Comparative genetics of selected Southern African Mountain Zebra (*Equus zebra zebra* and *Equus zebra hartmannae*) populations

By

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Dedicated to my Achan and Amma

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Declaration

I, SP Sasidharan, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisor, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for a degree at this university or any other university.

This dissertation is presented in partial fulfilment of the requirements for the degree MSc in Production Animal Studies.

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Signed ……

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Abstract

In recent years, South African conservation officials have noted the appearance of tumour like growths, very similar to equine sarcoids, in some Cape mountain zebra (CMZ) populations. In domestic horses, a genetic predisposition for this bovine papillomavirus-induced tumour is suspected. This investigation studied the levels of heterozygosity and population parameters such as inbreeding, within the tumour-affected populations. In comparison, CMZ populations with few or no tumours and Hartmann's mountain zebras (HMZ) from Namibia were analysed using similar techniques. This study utilised dinucleotide repeat genetic markers called microsatellites, originally isolated from domestic horse (*Equus caballus*), to amplify related segments in the mountain zebras. Sixteen such fluorescent-labelled markers were amplified using polymerase chain reactions run in multiplexes. A commercial genetic analyser was used to detect the amplified markers and resulting data was analysed using *STRand* software. Marker visualisation and genotyping was completed using specialised open-source software. Fifteen loci were repeatedly amplified with clarity within both mountain zebra subspecies. The lowest heterozygosity and allele polymorphism levels were detected in sarcoid-tumour affected populations. All CMZ populations analysed were highly related and substructured. By comparison, Hartmann's zebras were found to have highest levels of genetic diversity and polymorphism. The highest levels of inbreeding were found within the tumour-affected populations. High levels of heterozygote deficit found in CMZ populations, for the loci investigated, resulted in nonsignificant results when inbreeding values were analysed. This study indicates that the sarcoid tumour has been expressed in populations with the highest levels of consanguinity. The sarcoid tumour is a disease that is considered mutifactorial in aetiology and therefore other parameters such as immune status of tumour-affected populations and associated environmental variables warrant investigation. This study has simplified the archival and genotyping of individual mountain zebras. The study concludes that, among the populations tested, sarcoid tumours have been expressed in CMZ with highest levels of inbreeding. The establishment of a genetic database, incorporating information from polymorphic microsatellite markers, would assist in the conservation management of isolated CMZ populations by providing the information necessary to increase allelic diversity.

Chapter I

General introduction

The Cape mountain zebra (CMZ) (*Equus zebra zebra*) is among the most endangered mammals in the Republic of South Africa and the world (Penzhorn & Novellie 1991). The World Conservation Union (IUCN) red list (*Version 3.1*; Friedman & Daly 2004) lists *Equus zebra zebra* as vulnerable (criteria D1) and *Equus zebra hartmannae* as endangered. The Convention on International Trade in Endangered Species (CITES) listings of these two subspecies is Appendix I and II, respectively. The Cape subspecies is recognized as different from the Hartmann's mountain zebra (HMZ) (*Equus zebra hartmannae*), which is found along Namibia's western mountain ranges (Penzhorn 1988; Skinner & Smithers 1990; Friedman & Daly 2004).

The current CMZ population is around 1600, with the main population numbering approximately 400 animals in the Mountain Zebra National Park (MZNP), with seeded populations in other national parks and provincial reserves. Other original populations of CMZ are believed to be small populations in the Gamka Mountain and Kammanassie Nature Reserves (Novellie et al. 2002).

The Free State Department of Environmental Affairs and Tourism and South African National Parks officials have reported an increase in incidence of sarcoid-like tumours in some CMZ populations, with Free State conservation officials reporting an extreme manifestation of the tumour in the Gariep Dam Nature Reserve (GDNR) population. A high incidence of sarcoids has also been reported in Bontebok National Park (BNP). In addition, isolated cases have also been reported in Commando Drif (Eugene Bird personal communication), Gamka Mountain (Moodley 2002) and De Hoop Nature Reserves (Novellie et al. 2002). Incidence in Commando Drif, De Hoop and Gamka Mountain Nature Reserves have been isolated and restricted to few animals. This study focused on reports of a growing incidence of sarcoid tumours in two specific populations within GDNR and BNP. In GDNR, the current incidence is estimated to be approximately at 22% in a population numbering 83 animals (Nel unpublished data) and in the BNP, the incidence is 53% in a total population of 19 CMZ (Marais et al. unpublished data).

We could find no confirmed reports of sarcoid-like tumours in the relatively outbred Burchell's zebra population in Kruger National Park and HMZ populations in Namibia. A

literature review of such tumours suggests a genetic predisposition in Equidae to develop sarcoids. These observations strongly support the hypothesis that there is a relation between homozygosity in mountain zebra populations and expression of sarcoids.

The aims of this study were:

a) To establish and validate a microsatellite multiplex PCR panel composed of polymorphic domestic horse microsatellite loci for investigating genetic parameters in the mountain zebra.
b) To calculate and compare heterozygosity between and within selected Cape and Hartmann's mountain zebra populations.
c) To analyse possible fitness consequences of inbreeding in tumour-affected CMZ populations.

This dissertation consists of five chapters and has been written following the format of *Conservation Biology*.

Chapter II explores, from an evolutionary and genetic perspective, the validity of using domestic horse microsatellites for genetic studies in the mountain zebra. It describes the reasons for comparing these two specific subspecies and the validity of doing so. Chapter II reviews the known fitness consequences of inbreeding in captive and wild populations. It investigates whether the published literature supports the hypothesis that a correlation exists between homozygosity in equid populations and expression of a disease like sarcoid. Chapter II also details how genetic markers that are only indices of genomic variation and not object genes for natural selection and adaptation can serve as a tool to draw indirect correlations on population fitness.

