2002). From the 1960's, due to population growth in the Mountain Zebra National Park (MZNP), approximately 91% of CMZ were translocated to 75 different localities, including 18 reserves that are formally protected (Hrabar et al., 2016; Hrabar & Kerley, 2015). Currently, it is estimated that there are more than 4000 CMZ (Hrabar et al., 2016). In 2018, the Biodiversity Management Plan (BMP) for the CMZ was published (Birss et al. 2018). As a management strategy it was recommended that in order to maintain the current population growth rates while preventing the loss of genetic diversity, animals need to be further translocated into different areas. Although translocation is considered an important management tool for dispersal in isolated species (IUCN, 1987), it is also known as a mode for the spread of diseases (Fèvre et al., 2006; Woodford & Rossiter, 1994). Humans and wildlife in the areas receiving translocated animals are particularly at risk of infection (Kock et al., 2010). For example, plains zebra (*Equus quagga*) translocated from Namibia to Spain, is suspected as the mode of transmission of African Horse Sickness to domestic horses (Mellor et al., 1990; Rodriguez et al., 1992).

Equine piroplasmosis (EP) is a tick-borne disease of equids (horses, donkeys, zebra and mules) (*Equus caballus*; *E. asinus*, Malekifard et al., 2014; Laus et al., 2015; Schein et al., 2018) that is caused by haemoparasites, *Babesia caballi* and *Theilaria equi*. Piroplasm infections were first identified in plains zebra in 1905 (*Equus quagga burchelli*; Koch, 1905). Inoculation of blood from this zebra into a susceptible horse caused fever and parasitaemia, thus providing support that zebra are carriers of the parasite (Koch, 1905). Subsequently, *T. equi* has been identified in CMZ in South Africa (*E. zebra zebra*; Bhoora et al., 2010a; Zweygarth et al., 2002) and Babesia spp. has been reported in plains and Grevy's zebra (*Equus grevyi*) from East Africa (Brocklesby and Vidler, 1965). Clinical signs vary from asymptomatic to severe and in acute cases with fever, hemolytic anaemia, jaundice, hemoglobinuria, and death. Mortality rates are reported to vary between 10% to 50% (De Waal, 1992). However, the disease may be associated with other

non-specific clinical signs (De Waal, 1992). Nucleic acids of *T. equi* and *B. caballi* have been detected in several species including dromedary camels and domestic dog (Beck et al., 2009; Qablan et al., 2012). In a few cases, *T. equi* nucleic acids have been reported in sheep, goat, cow, South American tapir (*Tapirus terrestris*) and South American rodent (*Thrichomys foster*; Spickler, 2018i). In addition, organisms potentially related to *T. equi* have been observed in coati (*Nasua nasua*) (de Sousa et al., 2018), waterbuck (*Kobus defassa*; Githaka et al., 2014) and a Malayan tapir (*Tapirus indicus*; Spickler, 2018).

The occurrence of the disease is associated with the tick vector distribution (De Waal, 1992). Worldwide, ticks from the genera *Dermecentor, Hyalomma, Rhipicephalus* (De Waal, 1990; Mans et al., 2015; Scoles & Ueti, 2015) as well as experimentally infected *Amblyomma* (Scoles et al., 2011) have been identified as vectors for both parasites. In South Africa, *Rhipicephalus evertsi evertsi* and *Hyalomma truncatum* are the vectors of EP (Madder et al., 2013). The tick vectors are reported to be common within the Savannah and Grassland biomes of South Africa, which includes the natural distribution range of CMZ (Spickett, 2013) and prefer domestic and wild equids, although sheep, goats, cattle as well as a range of antelope species will be fed upon (Spickett, 2013, Horak et al. 2017).

Various methods can be used to detect the parasites including the use of blood or organ smears stained with Romanowsky-type stains. Serological tests may be used to measure antibodies and therefore exposure to the parasite. The most commonly used tests are the indirect fluorescent antibody (IFA) assay, enzyme-linked immunosorbent assay (ELISA) as well as the competitive-inhibition ELISA (CI-ELISA). However, there are limitations with serological assays due to problems with non-specificity, cross-reactivity and antibody detection limits (Bruning et al., 1997). Lastly, in cases where it needs to be determined if treatment has eliminated the parasites from a carrier, more labour intensive diagnostic methods may be employed such as *in vitro* culture (Spickler, 2018). Advances in molecular biological techniques have resulted in the improved detection, identification and genetic characterization of many haemoparasites (Caccio et al., 2000 and Nagore et al., 2004). Various PCR tests are available for the identification of *T. equi* and *B. caballi*, and are more sensitive than direct observation. (Bhoora et al., 2010b; Malekifard et al., 2014; Zweygarth et al., 2002). The real-time PCR (qPCR) test

has been described as being more sensitive than standard PCR analysis (Bhoora et al., 2010b; Laus et al., 2015).

In the present study, we investigated the prevalence of equine piroplasms in CMZ from three South African nature reserves in order to increase our knowledge of the epidemiology of equine piroplasmosis in this sub-species. This work will inform conservation management strategies as directed by the BMP of CMZ with regards to mitigating and managing the impact of current and emerging diseases.

5.3 Materials and methods

5.3.1 Ethical considerations

The samples used in this study were obtained through CapeNature and South African National Parks (SanParks) and the National Zoological Garden, South African National Biodiversity Institute (NZG SANBI). Ethical clearance was obtained through the University of the Free State's Animal Ethics Committee (Ref: UFS-AED2017/0011), The NZG Research Ethics and Scientific Committee (Ref: NZG/RES/P17/19) and approval was also granted under the Department of Agriculture Forestry and Fisheries Section 20 of the Animal Disease Act 1984 (Ref: 12/11/1/1/8).

5.3.2 Sample collection and DNA extraction

Whole blood was collected in Ethylene diamine tetractic acid (EDTA) vacuum tubes from a total of 137 CMZ at the two points, 2016 and 2018 (Supplementary Table 1). These Samples were collected from individuals from MZNP (n = 100) prior to translocation to a holding farm in the Eastern Cape for resale, and a private game reserve in the Western Cape respectively. In addition, samples collected from Karoo National Park (KNP, n = 10) (Figure 1) were obtained from the NZG Biobank. The blood was stored at the NZG Biobank at -20°C until use. DNA was extracted from 500 µl of whole blood using the Quick-DNA™ Universal Kit (Zymo Research) as per the manufacturer's instructions. The extracted DNA was stored at -20°C until analysis.

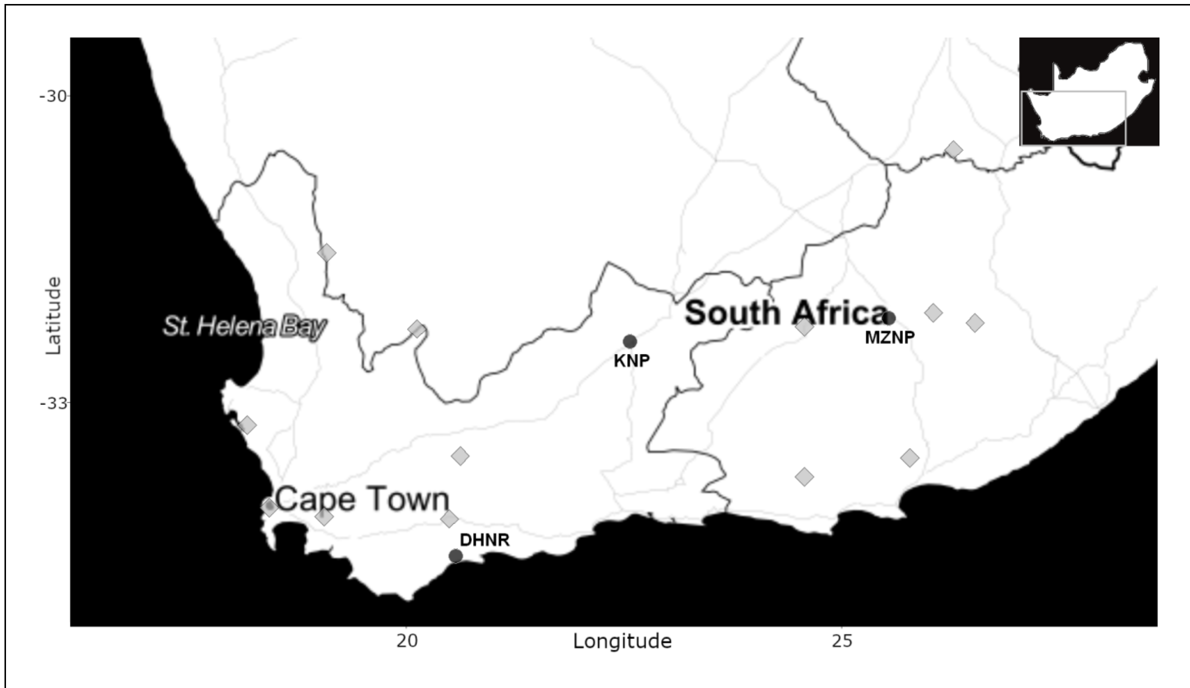

Figure 5.1: A map of south-western South Africa, showing the localities of the 3 reserves from which samples were obtained (black circles) namely Mountain Zebra National Park (MZNP), De Hoop Nature Reserve (DHNR) and Karoo National Park (KNP). The grey diamonds show other known municipal nature reserves and national parks which contain Cape Mountain zebra sourced from Cradock, Eastern Cape, South Africa.

5.3.3 Statistical analysis

Confidence intervals (CI) of the sample proportion were determined for a representative sample at a 95% confidence level for the populations based on Czaplewski et al., (1983). The Formula is as follows:

$$CI = Z\sqrt{\frac{pq(N-n)}{n(N-1)}}$$

Where: *CI* is the confidence interval, Z is the Z-index (Set to 1.96 for 95% Confidence), n is the sample size, *p* is the assumed proportion of population infected (Set at 0.5, for a conservative estimate), *q* is (1-*p*) and *N* is the total population size.

5.3.4 Quantitative PCR assay

The presence of *T. equi* and *B. caballi* was detected using the multiplex EP real-time PCR (qPCR) assay as described by Bhoora et al. (2018). The samples were run using Luna® Universal qPCR Master Mix (New England Biolabs) as per manufacturer's instructions. Briefly, reactions prepared in a final volume of 20 µl contained 10 µl Luna universal probe qPCR master mix (New England Biolabs), 2 µl of 10 x primer/probe mix and 5 µl of template DNA. Quantitative PCR assays were performed using the Quantstudio™ 5 Real-time PCR system (Thermo Fisher Scientific). Cycling conditions included enzyme activation at 95°C for 30 seconds, followed by 45 cycles of 10 seconds at 95 ºC, 45 seconds at 60 ºC. Data were analysed using QuantStudio™ Design analysis software.

5.4 Results

5.4.1 Quantitative PCR assays

All CMZ included in this study tested positive for *T. equi* and two specimens were identified to have a co-infection of *T. equi* and *B. caballi*. A summary of these results can be found in Table 5.1 and Supplementary Table 4. Quantification cycle (C_q) values of each of the animals are provided in Table 5.1. The multiplex EP qPCR assay detected *T. equi* parasite DNA in 100 % of samples tested; with C_q values between 19 and 31 indicating high levels of parasitaemia. In contrast, *B. caballi* was detected in only 2 of the 137 samples (~ 1.5%) and as a mixed infection with *T. equi*. One of these samples was detected at a C_q value below the reported 95% sensitivity of the assay. At higher C_q values, the sensitivity of the assay is highly compromised and therefore results below the reportable range of the assay are regarded as false-positives. The samples for MZNP and DHNR were shown to be statistically representative samples of the population determined through the confidence interval of the sample proportion. Based on these results we show that 100% of the populations MZNP (±9.38 CI) and DHNR (±16.57 CI) are infected with *T. equi*, and that only 2% (±9.38 CI) of MZNP is infected with *B. caballi*.

Table 5.1: Summary of the real-time PCR results obtained when screening the Cape Mountain zebra. Nd = nothing detected, CI = confidence interval at 95%.

			T. equi		*B. caballi*		
Reserve	**Population**	**Samples**	**No of (+)**	**Prevalence**	**No of (+)**	**Prevalence**	**CI (95%)**
MZNP	1191	100	100	100%	2	2%	±9.38
DHNR	115	27	27	100%	nd	0%	±16.57
KNP	842	10	10	100%	nd	0%	±30.82

5.5 Discussion

In this study, we report the prevalence of equine piroplasmosis in CMZ (*Equus zebra zebra*) from MZNP, KNP and DHNR. Molecular screening using qPCR detected *T. equi* parasite DNA in 100% of samples, while *B. caballi* infections could only be detected as a mixed infection in one of the samples (animal from MZNP). The results reported here are similar to previous studies where *T. equi* was detected in ten CMZ in the Bontebok National Park (100% tested positive; Bhoora et al., 2010a) and six CMZ from the KNP (100% tested positive, Zweygarth et al., 2002). Equine piroplasmosis is endemic in South Africa and the associated tick vectors have an overlapping distribution, thus it is not surprising that CMZ were found to be positive for *T. equi*. The zebra tested in our study were positive for *T. equi* with C_q values that range between 19 and 35. In a tick transmission study conducted by Ueti et al. (2005), the authors used a real-time PCR assay targeting the equi merozoite (*ema-1*) gene of *T. equi* to quantify the parasite load required for ticks to successfully transmit the parasite from chronically infected horses. The authors report that ticks infected with a minimum dose of $10^{5.5}$ parasites/ml were able to transmit the parasites. Determining the minimum parasite dose required by the vectors in South Africa to transmit these parasites does not fall within the scope of this study and therefore a tick transmission study was not conducted. However, we could infer that based on the 95% sensitivity of the *T. equi* qPCR assay (Bhoora et al., 2018), at a cut-off C_q value of 37, the multiplex EP assay could detect as little at $10^{5.1}$ parasites/ml. Given that the observed C_q values of *T. equi* detected in zebra, fall below the reported cut-off C_q of 37, we can conclude that the observed parasitaemia is zebra could allow for successful transmission by the tick vectors.

Previous studies also reported lower prevalence of *B. caballi* infections in horses (Bhoora et al., 2010b; Zweygarth et al., 2002). Lower prevalence of *B. caballi* infection has also been reported in zebra. A study conducted by Bhoora et al. (2010a) that included 70 plains zebra and ten CMZ samples, detected *T. equi* parasite DNA in 90% of the samples tested, while *B. caballi* could only be detected in 27% of the samples at high C_q values, indicative of low parasite parasitaemia. Zweygarth et al. (2002) reported two out of six CMZ obtained from KNP tested positive for *B. caballi* (Zweygarth et al., 2002). In addition, three animals tested positive for *B. caballi* from the Bontebok National Park (Zweygarth et al., 2002). It has been previously noted that *B. caballi* is typically present in their respective hosts at very low parasitaemia at 1%-0.01% (De Waal, 1992). In addition, *T. equi* has been found to remain as a lifelong infection, whereas *B. caballi* occurs in its host for approximately 1.5 years (Rüegg et al., 2008).

The CMZ sampled in this study showed no indications of chronic infection, thus it is highly likely that sub-clinical infection is common in CMZ. Previous reports have shown a prevalence of 26.4% of *T. equi* in clinically healthy horses from six geographical regions within Israel, thus providing support for sub-clinical infection (Steinman et al., 2012). In endemic areas (or enzootic) such as South Africa, constant infections with equine piroplasmosis induces stable immunity and therefore a state of endemic stability. This is similar to the observation in cattle, where super-infections with babesiosis have been reported to induce stable immunity (De Vos, 1979). Fenton and Perkins (2010) suggested that in certain cases, it may be advantageous for the host to tolerate the parasitic infection/s rather than attempt to clear them in order to maintain a more stable, controlled dynamic for the entire parasite community.

Cape mountain zebra are currently regarded as a single meta-population where dispersal is exclusively through translocation activities. It has been suggested that in order for the population to continue to expand and maintain genetic diversity, animals should be translocated to new suitable localities (Hrabar and Kerley, 2015). A molecular epidemiology study of equine piroplasmosis in South Africa has identified as many as 25 distinct *T. equi* sequences belonging to three distinct genotypes (Bhoora et al., 2010a). In addition, eleven distinct *T. equi* sequences have been observed in horses from Sudan (Salim et al., 2010) and genetic

variants within *T. equi* has been identified in Spanish horses based on 18S sequence data (Criado-Fornelio et al., 2004; Nagore et al., 2004). In addition, Githaka et al. (2014) reported an organism potentially related to *T. equi* was transmissible to waterbuck in Kenya. A study in Kenya conducted by Hawkins et al. (2015) reported infection in zebra (*Equus grevyi*) and due to their proximity to domestic donkeys, the authors suggested that transmission between the two species was likely. Thus, precautions pertaining to the movement of animals are recommended when new reserves are selected for founding CMZ populations, to ensure these vectors and novel genotypes are not introduced into non-endemic areas. In addition, the movement of zebra that test positive for equine piroplasms may be a risk to other equids and possibly to other animal species in proximity to their release location. In equids, particularly horses, the control of piroplasmosis infections is desirable in order to avoid abortions, intrauterine transmission and clinical disease which restrict the international movement of horses for equestrian sporting events.

In conclusion, this study provides additional data with regards to the epidemiology of equine piroplasmosis in CMZ. While CMZ are largely asymptomatic carriers, translocation on to new reserves may prove to be challenging for adjacent farms where domestic equids may be present. It will thus be useful to screen the other reserves from which translocations are set to take place. In addition, it is recommended that when founding new populations in regions where there are no wild equids, surrounding farms first be screened for the presence of equine piroplasms as well as to genotype these piroplasms so as to mitigate risks of transmission to animals that are not currently affected.

CHAPTER 6

Identifying sequence variation in the CATSPER genes 1 - 3 in the Cape mountain zebra (*Equus zebra zebra*)

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6.1 Abstract

Purpose:

The Cape mountain zebra (*Equus zebra zebra*) has recovered from near extinction over more than 8 decades. While their numbers have increased, populations remain isolated, with low genetic diversity. With more than 75 new populations founded and more than 4 800 extant animals, conservation management strategies are being implemented to mitigate risks of loss in genetic diversity and reproductive fitness. The current study was conducted to identify reproductive characteristics that may improve population growth. The calcium channel of sperm - (CATSPER) family 1-4 has been shown to play an important role in hyperactivation of sperm during fertilization. Mutations in these genes lead to reduced fertility or even infertility. We sampled 10 male zebras from a group that were translocated in 2016 as founders for a new population and identified single nucleotide polymorphisms (SNPs) in the CATSPER genes 1-3 via sequencing. Lack of variation was observed in all exons with four SNPs only being identified in the intronic regions, in close proximity to exons 1, 2, 7, 8, 9 of CATSPER1. In conclusion, these results may contribute to the pre-identification of males for new founder populations to ensure population growth and viability and may also be a useful tool for current low producing populations.