Chapter III describes the application and validation of domestic horse microsatellites to genotype the mountain zebra.

Chapter IV details the comparative genetics of different mountain zebra populations and describes various population parameters within analysed populations. This study tests whether these domestic horse markers are sufficiently polymorphic and informative enough to answer questions related to inbreeding and to potentially assign parentage in mountain zebras.

Chapter V concludes the dissertation and provides an analysis of the results obtained from comparing sarcoid affected mountain zebra populations with unaffected. Future applications of this genotyping technique and possible conservation measures are discussed.

Chapter II

Literature review

2.1 The Equidae and evolution

2.1.1 Genus *Equus*

The genus *Equus* can be divided into three subgenera (Groves & Ryder 2000; Oakenfull & Ryder 2002): *Equus*, which contains the horses, and does not occur naturally in Africa; *Asinus*, which contains the true asses, onager and hemiones, and is represented by a single species in Africa, the African wild ass (*Equus africanus*) and *Hippotigris*, which contains the zebras, all of which are African. The zebra group can be divided into three further subgroups; *Equus zebra*, the mountain zebra, including Cape mountain zebra (*Equus zebra zebra*) and Hartmann's mountain zebra (*Equus zebra hartmannae*); *Equus quagga,* the plains zebra, including the extinct true quagga (*Equus quagga quagga*) and Burchell's (plains) zebra (*Equus quagga burchellii*) and Grévy's zebra (*Equus grevyi*) of East Africa (Skinner & Smithers 1990). The taxonomic status of the subspecies of plains zebra is not yet well understood and experts do not agree on details. The Equid specialist group of the IUCN recognises five subspecies of plains zebras, plus one subspecies (Burchell's, *Equus burchellii burchellii*) that is extinct. The existing subspecies are Grant's (*Equus burchellii boehmi*), Upper Zambezi (*Equus burchellii zambesiansis*), Crawshay's (*Equus burchellii crawshayi*), Chapman's (*Equus burchellii chapmani*), and Damara (*Equus burchellii antiquorum*) zebras.

2.1.2 Phylogeny of the mountain zebra

A phylogenetic network of equid mitochondrial DNA (*mt*DNA) control region sequences provide evidence that mountain zebras split off first, then asses, Damara and Grant's zebras, followed by Grévy's zebras, the hemiones, and finally the horse (including Przewalski's horse) (Jansen et al. 2002). The present-day equine species are considered to have evolved from a common ancestor over the past 4 - 5 million years. The chromosome number of the extant equid species indicates that extensive karyotype divergence occurred during this evolution (Lear & Bailey 1997; Myka et al. 2003). It is suggested that among mammals, Equidae exhibit the highest rate of chromosomal evolution (Bush et al. 1977). In spite of their close relationship, the diploid number of equid chromosomes show a remarkable degree of variation amongst the various species; from Hartmann's mountain zebra (2n = 32), Burchell's zebra (2n = 44/45), Grevy's zebra (2n = 46) to the domestic horse (2n = 64) (Skinner &

Smithers 1990). Morphological as well as available chromosomal data, however, support the idea that all zebras share a recent common ancestor (Breen et al. 1995; Chopineau et al. 1999; Bowland et al. 2001). Studies on DNA from the extinct quagga also clearly indicate a close relationship with the plains zebras (Higuchi et al. 1984; Harley 1988).

2.1.3 Karyotype evolution in the mountain zebra

Ryder et al. (1978) state that despite differences in total chromosome numbers (2n = 32 to 66) and chromosome arm numbers (NF = 62 to 102), the total amount of DNA present per diploid cell in each equid species is roughly the same, indicating chromosomal rearrangement rather than gain or loss of genetic information. Classic Robertsonian fusion mechanisms have proven inadequate to explain the changes that might have occurred during this karyotypic evolution (Raudsepp et al. 2001). Comparative analysis of interstitial telomeric sites between other equids and Hartmann's mountain zebra have revealed that karyotype evolution in this species has also occurred by chromosomal rearrangements such as tandem or centric fusions, centromere related rearrangements and multiple reciprocal translocations and/or para- or pericentric inversions (Santani et al. 2002; Chowdhary et al. 2003; Yang et al. 2003). This proves that evolutionary conservation of chromosome segments exists across all *Equus* species, even beween domestic horses (2n = 64) and mountain zebras (2n = 32). Cape and Hartmann's mountain zebras have similar chromosome numbers and have been suggested to be conspecific (Heinichen, 1969). Both karyotypes have identical appearance, containing 13 meta to submetacentric pairs, two acrocentric pairs, a large metacentric X- and a small submetacentric Y- chromosome. Although Cape and Hartmann's mountain zebra populations have been accorded separate subspecies status based on morphological traits, recent data from the comparative sequencing of mitochondrial control regions of the two subspecies could find little variation (Moodley 2002). Feulner et al. (2004) argue that for populations to be treated as evolutionary and conservation units, genetic integrity rather than taxonomic status should take precedence. Pelagic and craniometric studies using multivariate analysis confirm that there is an absolute difference between the two subspecies (Groves & Bell 2004).