6.2 Introduction

Cape mountain zebras (CMZ) are a sub-species of Mountain zebra distributed only in South Africa (Novellie et al., 2002). These animals have recovered from a severe population bottleneck which took place in the 1930s (Hrabar and Kerley, 2013) to a current estimated population size of more than 4 800 individuals. Due to reported low genetic diversity (Moodley and Harley, 2005) a Biodiversity Management Plan (BMP) was developed that focusses on actions and strategies to strengthen the overall population performance, distribution and genetic diversity to ensure population fitness and resilience of the meta-population within the natural distribution range (Birss et al., 2018). Since the 1950s the number of CMZ has gradually increased through translocations to ensure continued population growth and genetic diversity (Novellie et al. 2002). To date, the overall population occurs in 75 localities that include 30 National and provincial parks, with 90% source animals from the Mountain Zebra National Park (MZNP).

Low reproductive success in CMZ mares (foaling rate = 32%) has been reported in MZNP (Penzhorn, 1985). In addition, abnormal sperm heads due to a weak head-neck junction has been identified in a CMZ stallion (Penzhorn and van der Merwe, 1988). Several genes control sperm motility of which the calcium channel of sperm - CATSPER is the most studied (Quill et al. 2001, Ren et al. 2001). The CATSPER protein family are specialized calcium (Ca^{2+}) channel proteins that are expressed exclusively in the sperm flagellum (Hildebrand et al., 2010) and thus directly involved in hyperactivation of the spermatozoa and penetration ability of the zona pellucida (Stauss et al., 1995), sperm motility and fertilizing ability. The CATSPER complex is reported to include four subunits (CATSPER 1-4) and three auxiliary subunits: CATSPERβ, CATSPERδ and CATSPERγ (Navarro et al., 2008; Wang et al., 2009; Chung et al., 2011). CATSPER 1-4 are expressed in spermatozoa and are functional on the principal piece of the sperm tail (Qi et al., 2007). This action is achieved through the use of Ca^{2+} ions that control the swimming behavior through the ion pump action located in the flagellum (Armon and Eisenbach, 2011). CATSPER has been identified as a necessary component for reproductive success in mice (Carlson et al., 2003; Qi et al., 2007; Ren et al., 2001), humans (Avenarius et al., 2009; Hildebrand et al., 2010; Saha et al., 2015; Strünker et al., 2011) and horses (Loux et al., 2013). Mutations leading to infertility have been

reported in all four subunits of CATSPER. In CATSPER 1, two insertion mutations (c.539-540insT and c.948-949insATGGC) are reported that lead to infertility in humans (Avenarius et al., 2009). Mutations in the CATSPER 2 gene also leads to low sperm counts in humans (Zhang et al., 2009) and a copy number variation was identified which caused infertility (Luo et al., 2019). CATSPER 3 and 4 mutations in mice were previously shown to cause infertility (Jin et al., 2007). Mutations which lies within the functional domain of CATSPER 3 (c.193T>C) and CATSPER 4 (c.247A>G, c.157T>C, c.992G>A) genes were identified in humans and are associated with asthenozoospermia (Visser et al., 2011). The current study was undertaken in order to screen the CATSPER 1-3 genes to determine nucleotide variations in CMZ as potential DNA markers associated with improved sperm motility. These markers may serve as a criterion when selecting individuals to be translocated to ensure population growth and viability.

6.3 Materials and methods

6.3.1 Ethical considerations and sample collection

Ethics submissions were approved by the University of the Free State Animal Ethics Committee (UFS-AED2017/0011), the Research Ethics and Scientific Committee of the National Zoological Garden, South African National Biodiversity Institute (NZG SANBI, NZG/RES/P17/19), and the Department of Agriculture, Forestry and Fisheries of South Africa granted a permit under Section 20 of the Animal Diseases Act of 1984 (Ref: 12/11/1/1/8). Samples were collected under a Threatened or Protected Species Regulations Permit (No. 07507) through the Department of Environmental Affairs of South Africa. Blood samples from 10 stallions were collected from the De Hoop Nature Reserve (DHNR). Two males were identified as foals, based on their size, presence of fluffy coat and their deciduous teeth; another two males were designated as sub-adult due to the presence of undescended testicles. Six males were identified as adult stallions.

6.3.2 Molecular methods

Reference sequences from the horse (*Equus caballus*) obtained from Ensembl were used to design primers and included: CATSPER 1 (ENSECAG00000024405), CATSPER 2 (ENSECAG00000020759) and

CATSPER 3 (ENSECAG00000014744). The primers were designed in flanking regions of each exon (Table 1). DNA was extracted from the whole blood using the Zymo Quick-DNA™ Universal (Zymo Research) as per the manufacturer's instruction for biofluid and cells. Extracted DNA was stored at -20°C until further analysis. The DNA fragments were amplified using Taq DNA polymerase Master Mix RED (Ampliqon) in 15 μl reactions and included forward and reverse primers (0.5 μM), 50 ng of genomic template and GC enhancer. The reactions were run under the following conditions: 95°C for 5 min, 35 cycles of 95°C for 30 seconds, annealing using the temperatures listed in Table 1 for 30 seconds, followed by elongation at 72°C for 30 seconds with a final elongation step of 72°C for 10 minutes. Polymerase chain reactions were carried out in a T100™ Thermal Cycler (Bio-Rad Laboratories Inc.). The amplified fragments were purified using Exonuclease I (Thermo Fisher Scientific inc.) and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific inc.) in a thermal cycler at 37°C for 15 minutes followed by 85°C for 15 minutes. Next, the fragments were used as a template for sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems inc.) as per the manufacturer's instructions. Briefly, 1 μl of BigDye™ Terminator v3.1 Ready Reaction Mix, 3.2 pmol of either the forward or reverse primer and 1X BigDye™ Terminator v1.1 & v3.1 5 X sequencing buffer was prepared in a mastermix with 5 μl of the amplified PCR product made up with nuclease-free water to 10 μl. Sequencing was conducted in a thermocycler using the following parameters: denaturation at 94°C for 2 minutes, 40 cycles of 85°C for 10 seconds, 53°C for 10 seconds and 60°C for 4 minutes. The resulting reaction was then purified using the BigDye® Xterminator™ sequencing purification kit as recommended by the manufacturer. The DNA products were sequenced on the ABI 3130 Genetic Analysis (Applied Biosystems inc.). The resulting outputs were analyzed with the sequencing analysis software v6.0 (Applied Biosystems Inc.).

The sequence files were visually inspected and the chromatograms were edited and assembled (forward and reverse sequences) using Geneious® v10.2.6 software (Biomatters Ltd., Kearse et al., 2012) and the default parameters. Low quality sections at the ends of the sequences were trimmed manually. A multiple sequence alignment was carried out for all ten samples. Horse sequences for each of CATSPER1-3 were used as a reference. The resulting alignment was

visually inspected for sequence variants such as insertions, deletions and base-pair variation. Single nucleotide polymorphisms between the horse reference sequence and zebra were not considered here.

Table 6.1: The primers used in the Polymerase Chain reaction for the amplification of Cape mountain zebra CATSPER genes 1–3.

Primer Name	Forward Sequence	Reverse Sequence	Annealing temperature (°C)
CAT1_1	AACCCTCGATGGCTGGAAAG	GGACGGTGAGCAAAGACTCA	62
CAT1_2	TCAGAAGCGCAAAGGTGAGT	GGCTCCCTGGTTCTTACCAC	60
CAT1_3	GCTGCACACCTTGTACCTCT	CAGTCCCATCCCTTGAGCAG	60
CAT1_4	CCTCTGCATCTACGTGGTGG	GGGGGTTGTTCGAACTGTGA	53
CAT1_5	CTTTACCCTGCTCACCCTGG	TTCACCCGGAAAGTCAGGTG	60
CAT2_1	TGAGTCTTAGTACAATGTGT	ATCCTACTCCAGGAGACA	55
CAT2_2	TCTGATCATTCTCTATCATTC	TGTTCCATTCCTGTATCTC	55
CAT2_3	TCTGAGAGGTTTAGATCTC	GAGCTGGGGAATTCTAAC	55
CAT2_4	CTACACTTCTGCTTCAGTAT	GTTTCATAGAAGGTGCTTGTA	55
CAT2_5	CCATATCTTGTAAATGTA	GTATGGATTTAGGGCAAT	55
CAT3_1	GCAGACTTTAGTTGCTAC	CATAGGGTCTGGACTATTC	55
CAT3_2	GCTCTGCAGCTTGATCTG	AGTCAGACACACCTTTCA	55
CAT3_3	GGCATGGCACTGGATACT	CAGCCCTGATTGTCCATC	55
CAT3_4	GGTGTCATCTTCTTATCATTGC	ACTGATGGTCTGGAGTCC	55