2.2 Cape and Hartmann's mountain zebra subspecies

2.2.1 Distribution and phenotype

Two mountain zebra subspecies have been described; Cape mountain zebra (CMZ; *Equus zebra zebra*), from Western Cape, Eastern Cape and portions of Northern Cape, and

Hartmann's mountain zebra (HMZ; *Equus zebra hartmannae*), of Namibia, Angola and north-western parts of the Northern Cape (Skinner & Smithers 1990). Phenotypically, compared to the widespread plains zebra (*Equus quagga*), mountain zebras are smaller and exhibit narrower and more numerous black stripes on their heads and bodies. They show no sign of "shadow" striping between the black stripes on the rump, with black body stripes mostly fading towards the lower parts of the flanks. Mountain zebras have a distinct dewlap, have white under parts, with a central narrow black stripe running from chest to belly. A characteristic feature of mountain zebras is the "gridiron" pattern formed by the black markings on the rump, from the front of ilea to the base of tail.

The Cape subspecies is relatively smaller in size and mass than its Namibian relative. The black stripes on the body are narrower than on the rump and do not continue on the under parts. The striping on the neck is broader than those on the body, and those on the head narrowest of all. The legs are distinctly black-striped down to the hooves and end in black patches just above the hooves. The tail consists of long black or blackish-brown hair that ends below the level of hock. Skinner & Smithers (1990) describe the hind legs of CMZ as having broader stripes than the forelegs, with the upper two or three black stripes on the rump being exceptionally broad. In HMZ, the black stripes are narrower than in the Cape subspecies and approximately equal in width, especially on the rump. The CMZ has a black muzzle tip and a characteristic orange-coloured suffusion immediately behind, on the top and sides, whereas HMZ has a black muzzle surrounded by black hairs interspersed with red hairs medially. The CMZ has characteristic-rounded ears that exhibit white tips and black margins when viewed from the front. When viewed from behind, they are white at the base, then black with white tips. The mane of the HMZ comes further forward between the ears than that of the Cape mountain zebra (Novellie et al. 2002).

2.2.2 Ecology

CMZ are non-territorial and gregarious animals, with breeding herds consisting of a stallion and a mean of 2.4 mares (range of 1 to 5) with their foals (Penzhorn & Novellie 1991). Bachelor herds are common with the occasional presence of fillies and there exists a distinct social hierarchy that is more flexible in structure than breeding herds. Breeding herds of both mountain zebra subspecies are maintained at between 2 and 13 animals and are stable over many years under natural conditions. Mares usually remain in breeding herds for life. New herds are formed when a stallion of five years or older acquires a mare or a filly from breeding herds or split-up herds. Average length of gestation for both the subspecies is around

a year, with mares producing their first foal at four to five years of age and a foaling interval of 25 - 30 months (Penzhorn 1988).

2.3 History of southern African mountain zebra populations

2.3.1 Establishment of the Mountain Zebra National Park

All South African CMZ are descended from no more than 30 individual animals originating from three populations: from the Mountain Zebra National Park (MZNP), and the Kammanassie and Gamka Mountain Nature Reserves (Bigalke 1952). MZNP was established in 1937 near Cradock, with a founder population of five stallions and one mare. At the time, the region in and around Cradock consisted of extensive private farms, where small CMZ populations had occurred for decades. These populations were confined within fenced areas for many generations and it is likely that they were considerably inbred. In 1950, Mr. H.J. Lombard donated five stallions and six mares to the MZNP. In 1964, the Michaus brothers donated another 30 animals to the park (Bigalke 1952). Since the original population perished without breeding, the latter group of animals formed the breeding nucleus from which the current extant Cradock CMZ population is derived. Only one stallion, *Tom*, introduced from the Kamanassie Nature Reserve in 1970, managed to form a breeding herd and sire foals (Penzhorn 1988). The population at MZNP is currently maintained at around 300 to 400 animals and as many as 40 zebras are removed annually to re-establish breeding herds elsewhere within their original range (Penzhorn 1993). Animals have been translocated to a multitude of places, including 6 national parks, 10 provincial and 17 private reserves (Novellie et al. 1996; 2002).

2.3.2 Mountain zebra behaviour and inbreeding

Inbreeding in free-living CMZ populations is controlled by animal-avoidance behaviour adaptations, especially dispersal. Fillies and colts usually leave the maternal herd at puberty and there is individual recognition of sibling or closely related zebra by stripe-pattern association (Penzhorn & Novellie 1993). Such behaviour reduces contact between kin and aids in avoidance of mating with close kin (Blouin & Blouin 1988). Though specific instances of incest-avoidance have been documented and reported (Rasa & Lloyd 1994), it is likely that inbreeding avoidance mechanisms are not as effective in fenced-in populations. Animal behaviour patterns such as mate-choice actively favouring non-kin and where differential dispersal by sex actively separates zebra siblings, was impaired for decades in CMZ. Stripe-

pattern recognition mechanisms and other natural adaptations are possibly less functional under intensively managed and artificially fenced-in conditions (Penzhorn 1979; Penzhorn & Novellie 1991). More than 30 years back, Young & Zumpt (1973) studying the parasites and diseases of the CMZ in MZNP, made the following visionary statement:

> "*Inbreeding could already have reduced the inherent resistance of these animals to diseases and parasites by now and may even become a bigger problem in the future if the necessary provision is not made for the introduction of sufficient new genetic material*."