6.4 Results and Discussion

Here, we report for the first time, sequence analysis of CATSPER 1-3 in CMZ in order to identify possible nucleotide variations associated with sperm motility. Sequence data covered a range of exons as listed in Table 2, which provided complete and in some cases partial coverage for some of the exons. Partial fragments were obtained either due to the selected primer regions or due to target regions being too long for the sequencing method used. This study revealed that the CMZ males have exons that are highly conserved within the sample set with an absence of SNPs within exons of CATSPER genes 1-3. The role of CATSPER genes in sperm motility is widely reported and the products of these genes are recognized as the most important calcium channels required for the fertility in mammals (Singh & Rajender, 2015). Single nucleotide polymorphisms have been identified in Vrindavani cattle which are associated with sperm motility (Sivakumar et al., 2018). SNPs within the CATSPER genes have been reported to be closely correlated to reduced sperm hyperactivation and motility (Qi et al., 2007). In addition, knock-out studies have demonstrated that CATSPER may cause

infertility in humans without affecting normal sperm production (Singh & Rajender, 2015). The absence of SNPs in this study may be attributed to the number of animals studied. Sivakumar et al. (2018) identified seven bulls out of 122 randomly selected Vrindavani cattle that demonstrated SNPs in CATSPER. Since only a small sample set from a single population was used here, it would be useful to compare variation between each of the isolated populations of CMZ. In addition, lack of SNPs in CATSPER may indicate that these exons are highly conserved in this species and suggests that polymorphisms in coding regions of other genes may be responsible for sperm motility. Lastly, the absence of SNPs identified here, may indicate other ecological reasons for the slow increase in population growth of CMZ. It has been reported that high grass abundance was associated with higher population growth rates and zebra density and less skewed adult sex ratios (Lea et al., 2016). The DHNR was found to have a large proportion of unsuitable habitat, which the majority of the animals grazing on only 30% of the total area of the reserve (Smith et al., 2007).

Table 6.2: Coverage of the exons obtained from the primers that were designed in this study.

Gene	Fragment	Exon covered	Partial/Full	Coverage (%)
CATSPER 1	CAT1_1	Exon 1	Partial	88
	CAT1_2	Exon 2	Full	100
	CAT1_3	Exon 3	Partial	41
	CAT1_3	Exon 4	Full	100
	CAT1_5	Exon 7	Full	100
	CAT1_5	Exon 8	Full	100
CATSPER 2	CAT2_1	Exon 1	Full	100
	CAT2_1	Exon 2	Full	100
	CAT2_1	Exon 3	Full	100
	CAT2_2	Exon 4	Full	100
	CAT2_2	Exon 5	Full	100
	CAT2_2	Exon 6	Full	100
	CAT2_3	Exon 7	Full	100
	CAT2_3	Exon 8	Full	100
	CAT2_5	Exon 9	Full	100
	CAT2_5	Exon 10	Full	100
CATSPER 3	CAT3_1	Exon 2	Full	100
	CAT3_2	Exon 3	Full	100
	CAT3_3	Exon 4	Full	100
	CAT3_3	Exon 5	Full	100
	CAT3_3	Exon 6	Full	100
	CAT3_4	Exon 7	Partial	59
	CAT3_4	Exon 8	Full	100

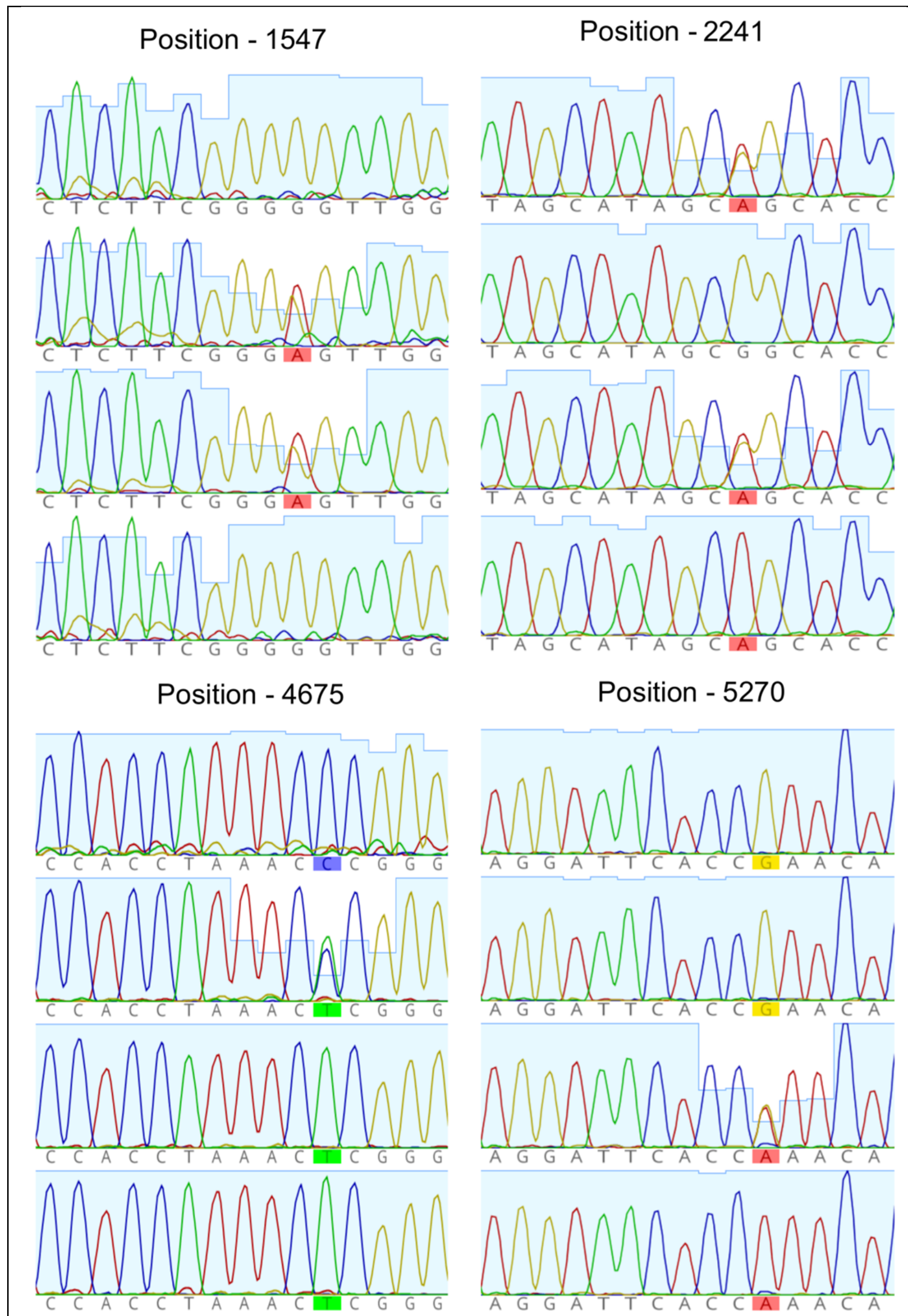

Figure 6.1: Representative sequence chromatograms showing the positions of Single Nucleotide Polymorphisms identified in the intronic regions of the CATSPER 1. The positions showed are (a) G1547A, (b) G2241A, (c) C4675T and (d) G5270A.

Here, portions of introns were also sequenced and we report four SNPs identified within the intronic regions of 1, 7 and 9 of CATSPER 1 (Figure 1). The four SNPs identified are G1547A which is 89 bp downstream from exon 1, G2241A located 126 bp upstream from exon 2, C4675T found 43 bp downstream from exon 7 and

G5270A that was located 206 bp downstream from exon 9. Studies have shown that SNPs within the introns of genes play a role in mRNA expression (Nott et al., 2003; Wang et al., 2011; Zhang et al., 2014) as well as determining the phenotypic expression of certain traits, such as eye color in humans (Sturm et al., 2008). Thus, these SNPs may have a role in the expression of the CATSPER 1 protein in the mid-piece of the sperm tail. Additional analysis is required in order to determine whether the SNPs in these regions influence the expression of the CATSPER 1 gene.

Unforeseen environmental stochasticity may necessitate artificial fertilization techniques as a tool in CMZ in the future to increase the reproductive output in key reserves for the purpose of maintaining genetic diversity and population viability. Identifying SNP variations within the introns and exons of genes associated with fertility may provide a criterion for selecting suitable candidates. Further studies on a larger sample set, could include additional genes such as Glutamine-rich protein 2 (QRICH2) and/or A-kinase anchoring protein 4 (Akap4) which have been reported to identify a loss of sperm function (Shen et al., 2019) or reduced sperm motility (Moretti et al., 2007). Once functional correlations are determined, understanding structural changes in the protein could be determined. In future comparative studies between zebra species characterizing sperm and parameters may be useful to diagnose potential defects in stallions, should semen samples become available.

CHAPTER 7

Conclusion

Climate change and anthropogenic factors are the biggest threat that wildlife face in this era (Hunter et al., 2010; Keith et al., 2008; Muñoz et al., 2015; Thomas et al., 2004). However, conservation efforts have increased and endeavour to preserve what biodiversity is left. Conservation efforts by agencies such as South African National Parks, CapeNature, other provincial government agencies, and private landowners, have had an invaluable role in the recovery of the Cape mountain zebra (CMZ, *Equus zebra zebra*). Cape mountain zebra were driven to the brink of extinction but have made a significant recovery, doubling in number since 1999 (Hrabar and Kerley, 2015; Moodley and Harley, 2005). Conservation agencies have acknowledged that the historic bottleneck and the continued isolation of CMZ have left these animals vulnerable to stochastic changes in the environment (Hrabar et al., 2016). Populations such as those from the Gamkaberg Nature Reserve (GNR) and the Kammanassie Nature Reserve (KNR) account for two-thirds of the extant genetic diversity of the sub-species (Moodley and Harley, 2005) but this diversity is slowly being lost due to factors that could be avoided through human intervention. A biodiversity management plan (BMP) was gazetted which aims to address these challenges (Birss et al., 2018).

The present study has contributed to key objectives of the BMP in order to guide conservation management decisions by providing scientific data. This was achieved in the following ways:

- An assessment of conservation efforts was carried out to determine how past management decisions have affected the current genetic diversity (Chapter 3). This was conducted using 14 microsatellite markers in three key reserves. This study is in line with activity 1 of Action 5.1.2.1 of the BMP, which was to *"research and develop suitable genetic markers using modern technologies to conduct analyses of genetic diversity within sub-populations and consequences of implemented and proposed management actions."* This study enabled the first investigation into changes in genetic diversity over a temporal period of one generation. Declines in key diversity

measures were reported and a shift in population structure was observed in De Hoop Nature Reserve (DHNR). These data emphasized the urgency to implement measures to preserve allelic richness in the key populations as well as to regularly measure changes in genetic diversity in the populations using F-statistics, estimating effective population size and determining population structure.

- Single nucleotide polymorphism (SNP) variation was investigated in the Toll-like receptor 9 gene (TLR9) (Chapter 4). The resulting amino acid changes were then characterised to determine if protein function was affected. This gene is associated to adaptive potential and disease risk, which contributes to activity 3 in Action 5.1.2.4 in the BMP which was to *"Research and develop the screening of innate immunity genes in Cape mountain zebra to identify disease associated mutations as well as determine diversity of these genes"*. SNP variants at varying proportions were identified in three CMZ populations. One of these SNPs resulted in an amino acid substitution, though this did not affect protein function. Two reserves - DHNR and the private reserve outside of Grahamstown (GHT) - has reduced genetic variation at these loci. Moreover, the genetic variation in the TLR9 was compared to data obtained from neutral markers. This study thus provided insights into the genetic variation at a gene which is under selection in three populations. Moreover, these results demonstrated how in the future, additional Toll-like receptor genes can be screened for mutations and functional changes which could affect adaptive potential. This information will inform conservation agencies in prioritising augmentation of populations that may have deleterious SNPs.
- The effects of translocations on the increased spread of equine piroplasms, and their prevalence at their sourced localities were investigated (Chapter 5). Translocations and reintroductions of CMZ into new areas are a planned activity as per the Action 5.1.2.4 of the BMP; to *"conduct research to quantify the extent and severity of possible disease occurrence in Cape mountain zebra"*. Increased gene flow between reserves is necessary for the preservation of genetic diversity. Thus, understanding how the movement of disease occurs is an important consideration. All animals screened in this study tested positive for *Theileira equi* infection and 2% of

all samples had a mixed infection with both *T. Equi* and *Babesia caballi*. Two of these sampled groups were translocated to new reserves. It is still unclear which genotypes were present in these animals. However, the prevalence of piroplasms in three reserves was reported and based on these findings, it was recommended that when founding new populations of CMZ as well as equids on adjacent farms, are screened prior to the reintroduction.

- Potential genetic factors that affect population growth and reproductive fitness were investigated (Chapter 6). The cation channel sperm associated genes (CATSPER) 1-3 were screened for SNPs that may affect gene function. This work contributed to activity 1 of action 5.1.2.5 of the BMP which was *"Opportunistic research and develop reproductive fitness assessment of Cape mountain zebra: conduct fundamental and applied research to further knowledge and understanding of Cape mountain zebra reproduction and integrate results into management recommendations".* Exons of all the CATSPER genes screened were highly conserved between individuals. Only 4 intronic SNPs identified within the species. These results may be used in conjunction with data for other genes to assist in conservation management actions as a method for identifying males for new founder populations, to ensure population growth or as criteria to select males to reinforce populations with low production.

7.1 Temporal changes in genetic diversity of the Cape mountain zebra.

In a previous study by Moodley and Harley (2005), the CMZ was determined to have low genetic diversity. The diversity of the species was largely contained within the three relict populations: Mountain Zebra National Park (MZNP), GHT and KNR. The De Hoop Nature Reserve (DHNR) population founded using animals from the two relict populations, MZNP and KNR, had the most genetic diversity of all the populations. This was the first temporal study of genetic diversity in CMZ. Genetic-based effective population size (N_e) using the temporal method was determined. Multiple tools were used to investigate the shift in genetic structure in DHNR which more closely resembled KNR. A loss of genetic diversity was observed for all populations, and a significant loss was detected of observed Heterozygosity in DHNR. Losses in genetic diversity and shifts in population

structure took place in the span of one generation (~14 years). These results show that continued isolation will increase the loss of genetic variation through genetic drift. Moreover, establishing conservation measures that would lead to an increase of Ne to greater than 50 would prevent the worst effects of inbreeding over the long-term.

Since only one generation interval between samples was available, the power of predictions for the temporal method of N_e estimations employed here was reduced. In future studies, additional temporal periods should be included. Additionally, only a small number of samples were obtained for KNR which limited conclusions that could be reached using the genetic data. Nevertheless, a recent drought in 2016 resulted in a population decline for KNR and the vulnerability of the population can be reasonably assumed without empirical N_e or Bottleneck estimates. For future studies, it is recommended that a minimum of 20 samples are collected at each generation for multiple reserves. Incorporating next-generation sequencing techniques such as restriction-associated DNA (RAD) capture sequencing could improve genetic diversity estimations (Ali et al., 2016). Models such as those used in BOTTLENECK software tests for an excess of heterozygosity (Piry et al., 1999). Thus, in the event of admixing with outbred individuals, heterozygosity will be affected in a way that would violate the core assumptions of the program and thereby lead to inaccurate conclusions (Piry et al., 1999). Controlled reintroductions are planned as per the BMP (Birss et al., 2018). Such breeding programs at dedicated localities, aimed at understanding the affected of admixing CMZ from relict populations, paired with studbooks, may provide a controlled environment to develop models that will assist for this purpose. This work may also benefit other species that are endangered. Forward simulations using software such as DIYABC is another method that will enable better predictions of potential outcomes of admixture and hybridization events in the CMZ populations. This program approximates Bayesian computations through inference of past history of populations of species (Cornuet et al., 2014). Furthermore, applying artificial intelligence or machine learning algorithms to predicting conservation outcomes may be a further step in ensuring sound management strategies.

7.2 Genetic diversity and molecular characterization of the Toll-like receptor gene 9

Genes with adaptive significance enable a better understanding of the adaptive potential of a species. Historically, only neutral markers were used for this purpose (Kirk and Freeland, 2011; Ouborg et al., 2010). In the current study, TLR9 (which signals the activation of the innate immune response upon recognition of pathogen-associated molecules (Bauer et al., 2002), was identified as a candidate to measure adaptive potential in CMZ. The three populations - MZNP, DHNR and GHT - were studied to identify genetic variation in the gene and protein sequences of the three populations. A total of six SNPs located in exon 2 of the protein were identified. MZNP populations showed a higher prevalence of each variant of these SNPs. In the translated protein, only one out of the six mutated SNPs resulted in an amino acid substitution within the protein. In addition, two amino acid substitutions exclusive to CMZ were identified, after aligning sequences to horse and donkey breeds. The amino acid substitutions were predicted to not affect the function or influence protein activity. Notwithstanding the lack of adaptive effect, there were clear losses in genetic diversity for the two smaller populations, DHNR and GHT, which are likely a result of the founder effect.

Future work should include screening of additional genes that progress preventative actions towards disease and disease risk in the CMZ. There are a total of 10 Toll-like receptors in equids. Thus, identifying the genetic diversity within the species will reinforce the findings in the present study in enabling more informed actions to be implemented in the BMP. Interspecific genetic variation in the major-histocompatibility complex; a gene involved in adaptive pathways, has been reported previously for multiple zebra species (Kamath and Getz, 2012). A more in-depth study of genetic variation in the ligand binding sites of this protein complex, between multiple CMZ populations will be valuable in producing a more complete understanding of genetic diversity in the metapopulation.

7.3 Translocation and the movement of piroplasms

A key objective in the BMP is to increase gene-flow and population growth through translocations and the founding of new populations (Birss et al., 2018). This is a necessary step since many of the reserves that currently stock CMZ are not able to accommodate additional growth. A previous study by Lea et al. (2016) showed this to be the case for multiple nature reserves, which includes the DHNR, where large tracts of reserves are not used by CMZ. Mountain catchment areas around the Western Cape that lay within the natural distribution range of CMZ constitutes of 855 940 ha of land have been identified as prospective sites for founding new populations. Translocation to these locations may encourage population growth as a result of reduced competition for limited grazing areas. Such competition has been reported in reserves such as KNR, where farmers continue to prevent CMZ to access palatable grass (Watson and Chadwick, 2007). However, it was initially unclear how the increased movement of the CMZ will affect the spread of disease. Wildlife is generally thought of as reservoirs of disease and international control of pathogens is a major challenge for conservationists (IUCN, 2012). Though equine piroplasms are endemic to South Africa (De Waal, 1992), there are regions where the pathogen or specific genotypes are absent and therefore equid populations in these regions could be at risk of their introduction which may lead to economic loss (Bhoora et al., 2010).