Cornuet & Luikart (1996) argue that researchers tend to rely on historical population sizes and therefore tend to overemphasise the effect of population bottlenecks on genetic variation. In the case of the CMZ, however, all available evidence indicates that this subspecies did indeed go through a severe genetic bottleneck. Environmental barriers are key factors for the differentiation of populations (Gerlach & Musolf 2000). The ability of an individual to disperse naturally is restricted by manmade barriers and is significant in reducing geographical distribution. Such fragmented populations are subject to genetic distortions such as accelerated genetic structuring, which actually is a reflection of the genetic material exchanged between populations. Conservation biologists have voiced concern about inbreeding as an inevitable consequence in isolated populations (O'Brien et al. 1985; Lande 1988).

2.3.3 History of Bontebok, Gariep Dam, Karoo National Park and Karoo Nature Reserve populations

The CMZ in Bontebok National Park (BNP) originated from the MZNP population. The habitat is less than optimal here and CMZ have to compete with other grazers for the limited resources available. The Gariep Dam Nature Reserve (GDNR) population originated from a breeding nucleus numbering six or seven CMZ that were translocated from the Cradock area. One or two breeding stallions probably formed the core of the herd. All additions to these two populations came from either the Karoo National Park (KaNP) or MZNP (Table 2.1).

The Karoo National Park was established in 1978 by introduction of zebras from MZNP. It is among the few seeded populations where a high population growth has been recorded (Novellie et al. 2002). Karoo Nature Reserve (KaNR) was seeded with 20 animals from

MZNP in 1981 and has also exhibited a good population growth to reach a total of over 120 animals.

***Table 2.1*: History of Cape mountain zebra reintroductions in the Bontebok National Park and Gariep Dam Nature Reserve populations**

Population	Date	Origin of animal	No.	Sex		Remarks
				M	F	
Bontebok National Park	1986	Mountain Zebra National Park	3	3	-	Only two survived
	1990	Karoo National Park	9	4	5	Two stallions moved subsequently
	1993	Karoo National Park	2	-	2	One foal
	1997	Mountain Zebra National Park	6	-	-	Data not available
Gariep Dam Nature Reserve	1985	Mountain Zebra National Park	7	3	4	One subadult mare
	1987	Mountain Zebra National Park	5	-	-	Data not available
	1989	Karoo National Park	10	-	-	Data not available
	1997	Mountain Zebra National Park	5	4	1	Data not available

2.3.4 History of Namibian mountain zebra populations

Mountain zebras in Namibia did not go through a period of population reduction like their Cape cousins. Populations of HMZ total between 20,000 and 30,000 and are maintained in state-protected areas, conservancies in communal land, private farmland and other state-owned land (Novellie et al. 2002). It is estimated that about a quarter of the total population occurs within formally proclaimed conservation areas, and principally within the Naukluft part of the Namib-Naukluft Park. Conservancies in communal lands account for 25 % of the total population, with the remainder on commercial livestock and game farms. The HMZ is still found throughout its historical range and the widespread establishment of artificial water points has allowed it to occupy previously unsuitable habitat (Moodley 2002). The recent fencing of large areas, especially in the central Namibian areas, has put pressure on surviving populations by disrupting migration of animals between north and south.

2.4 Microsatellites and genetic markers

2.4.1 Microsatellites and function

Microsatellites or short tandem repeats (STR) are tandem repetitive stretches of short (2 - 4 base pair) motifs (e.g., CACACACACACA). They belong to a class of sequences termed variable number of tandem repeats (VNTR), referring to any tandem repetitive DNA that shows length polymorphism (Ellegren 2000). These tandem arrays of short stretches of nucleotide sequences are usually repeated between 15 and 30 times and along with the flanking regions, range in size, with a mean of about 100 base pairs (bp).

Microsatellites differ from most other types of DNA sequences in their unusual degree of polymorphism, making them attractive as genetic markers. They have been widely used in a variety of fields, including conservation genetics, population genetics and forensics (Goldstein & Schlötterer 1999). The exact function of such apparently 'junk' DNA sequences is still under debate. A growing number of reports suggest that changes in microsatellite repeat numbers might cause quantitative variation in protein function and gene activity, thus effecting physiology and development (Koreth et al. 1996; Li et al. 2002; 2004). Accordingly, microsatellite mutations within genes have recently been demonstrated to contribute to change in bacterial pathogenicity and adaptation (Bayliss et al. 2004).

Microsatellite analyses have been applied widely in the field of animal genetics and ecology. These genetic markers have been used to:

- Detect inter-species hybridization
- Study population history
- Distinguish demographic factors affecting present day allele frequencies
- Study population bottlenecks and potential inbreeding
- Assess the impact of reproductive behaviour, social structure and dispersal on genetic structure of endangered populations (Goldstein & Schlötterer 1999)

2.4.2 Microsatellites in animal conservation

Over the years, microsatellites have been preferred over other genetic markers in the field of conservation genetics. In many species they are relatively easy to obtain, either through the direct isolation of species-specific markers, involving the construction of a genomic DNA library, or by the application of markers originally isolated from related species. They can be amplified by polymerase chain reaction (PCR) and can be used on non-invasively sampled material (Ellegren 2000). They are comparatively easy to automate, with multiplex amplification of many loci possible in a single PCR reaction. Currently, the highly optimized commercial systems that are available offer multiple loading coupled with special DNA fragment analysis software, making very high throughputs possible (Maudet et al. 2002).