In the present study, we investigated the prevalence of equine piroplasms in CMZ that were being translocated to new locations. The piroplasm *T. equi* was near ubiquitous in the sample set with only 2 cases of *B. caballi*. Historically, CMZ were only found in three localities but the species has now spread to more than 75 localities, 91% of which were sourced from MZNP (Moodley and Harley, 2005). Various genotypes of piroplasms have been discovered (Bhoora et al., 2010), therefore, with the increased movement of CMZ being planned, there is a risk that additional genotypes may be introduced into regions where the tick vectors are present. It was recommended that samples be tested from both zebra from the source population and domestic equids to determine whether the CMZ introductions will pose an economic risk to farmers through the introduction of novel genotypes. Future work will constitute genotyping samples from CMZ reserves that will be selected for translocation, to identify the strains present.

Screening of domestic equid populations to determine the prevalence of equine piroplasms genotypes will also be carried out. This will reduce the risk of economic losses as a result of infection with potentially more virulent strains of the pathogen. Future studies will investigate the interface between domestic equids and CMZ, to monitor how this interaction affects the health of both domestic animals and wildlife. In addition, CMZ samples will be screened to determine which genotypes are present in the population on various reserves, which will inform activities to reinforce existing herds.

7.4 Reproductive fitness of the Cape mountain zebra

The DHNR was founded using individuals from two relict populations and is the only admixed population of CMZ (Hrabar and Kerley, 2013). Initial reports of the genetic diversity in the population indicated that it is the highest of all extant populations (Moodley and Harley, 2005). Moreover, it was found that the population had low fecundity and a low proportion of breeding herds, with a skewed sex ratio (Smith et al., 2007) which is still a persistent challenge to the population (Birss, unpublished). The current study screened exons and partial introns from the CATSPER 1-3 genes for 10 males from a group of DHNR individuals that were set to be translocated to a new reserve (four of which were dominant stallions). Analysis showed that the exons studied were well conserved between individuals, though 4 SNPs were identified in the group. More work will need to be carried out to determine if these SNPs affect protein expression, as was shown for other genes such the intronic SNP that influence expression in the CYP3A4 gene (Wang et al., 2011).

Due to threats of climate change as well as the influence of other anthropogenic factors, it may be necessary to obtain semen for artificial insemination. Intronic mutations at introns, 1, 7 and 9 may contribute to the ability of sperm to withstand freezing. Similar work is being carried out in cattle to improve sperm for durability during freezing (Geetha et al., 2011). Moreover, work will need to be done to screen the remaining exons in the CATSPER genes as well as the CATSPER4 gene and a larger sample size should be used from multiple reserves to improve the confidence level of results obtained. Comparisons with sperm can be used to test a variety of genes that are associated with reproductive health and production,

will allow for better-managed populations. Genes such as the androgen receptor gene, associated with behaviour such as aggression in dogs would also be a useful gene to study (Konno et al., 2011). The androgen receptor gene may be linked to the ability of male stallions to compete for dominance over harems. A Microsatellite locus in the zebra androgen receptor gene was identified by Ito et al., (2015), though it was not determined how this affected animal behaviour or physiology. As more data are obtained regarding the genetic mechanisms of fertility in CMZ, less invasive or minimally invasive sampling techniques can be developed that are still able to identify candidate animals that may be valuable when founding of new populations. Determining whether dominant stallions are able to consistently sire offspring may be a manner of ensuring that there is no waste of reproductive effort.

7.5 The impact of translocations of the genetic diversity of Cape mountain zebra

During the course of this study, an assessment was performed by Hrabar et al., (2016); evaluating the vulnerability of CMZ, and this research determined that the status of the species as per the IUCN should be downgraded to least concern. The condition for this assessment was that continued action in improving the biodiversity is ongoing. The basis for which reduced risk was determined was that if the largest population, MZNP, was to lose all CMZ, the remaining number of CMZ would still exceed the minimum threshold of 1000 (Hrabar et al., 2016). In the past, translocation activities were carried out injudiciously, with as much as 50% of founding populations using less than the recommended number of animals (Birss et al., 2018; Hrabar and Kerley, 2015). Techniques for translocation have since become refined and more successful. The incorporation of a genetic basis for conservation has been a key concern internationally (Ralls et al., 2018). South African conservation agencies have adopted this approach and incorporated it into the BMP for CMZ. However, various interventions were recommended to allow for better gene-flow since 2001 has not been successfully implemented (Birss et al., 2018; Hrabar and Kerley, 2015, 2013; Moodley and Harley, 2005; Novellie et al., 2002; Watson and Chadwick, 2007). Increased efforts, based on sound scientific knowledge, are now enabling progress in conservation of the CMZ.

The current state of genetic diversity within the metapopulation was unknown and this study was able to elucidate the genetic status of populations in three key reserves that were intended for translocations with the purpose of reinforcing herds and founding new populations. The nature of historic translocation activities, which was primarily done to found new populations, was not enough to preserve genetic diversity in populations. Moreover, the TLR9 gene in the populations sourced from MZNP, were less diverse than MZNP. These data emphasize the need for urgent action towards augmenting existing herds. Interestingly, in a study using the model species, *Drosophila sp.*, researchers found that as little as one outbred individual can improve the genetic diversity by as much as 50% (Spielman and Frankham, 1992). This may however not be implemented as readily in mountain zebra due to their complex social orders (Penzhorn and Novellie, 1991).

7.6 Future work

In addition to the work reported on in this thesis, other research objectives have been identified in the BMP. Hybridization between zebra species is a major concern for the preservation of the species (Dalton et al., 2017). In this regard, work is currently being carried out which will support the conservation actions of the BMP, but additional work on this can be done using genes such as the CATSPER channel genes which can be used to determine how hybridization affects reproduction associated genes. Sperm could be collected from hybrids to identify changes to morphology and motility, and SNP mutations associated to these changes, which could complement genetic measures of hybrid fertility as carried out previously (Dalton et al., 2017).

More work should be done regarding parasite load and host-pathogen interactions. While some of this work was addressed in the present study, additional studies should include the study of the prevalence of the pathogens. As many as 53% of CMZ in the Bontebok National Park and 25% in Gariep Dam Nature Reserve had sarcoid tumours (Marais et al., 2007; Sasidharan et al., 2011). Investigating the prevalence of the causative agent, bovine papillomavirus, in other reserves will enable a better understanding of which populations are most at risk of the disease. This action will assist in determining which populations can be translocated without increasing the prevalence of sarcoids within populations. Small inbred populations

have been particularly susceptible to sarcoidosis (Marais et al., 2007; Sasidharan et al., 2011). Additional pathogens such at the equine herpes virus 1, which has been isolated from the related plains zebra (*Equus burchellii*) and Hartmann's mountain zebra (*Equus zebra hartmanne*) (Abdelgawad et al., 2014; Borchers and Frölich, 1997; Guo et al., 2014), will be screened for in CMZ populations. The pathogens' impact on population health will also be assessed. New methods for less invasive sampling methods should be developed using biomaterials such as hair, faeces or using other sample collection techniques that do not require the capture and sedation of the animals. Nevertheless, capture and sedation is a necessary step during translocations, which means that this will method will likely remain a valuable opportunity for sampling.

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SUPPLEMENTARY TABLE 1

Primers used in this study adapted from Horse (*Equus caballus*). T(a) indicates annealing temperature.

Locus	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	T(a)	Repeat
HMB1	GTGTGTATGCTTCCCAACCCTT	GTTATAAAGCACTATGATCTCA	58	$(GT)_{18}$
HTG 14	CCAGTCTAAGTTTGTTGGCTAGAA	CAAAGGTGAGTGATGGATGGAAGC	55	$(TG)_{14}$
HTG09	TGTGGGAAGAGTGTCAATAGCTGT	AGGCATCTGGTTTGCTGCAATTTC	55	$(GT)_{17}$
HTG11	CAATGATGGTACTTTGCATATTAA	ATCGGCATGCACACTCATAGGTAG	59	$(GT)_{15}$
HTG15	TCTTGATGGCAGAGCCAGGATTTG	AATGTCACCATGCGGCACATGACT	55	$(TG)_{14}$
HTG7	CCTGAAGCAGAACATCCCTCCTTG	ATAAAGTGTCTGGGCAGAGCTGCT	55	$(TG)_{19}$
LEX20	GGAATAGGTGGGGGTCTGTT	AGGGTACTAGCCAAGTGACTGC	55	$(TG)_{19}$
LEX52	GGAACGGAAGAGTGTAGTTTT	CATTTATTCATCAGCGATTTG	55	$(TG)_{13}$
TKY273	GATCACTGGCGAGGGTAAGC	TATGTTCCCGATTCGCAAGC	55	$(CA)_{22}$
UCDEQ505	ATCACTCTCTTGTTGAGATAAC	GGGATTTCCTTCTTTCTC	55	$(GT)_{17}$
VHL47	GTTTGCTGTGGTTACCAGGCAGA	GCAAATTGAATATTTGAAGTTGAGAC	55	$(CA)_{13}$
COR014	CTATCATGTCAGGGACCAGG	CTGCCCTAGTTAGCAACCAA	58	$(GT)_{21}$
AHT21	TCCAAGTTGCTGAATGGATC	ACGGCCTGATTCTCTCTTTG	60	$(GT)_{21}$
HTG3	TAACCTGGGTGCAAAGCCACCCAT	GTCAGGGCCAATCTTCCTCAC	63	$(TG)_{16}$
HTG5	TGCTAAGCCTCAGCACATACA	TGGAAATAAGGTTAGCAGGGATGC	63	$(TG)_{15}$

SUPPLEMENTARY TABLE 2

Null allele estimations for three Cape mountain zebra populations at two temporal periods. The data was tested using the methods from Oosterhout and Chakraborty.