2.4.3 Choosing the correct markers: allozymes vs. microsatellites

Microsatellites have been proven to have comparative advantages over other genetic markers (Table 2.2). Historically, allozyme markers have been used to study correlations between

inbreeding and fitness and the level of inbreeding measured as the level of heterozygosity (Goudet & Keller 2002). The use of genetic markers such as allozymes and *mt*DNA to answer questions relating to population dynamics has been questioned (Davies et al. 1999). Apart from their relatively low variability, the validity of application of such markers to relatively young populations as compared to established ancestral populations, is suspect. In such nascent populations it is probable that assumptions of equilibrium, upon which many of the population genetic parameters are calculated, have been violated.

Allozymes have been used for genetic studies in Equidae as recently as 2001 (Cothran et al. 2001). Allozyme and protein electrophoresis has been the method of choice in previous studies undertaken on population parameters of endangered equids from this continent (van Dyk et al. 1997; Cothran & van Dyk, 1998). Among other disadvantages, this method is less effective for the characterisation of changes in genetic variation in response to recent range fragmentation and population bottlenecks as well as the examination of microgeographic variation and gene flow patterns (Goldstein 1999).

Table 2.2: **Comparative advantages of microsatellites as genetic markers**

Character	***mt*DNA**	**AFLP**	***r*DNA**	**Allozyme**	**RAPD**	**Mn-st**	**Mc-st**
PCR assay	Few	Yes	Yes	No	Yes	Few	Yes
Single locus	Yes	No	No	Yes	No	Yes	Yes
Allele genealogy feasible	Yes	No	No	Rarely	No	Rarely	Yes
Rapid transfer to new taxa	Yes	Yes	Yes	Yes	Yes	Few	Some
Codominance	-----	-----	-----	Yes	No	-----	Yes
Neutrality	-----	-----	-----	Dubious	Yes	-----	Yes
Stage scorable: Embryo Young Adult	 ----- -----	 ----- -----	 ----- -----	 Rarely Yes	 Yes Yes	 ----- -----	 Yes Yes
Variable loci analysed:	Single	Many	Few	1 - 5	2	Moderate	1-50
Molecular information (Structure, mutation)	-----	-----	-----	Rarely	Rarely	-----	Available
Individuals scorable / unit effort	-----	-----	-----	1	1	-----	0.2 - 0.4
Relative cost per individual	-----	-----	-----	1	1	-----	3 - 4

Adapted from Jarne & Lagoda (1996); Sunnucks (2000). [*mt*DNA: mitochondrial DNA; AFLP: Amplified Fragment Length Polymorphism; *r*DNA: ribosomal DNA; RAPD: Restriction fragment length polymorphisms; Mn-st: Minisatellite; Mc-st: Microsatellite]

Allozyme based studies are also hampered by the relatively small number of polymorphic loci and small numbers of segregating alleles. This might lead to the formation of homozygous loci even in the absence of inbreeding. There are differences of opinion regarding the level and cause of selective differences between heterozygotes and homozygotes for allozyme loci (David 1998). On the other hand, the majority of microsatellites are in non-coding regions and consequently neutral, whereas allozymes are indisputably in coding regions. The argument

against allozyme markers is also whether differences in fitness seen in populations investigated are the result of intrinsic genotypic effects or the result of associated loci, either because of linkage disequilibrium or genotypic associations.

Microsatellites have the advantage of being far more polymorphic than allozymes. They exhibit higher heterozygosity levels and individual heterozygosity is more closely related to the degree of inbreeding (Balloux et al. 2004; Slate et al. 2004). Thus allozyme markers have now almost completely been replaced by the more variable and abundant microsatellites. Tsitrone et al. (2001) state categorically that microsatellite loci are ideal to investigate fitness consequences of short-term inbreeding. Under close inbreeding, the correlation between fitness and heterozygosity is higher for markers with high mutation rates, meaning that microsatellites are better suited for such studies. This indicates that for populations with short divergence times (few hundred to few thousand generations), microsatellite markers with high mutation rates would be better suited. Some reviewers have suspected under-reporting of non-significant results in microsatellite-based analysis of populations (Pemberton et al. 1995; Maudet 2002). Until the emergence of a better genetic marker, the preferred use of microsatellites in conservation genetics is likely to remain unchanged.

2.4.4 Cross-specific utilisation of microsatellites

2.4.4.1 Stepwise mutation model

An understanding of the mechanism of base-pair sequence changes over time, within genetic markers, is crucial to its utilisation in population genetic studies. The stepwise mutation model (SMM) describes mutation of microsatellite alleles by addition or deletion of one or more repeated motifs or single tandem repeats, and hence alleles may possibly mutate toward allele states already present in the population (Ellegren 2002). Originally introduced to model electrophoretically detectable enzyme variation in finite populations, SSM has become the mainstay in statistical evaluation and evolutionary interpretation of microsatellite polymorphisms (Weber & Wong 1993; Brinkmann et al. 1998; Di Rienzo et al. 1998; Xu et al. 2000; Balloux & Goudet 2002).

2.4.4.2 Variations in polymorphism

Microsatellite loci are generally assumed to be more polymorphic in the species from which they are cloned than in related species. Ellegren (1995) notes that loci chosen on the basis of

high polymorphism in one species, often exhibit shorter repeats in a related species.

2.4.4.3 Review of microsatellite marker based studies in non-equids

A significant percentage of microsatellite loci isolated and characterized in cattle (*Bos taurus*), sheep (*Ovis aries*) and a multitude of other ungulates have been determined as extensively conserved across other species (Pepin et al. 1995; Engel et al. 1996). Microsatellites isolated from related species have been used for genetic studies in red deer (*Cervus elaphus*; Kühn et al. 1996; Slate et al. 1998), North American elk (Wapiti; Talbot et al. 1996), caribou (*Rangifer tarandus*; Wilson et al. 1997), Scandinavian cervids (Roed 1998), reindeer, (*Rangifer tarandus*; Roed & Midthjell 1998) and Alpine ibex (*Capra ibex*; Maudet et al. 2002).