Mountain zebra National Park							
1999-2001				**2015-2016**			
Locus	**Null Present**	**Oosterhout**	**Chakraborty**	**Locus**	**Null Present**	**Oosterhout**	**Chakraborty**
HTG07	no	0.1104	0.122	HTG07	no	-0.0341	-0.0351
HTG15	no	0	0	HTG15	no	0	0
HMB1	no	0.0787	0.0795	HMB1	no	-0.0216	-0.024
LEX20	no	0.1619	0.1538	LEX20	no	-0.015	-0.0075
VHL47	no	0	0	VHL47	no	0.1016	0.1527
UCDEQ505	no	0	0	UCDEQ505	no	-0.0426	-0.0382
COR014	**yes**	**0.3416**	**1**	**COR014**	**yes**	**0.1024**	**0.1603**
LEX52	no	0	0	LEX52	no	0	0
AHT21	no	0	0	AHT21	no	0.0051	-0.0096
TKY273	no	0	0	TKY273	no	-0.0134	-0.0067
HTG14	no	0.1469	0.1111	HTG14	no	0.0888	0.2338
HTG03	no	0	0	HTG03	no	0	0
HTG9	no	0.1142	0.1416	HTG9	no	0.0343	0.04
HTG11	no	0.2296	0.4286	HTG11	no	0.038	0.047

De Hoop Nature Reserve							
1999-2001				**2015-2016**			
Locus	**Null Present**	**Oosterhout**	**Chakraborty**	**Locus**	**Null Present**	**Oosterhout**	**Chakraborty**
HTG07	no	0.0922	0.0947	HTG07	no	0.1041	0.1099
HTG15	no	0	0	HTG15	no	0	0
HMB1	no	0.1945	0.4667	HMB1	no	0	0
LEX20	no	-0.3767	-0.2329	LEX20	no	-0.077	-0.0385
VHL47	no	-0.1056	-0.0526	VHL47	no	-0.077	-0.0385
UCDEQ505	no	-0.1162	-0.0959	**UCDEQ505**	**yes**	**0.3411**	**0.6981**
COR014	no	-0.0531	-0.049	COR014	no	0.1826	0.25
LEX52	no	0	0	LEX52	no	0	0
AHT21	no	-0.0259	-0.0365	AHT21	no	-0.0388	-0.0371
TKY273	no	0.0246	0.0255	TKY273	no	-0.2404	-0.1183
HTG14	no	-0.0364	-0.0345	HTG14	no	-0.0889	-0.0781
HTG03	no	0	0	HTG03	no	0	0
HTG9	no	-0.1093	-0.0909	HTG9	no	-0.3118	-0.1321
HTG11	no	0.1547	0.25	HTG11	no	-0.0187	-0.0093

Kamanassie Nature Reserve							
1999-2001				**2015-2016**			
Locus	**Null Present**	**Oosterhout**	**Chakraborty**	**Locus**	**Null Present**	**Oosterhout**	**Chakraborty**
HTG07	no	0.1046	0.1045				
HTG15	no	0	0				
HMB1	no	0.0787	0.0924				
LEX20	no	-0.1835	-0.0909				
VHL47	no	-0.1181	-0.0588				
UCDEQ505	no	0	0		Insufficient sample size		
COR014	no	0	0				
LEX52	no	0	0				
AHT21	no	0	0				
TKY273	no	0.1498	0.2174				
HTG14	no	-0.3333	-0.1613				
HTG03	no	0	0				
HTG9	no	0	0				
HTG11	no	0.3797	1				

SUPPLEMENTARY TABLE 3

Genetic diversity estimates per marker for three populations; Kammanassie Nature Reserve, Mountain Zebra National Park and De Hoop Nature Reserve over two temporal periods. Ar = the Allelic richness for which is corrected for sample size, Ae = number of effective alleles, I = Shannon's information index, Ho = Observed heterozygosity, He = expected heterozygosity, F = fixation index, HWE = deviation from Hardy-Weinberg Equilibrium (* = Significant value $p < 0.05$).

Population	Locus	A_r	A_e	I	H_o	H_e	F	HWE
	HTG07	3.2112	3.574	1.483	0.5313	0.732	0.262	0.0142
	HMB1	2.4141	2.481	1.117	0.2647	0.606	0.557	**0.0000***
	LEX20	2.1543	1.755	0.766	0.4857	0.436	-0.129	0.0545
	VHL47	1.4668	1.635	0.577	0.1389	0.394	0.643	**0.0003***
	UCDEQ505	16663	2.190	0.850	0.3125	0.552	0.425	**0.0127***
	COR014	1.6656	2.253	0.883	0.2188	0.565	0.607	**0.0000***
	AHT21	1.9428	2.034	0.812	0.4063	0.516	0.201	0.2466
	TKY273	1.5575	1.619	0.571	0.2727	0.388	0.287	0.1561
	HTG14	2.4252	2.187	0.897	0.4722	0.550	0.130	0.0737
	HTG9	2	2.033	0.862	0.3824	0.516	0.248	**0.0017***
1999-2001	HTG11	2.2631	1.552	0.662	0.1154	0.363	0.676	**0.0001***
		2.069	**2.120**	**0.862**	**0.327**	**0.511**	**0.355**	
	HTG07	2.9511	2.679	1.091	0.621	0.630	0.009	0.1494
	HMB1	1.756	2.175	0.842	0.353	0.543	0.347	**0.0000***
	LEX20	1.3707	1.063	0.137	0.061	0.060	-0.032	1.0000
	VHL47	1.5868	1.227	0.331	0.147	0.186	0.204	0.0688
	UCDEQ505	1.6557	1.679	0.594	0.271	0.406	0.330	**0.0022***
	COR014	1.6625	1.540	0.571	0.188	0.352	0.463	**0.0000***
	AHT21	2.3081	1.739	0.730	0.390	0.427	0.081	**0.0043***
	TKY273	15594	1.142	0.245	0.133	0.125	-0.071	1.0000
	HTG14	1.7986	1.418	0.471	0.184	0.296	0.374	**0.0005***
	HTG9	2.4241	2.353	0.956	0.554	0.578	0.036	0.3008
2015-2016	HTG11	1.3906	1.135	0.237	0.108	0.120	0.096	0.3348
		1.86033	**1.650**	**0.564**	**0.274**	**0.338**	**0.167**	

SUPPLEMENTARY TABLE 4

List of the quantification cycle (Cq) values obtained from the Cape mountain zebra samples. MZNP = Mountain Zebra National Park, DHNR = De Hoop Nature Reserve and KNP = Karoo National Park.

Number	Date of sampling	Reserve	Agency	*T. equi*	*B. caballi*
1	Oct 2018	MZNP	SANParks	27,07	
2	Oct 2018	MZNP	SANParks	28,67	
3	Oct 2018	MZNP	SANParks	28,68	
4	Oct 2018	MZNP	SANParks	27,84	
5	Oct 2018	MZNP	SANParks	22,89	
6	Oct 2018	MZNP	SANParks	26,32	
7	Oct 2018	MZNP	SANParks	29,14	
8	Oct 2018	MZNP	SANParks	28,66	
9	Oct 2018	MZNP	SANParks	24,21	
10	Oct 2018	MZNP	SANParks	28,52	
11	Oct 2018	MZNP	SANParks	28,88	
12	Oct 2018	MZNP	SANParks	29,61	
13	Oct 2018	MZNP	SANParks	26,97	
14	Oct 2018	MZNP	SANParks	25,05	
15	Oct 2018	MZNP	SANParks	27,17	
16	Oct 2018	MZNP	SANParks	26,02	
17	Oct 2018	MZNP	SANParks	24,92	
18	Oct 2018	MZNP	SANParks	26,21	
19	Oct 2018	MZNP	SANParks	26,82	
20	Oct 2018	MZNP	SANParks	28,05	
21	Oct 2018	MZNP	SANParks	27,06	
22	Oct 2018	MZNP	SANParks	25,99	
23	Oct 2018	MZNP	SANParks	28,81	
24	Oct 2018	MZNP	SANParks	29,19	
25	Oct 2018	MZNP	SANParks	26,17	
26	Oct 2018	MZNP	SANParks	25,82	
27	Oct 2018	MZNP	SANParks	30,06	
28	Oct 2018	MZNP	SANParks	24,56	
29	Oct 2018	MZNP	SANParks	25,89	
30	Oct 2018	MZNP	SANParks	29,73	
31	May 2016	MZNP	SANParks	28,73	
32	May 2016	MZNP	SANParks	29,21	
33	May 2016	MZNP	SANParks	28,73	
34	May 2016	MZNP	SANParks	27,19	
35	May 2016	MZNP	SANParks	27,68	
36	May 2016	MZNP	SANParks	25,77	
37	May 2016	MZNP	SANParks	24,23	
38	May 2016	MZNP	SANParks	30,27	
39	May 2016	MZNP	SANParks	26,77	

Number	Date of sampling	Reserve	Agency	*T. equi*	*B. caballi*
40	May 2016	MZNP	SANParks	31,75	
41	May 2016	MZNP	SANParks	25,08	
42	May 2016	MZNP	SANParks	28,92	
43	May 2016	MZNP	SANParks	23,12	
44	May 2016	MZNP	SANParks	25,94	
45	May 2016	MZNP	SANParks	24,75	
46	May 2016	MZNP	SANParks	25,46	
49	May 2016	MZNP	SANParks	25,05	
50	May 2016	MZNP	SANParks	28,99	
51	May 2016	MZNP	SANParks	23,44	
52	May 2016	MZNP	SANParks	26,7	
53	May 2016	MZNP	SANParks	27,3	
54	May 2016	MZNP	SANParks	25,9	
55	May 2016	MZNP	SANParks	29,11	
56	May 2016	MZNP	SANParks	26,01	
57	May 2016	MZNP	SANParks	26,77	32,76
58	May 2016	MZNP	SANParks	25,74	
59	May 2016	MZNP	SANParks	26,48	
60	May 2016	MZNP	SANParks	28,18	
61	May 2016	MZNP	SANParks	28,52	
62	May 2016	MZNP	SANParks	24	
63	May 2016	MZNP	SANParks	27,94	
64	May 2016	MZNP	SANParks	28,43	
65	May 2016	MZNP	SANParks	26,98	
66	May 2016	MZNP	SANParks	23,82	
67	May 2016	MZNP	SANParks	27,13	36,39
68	May 2016	MZNP	SANParks	28,72	
69	May 2016	MZNP	SANParks	26,53	
70	May 2016	MZNP	SANParks	28,16	
71	May 2016	MZNP	SANParks	28,43	
72	May 2016	MZNP	SANParks	25,29	
73	May 2016	MZNP	SANParks	26,46	
74	May 2016	MZNP	SANParks	24,89	
75	May 2016	MZNP	SANParks	26,69	
76	May 2016	MZNP	SANParks	23,64	
77	May 2016	MZNP	SANParks	27,48	
78	May 2016	MZNP	SANParks	28,88	
79	May 2016	MZNP	SANParks	29,88	
80	May 2016	MZNP	SANParks	25,83	
81	May 2016	MZNP	SANParks	23,95	
82	May 2016	MZNP	SANParks	24,37	
83	May 2016	MZNP	SANParks	26,91	
84	May 2016	MZNP	SANParks	24,45	
85	May 2016	MZNP	SANParks	24,88	
86	May 2016	MZNP	SANParks	24,34	

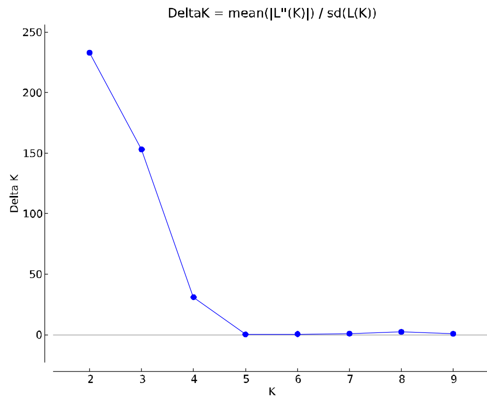
Number	Date of sampling	Reserve	Agency	*T. equi*	*B. caballi*
87	May 2016	MZNP	SANParks	24,72	
88	May 2016	MZNP	SANParks	22,78	
89	May 2016	MZNP	SANParks	26,74	
90	May 2016	MZNP	SANParks	28,04	
91	May 2016	MZNP	SANParks	28,64	
92	May 2016	MZNP	SANParks	26,74	
93	May 2016	MZNP	SANParks	24,77	
94	May 2016	MZNP	SANParks	27,64	
95	May 2016	MZNP	SANParks	27,82	
96	May 2016	MZNP	SANParks	24,53	
97	May 2016	MZNP	SANParks	28,5	
97	May 2016	MZNP	SANParks	30,61	
98	May 2016	MZNP	SANParks	27,31	
99	May 2016	MZNP	SANParks	28,62	
100	Sept 2016	DHNR	CapeNature	25,93	
101	Sept 2016	DHNR	CapeNature	26,2	
102	Sept 2016	DHNR	CapeNature	24,14	
103	Sept 2016	DHNR	CapeNature	28,24	
104	Sept 2016	DHNR	CapeNature	26,76	
105	Sept 2016	DHNR	CapeNature	26,07	
106	Sept 2016	DHNR	CapeNature	24,55	
107	Sept 2016	DHNR	CapeNature	26,46	
108	Sept 2016	DHNR	CapeNature	24,1	
109	Sept 2016	DHNR	CapeNature	24,08	
110	Sept 2016	DHNR	CapeNature	24,47	
111	Sept 2016	DHNR	CapeNature	28,72	
112	Sept 2016	DHNR	CapeNature	28,49	
113	Sept 2016	DHNR	CapeNature	26,87	
114	Sept 2016	DHNR	CapeNature	27,7	
115	Sept 2016	DHNR	CapeNature	28,88	
116	Sept 2016	DHNR	CapeNature	27,28	
117	Sept 2016	DHNR	CapeNature	23,67	
118	Sept 2016	DHNR	CapeNature	26	
119	Sept 2016	DHNR	CapeNature	24,59	
120	Sept 2016	DHNR	CapeNature	26,76	
121	Sept 2016	DHNR	CapeNature	28,98	
122	Sept 2016	DHNR	CapeNature	24,91	
123	Sept 2016	DHNR	CapeNature	25,03	
124	Sept 2016	DHNR	CapeNature	Positive	
125	Sept 2016	DHNR	CapeNature	27,6	
127	2016	KNP	SANParks	22,73	
128	2016	KNP	SANParks	27,77	
129	2016	KNP	SANParks	29,27	
130	2016	KNP	SANParks	29,87	
131	2016	KNP	SANParks	26,77	

Number	Date of sampling	Reserve	Agency	*T. equi*	*B. caballi*
132	2016	KNP	SANParks	30,13	
133	2016	KNP	SANParks	25,29	
134	2016	KNP	SANParks	27,73	
135	2016	KNP	SANParks	26,14	
136	2016	KNP	SANParks	25,55	
137	2016	MZNP	SANParks	19,09	

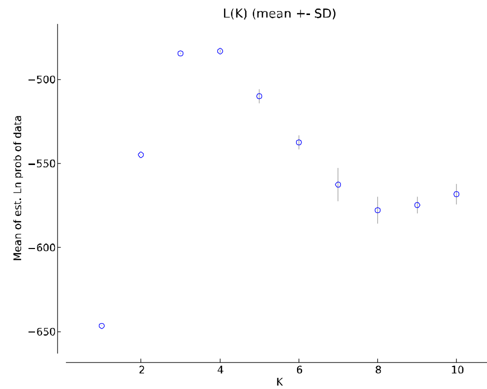
SUPPLEMENTARY FIGURE 1

Supplementary Figure 1: Structure Harvester results indicating the ΔK values (a and c) and log-likelihood (b and d) values which suggest the most likely K-value for among three Cape mountain zebra populations, MZNP, DHNR and KNR for the periods 1999-2001 (a) and (b) and 2015-2016 (c) and (d).

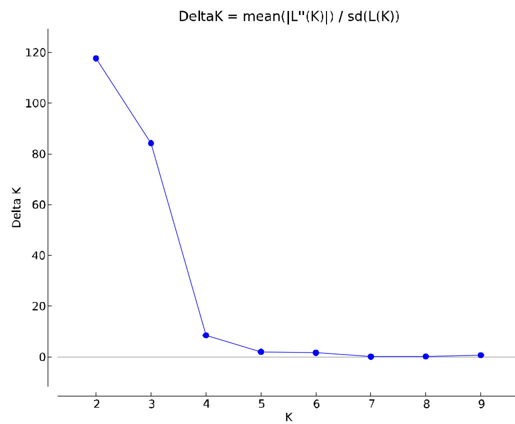
(a)



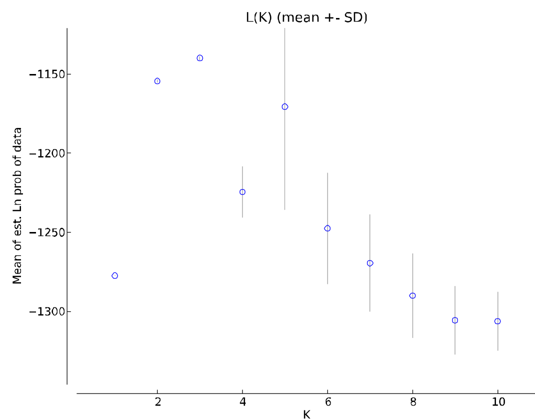
(b)



(c)



(d)



SUPPLEMENTARY FIGURE 2

Supplimentary Figure 2: Structure results showing a comparison between the K=3 and K=2 before structure harvester.