Primers specific for Y-chromosome microsatellites in cattle amplified in male but not in female African buffalo (*Syncerus caffer*), indicating chromosomal conservation across these two species (van Hooft et al. 2002). O'Ryan et al. (1998) utilized seven microsatellite loci isolated from *Bos taurus*, to study the levels of heterozygosity, allelic diversity and genetic differentiation in fragmented South African buffalo (*Syncerus caffer*) populations.

The genetic variability of the African wild cat (*Felis lybica*), compared to the domestic cat, was studied using microsatellite loci first isolated from domestic cats (Wiseman et al. 2000). Recent genetic studies in jaguars (*Panthera onca*) used 35 microsatellite loci originally developed from mapping the domestic cat genome (Eizirik et al. 2001).

Furthermore, microsatellites developed for American mink (*Mustela vison*) were used to genotype a related species, the European mink (*Mustela lutreola*). The microsatellite polymorphism detected was compared to that of a closely related species, the European polecat (*Mustela putorius*; Peltier & Lodé 2003). Ruiz-Garcia (2003) used five microsatellites that were developed for black bear (*Ursus americanus*) in studies on spectacled black bears (*Tremarctos ornatus*). Altmann et al. (1996) used 10 polymorphic microsatellite markers from the human genome for analysis of behavioural studies in baboon troops.

These studies illustrate that a multitude of well-characterized microsatellites derived from domestic and wild species can be characterised and optimised in related species. Cross-species use of microsatellite loci saves time and effort, allowing rapid progress of genetic studies in several close species (Slate et al. 1998; Luikart et al. 1998).

2.4.4.4 Review of microsatellite marker based studies in equids

Bowling & Ruvinsky (2000) reviewed the baseline polymorphism levels reported for different horse breeds, using microsatellites and other genetic markers. Genetic studies in Equidae other than *Equus caballus* have used sets of microsatellite loci isolated from the domestic horse. Successful applications were reported in Hartmann's zebra (Breen et al. 1995) and Catalonian donkeys (Jordana et al. 1999). A study of five Spanish donkey breeds reported the use of 15 horse microsatellites for the analysis of hierarchical population structure (Aranguren-Mendez et al. 2002). Of these, 13 loci amplified well and were polymorphic in donkeys. Primers flanking polymorphic microsatellite loci of *Equus caballus* Y chromosome have recently been shown to have homologous loci in other equine species (Wallner et al. 2004). Over the years, numerous polymorphic microsatellite markers have been isolated in the domestic horse. Consequently, a genomic DNA library has been developed for the species (Breen et al. 1997; Swinburne et al. 2000). Many of these markers are used routinely in over 86 international laboratories across the world for applications such as identification and parentage verification of individual horses, and genetic analysis across breeds (Cunningham et al. 2001). The horse parentage verification panel can be amplified together under multiplex PCR conditions and exhibits moderate levels of polymorphism across breeds. Size ranges within a single lane of gel electrophoresis are easly accommodated (Bowling et al. 2001; Tozaki et al. 2001). These highly polymorphic horse microsatellite markers have replaced previously published genetic markers in population studies of Equidae (e.g., Cothran et al. 2001). The laboratory methods used allow a high degree of automation and are free of any possibility of radiation exposure (Moodley 2002).

2.5 Bottlenecks and historical genetic variation

The number of alleles remaining after a severe population depletion event is important for the long-term response to selection and for survival of the population (Allendorf 1986). Pre-existing genetic variation is a critical factor for short-term evolutionary change, with such changes possibly being triggered by factors such as diseases, parasites, predators, competitors, pollutants and a multitude of other environmental stressors (Frankham 1997). It is therefore essential for population sizes to be high for favourable mutations to establish. An evident exacerbation of fitness levels, however, may not immediately be detected in populations, where all the individuals have low fitness due to past inbreeding or genetic drift (Hedrick & Kalinowski 2000). In previously depleted populations, the collective genetic load might

include high numbers of deleterious alleles, with eventual inbreeding depression and lowered fitness being caused by mildly deleterious alleles.

Where populations are deviating from mutation drift equilibrium, allelic diversity reduces more rapidly than heterozygosity so that the number of alleles observed is usually less than expected from the observed heterozygosity (Cornuet & Luikart 1996). The fact that extant CMZ populations exhibit good population growth under optimum conditions and show no evident reduction in any commonly indexed fitness trait suggests that the majority of lethal alleles were probably purged from the population during the period of extreme population reduction. Recessive alleles with only small negative effects, however, are purged from the population at a much lower rate (Hedrick 1994) and probably survived the bottleneck.

Reintroduction or reseeding by translocation, as was the case in CMZ, would possibly have caused a second bottleneck. The various consequences of a second bottleneck in already inbred populations are due to the difference in purging of slightly deleterious or detrimental alleles (Wang et al. 1999). The survival rate and population growth after this event are smaller when the initial population size or the carrying capacity is low (Thévenon & Couvet 2002). Harmful effects of the second bottleneck are proportional to inbreeding levels and would cause purging of mutational load to be ineffective. These parameters fit the historical profiles of the CMZ populations investigated.

2.6 Heterozygosity as an indicator of fitness levels

Heterozygosity is a factor that is commonly used to measure genetic variation and loss thereof (David 1998; Balloux 2004; Slate 2004). It can be generally defined as the proportion of heterozygous individuals at a particular locus, and is widely quoted in research since it is proportional to the amount of genetic variation at that locus. Another advantage of this measure is that it can also easily be adapted for theoretical considerations of the effect of limited population size on genetic variation. Its disadvantage is the insensitivity to the actual number of different genotypes at a locus (Allendorf, 1986). Several studies have revealed positive heterozygosity-fitness correlations, using restriction fragment length polymorphism (RFLP) markers (Pogson & Fevolden 1998) and microsatellite markers (Bierne et al. 1998; Coltman et al. 1998; Coulson et al. 1998). A recent review points out the likelihood of null results being under-represented in studies reporting multilocus heterozygosity-fitness correlations (Hansson & Westerberg 2004).

2.6.1 Measures of heterozygosity: heterozygosity (*H*) vs. mean d^2

Coulson et al. (1998) proposed the use of a measure denoted mean d^2, which is the measure of variance in average allele lengths within an individual. It uses the squared difference in number of repeats for two alleles at a locus within an individual, to identify the levels of inbreeding and outbreeding. While individual heterozygosity would reflect recent mating between relatives, mean d^2 is strongly influenced by variation in allele length. This factor thus reflects events like intra-population variation due to migration between populations that have diverged, that are deeper within the pedigree. In an analysis of inbreeding and outbreeding in a well-defined captive wolf population, it was found that mean d^2 was actually less predictive of the known inbreeding coefficient than microsatellite heterozygosity (Hedrick et al. 2001). The usefulness of this measure in inbred populations is questioned, where inbreeding has occurred for only a few generations and where mutation plays an insignificant role. Analysing the costs of parental similarity and fitness in wild populations of seals, whales and albatross, Amos et al. (2001) demonstrated that mean d^2 could not explain the significant variation in fitness. Slate et al. (2000) concludes that until the microsatellite mutation process is fully understood, the parameter measured by mean d^2 would remain dubious. Tsitrone et al. (2001) demonstrates that assuming stepwise mutation process and under close inbreeding, fitness was more closely correlated with heterozygosity than mean d^2. Mean d^2 is more useful when individuals of hybrid origin are examined, where there are alleles with larger size differences at each locus than in individuals whose parents are from the same subpopulation. Citing this to be an important development in the short history of microsatellite analysis, Goudet & Keller (2002) call for the use of heterozygosity over d^2 as the preferred measure for recent inbreeding. Since mean d^2 is based on long-term mutational divergence between alleles, has a large variance and will be best suited to situations where population admixture has occurred, it is probably not the best measure to apply to CMZ, considering the history of the populations.

2.6.2 Heterozygosity-fitness correlations

Individuals with low allozyme heterozygosity and/or high number of lethal equivalent alleles exhibit higher susceptibility to factors that may not affect more heterozygous individuals (Pierce & Mitton 1982; O'Brien et al. 1985; Ralls et al. 1988). Ralls et al. (1988) calculated the median number of lethal equivalents as 3.14 in a survey of 40 captive species, although for a number of species the lethal equivalent was not significantly greater than zero. Though such effects are suspected to be partly the result of genetic associations between the markers

and fitness genes (assertive overdominance), the majority of these effects are probably not due to the direct effects of marker genes on the phenotype (David 1998). Linkage disequilibrium is a probable significant cause of such physical linkage between fitness genes and marker genes in small populations that have been subjected to genetic drift (Pamilo & Palsson 1998). Charlesworth & Charlesworth (1999) suggest that identity disequilibrium due to variance in inbreeding, generated by the correlation between homozygosity of marker loci and low fitness due to inbreeding depression, might be a source of association.

2.6.2.1 Calculation and interpretation of equilibrium and linkage

Evolutionary selection acts best on populations that have high levels of polymorphism. For determining the action of migration, mutation, drift and selection on levels of polymorphism, genetic diversity of markers, their allele frequencies and expected proportions of these markers under a Hardy-Weinberg hypothesis are relied upon (Hartl & Clark 1997). Measurement of deviations from equilibrium is preferably done using exact tests based on a Markov chain algorithm for departure from Hardy-Weinberg proportions (Guo & Thompson 1992) and linkage disequilibrium. Population substructuring, selection acting on linked loci, biased genotyping, presence of null alleles or sex-linked locus and other locus–specific causes result in deviations from equilibrium conditions.

GENEPOP (*Version 3.3*) (Raymond & Rousset 1995) uses Weir & Cockerham's (1984) *F* and Robertson & Hill's (1984) *f* statistics to estimate whether the populations analysed conform to Hardy-Weinberg (H-W) equilibrium. The null hypothesis (H_0) tests the existence of random union of gametes. The *P*-value associated with H_0 (i.e. H-W equilibrium) and the standard error (*S.E.*) of this estimate is estimated. Exact probabilities are estimated using a Markov chain method to estimate without bias, the exact *P*-value. Genotypic disequilibrium is calculated using Markov chain and Fisher's exact procedures as implemented in the program GENEPOP. The H_0 that is tested here is: "*Genotypes at one locus are independent from genotypes at the other locus*". The settings for the exact probabilities are the same as used for testing Hardy-Weinberg equilibrium, with 5000 dememorisation steps, 1000 batches and 10,000 iterations per batch.

2.7 Inbreeding in fragmented populations

Conservation managers of small populations, similar to that of the CMZ, face multiple numbers of issues. Apart from demographic stochasticity and environmental variations, genetic factors such as decreasing population fitness due to inbreeding depression, expression

of deleterious recessive alleles, allele loss and lowered heterozygosity also impact conservation measures undertaken. The manifestation of these genetic factors could potentially lead to eventual fixation of deleterious mutations and subsequent reduction in adaptability and evolutionary potential of populations (Wright 1978; Ralls et al. 1988; Lacy 1993; Jimenez et al. 1994; Lacy 1997; Bowland et al. 2001; Higgins & Lynch 2001). Inbreeding depression is thought to arise as a consequence of genetic mechanisms that result in decreased heterozygosity during the inbreeding process (Charlesworth & Charlesworth 1987). It is usually expressed as a proportionate reduction of a fitness trait relative to the level found in a non-inbred population. The majority of observed inbreeding depression is thought to involve expression of deleterious alleles (*dominance* hypothesis), though the declining heterozygosity among loci exhibiting heterozygote superiority (*overdominance* hypothesis) might also result in inbreeding depression. There is, however, a decrease in population fitness parameters, irrespective of the genetic mechanism involved.

2.7.1 Inbreeding coefficient values: F_{ST} and R_{ST}

Sewall Wright (1951) developed the inbreeding coefficient (F) and several associated indices (F_{IS}, F_{IT} and F_{ST}), as an estimate of inbreeding on gene dynamics, expressing it as the mean correlation of genes within individuals. Over time, the F value in populations under Hardy-Weinberg equilibrium increases, depending on the rate of inbreeding and number of generations that has passed since the founding of respective populations. Among the three indices that Wright developed (F_{IS}, F_{IT} and F_{ST}), F_{IS} indicates deviation in rate of inbreeding in a genetic subpopulation from the rate of inbreeding expected under random mating in an ideal subpopulation. F_{IT} indicates deviation of inbreeding from that expected in a randomly mating total population whereas F_{ST} indicates the amount of genetic variation in total population that is partitioned into subpopulations or the deviation of population subunits from the expectation of a randomly mating total population. F_{ST} is considered to be the most informative statistic for examining the overall level of genetic divergence among subpopulations (Hartl & Clark 1989).

Wright (1978) suggested that an F value range of 0 - 0.05 might be considered as indicating 'little' genetic variation whereas 0.05 - 0.15 indicates 'moderate' genetic differentiation. An inbreeding coefficient range of 0.15 - 0.25 would indicate 'great' genetic differentiation and any value above 0.25 would be indicative of 'very great' genetic differentiation. The deviation of F_{IS} and F_{IT} from zero indicates the promotion (high positive values) or minimisation (high

negative values) of inbreeding relative to the subpopulation or total population. Negative values are also caused due to other factors, as will be discussed later.

R_{ST} (Slatkin 1995) is an analogue of F_{ST} but assuming a strict stepwise mutation model. Although both *F*-statistics and *R*-statistics have been widely reported in studies using microsatellite markers, the estimates often differ significantly (Balloux & Lugon-Moulin 2002). Balloux & Goudet (2002) evaluated the statistical performance of F_{ST} with R_{ST} and concluded that F_{ST} is better for population differentiation estimates in cases where a high level of gene flow is present. R_{ST}, on the other hand, is better suited for populations characterised by very low gene-exchange. R_{ST} values are based on allele size changes in populations analysed. Since the exact allele sizes of horse microsatellites that will be amplified in the mountain zebra will be unknown unless they are sequenced, calculations based on R_{ST} will not be feasible in this study.

2.7.2 Calculation and interpretation of *F*-statistics

2.7.2.1 Estimating levels of population differentiation

The *f*- statistic value, analogous to Wright's (1965; 1978) F_{IS} statistic for each locus, is generally measured according to the method described by Weir & Cockerham (1984). In FSTAT (*Version 2.9.3.2*), it is a measure of the deficit or excess of heterozygotes that could exist in populations. Significance levels are determined from permutation tests with the sequential Bonferroni procedure (Hochberg 1988). The within population inbreeding estimate ($f = F_{IS}$) average is obtained by jackknifing over loci. Genetic differentiation between populations, as defined by Wright's *Fst*, is commonly estimated as θ (Weir & Cockerham 1984) and 95% confidence intervals of θ calculated by bootstrapping (1000 replicates or more). Populations can be tested for significant departure from zero by permutation (1000 replicates or more) of individual genotypes between samples. Pairwise tests of differentiation can be attempted, where for each pair of samples, multi-loci genotypes are randomised between the two samples.

Nei's (1972) genetic standard distances are useful to further quantify population differentiation. *G*- statistics, as originally formulated by Nei (1972) are derived from Wright's *F*-statistics (Wright 1951) and assumes similar definitions in terms of frequencies of identical pairs of genes. Cockerham & Weir (1993) state that the estimates of both parameters are essentially unbiased and that the statistical calculations for deriving both values are similar, although different parameters are tested. Weir and Cockerham's (1984) estimates of F_{IT}, F_{ST}

and F_{IS}, are calculated for each allele, locus and overall with allele frequencies weighed according to sample sizes, as would be done in an analysis of variance. Among the differences, *G*- statistics assumes that all samples carry equivalent weight, irrespective of sample size. The variance components calculated in *F*-statistics and *G*- statistics would be similar if all samples have the same size. However, when sample sizes vary a lot, this can lead to large differences between the two families of estimators. In the case of a completely monomorphic locus, G_{IS} and G_{ST} (and $G_{ST'}$) have values equivalent to zero, while Weir and Cockerham (1984) consider that the estimators cannot be defined.

The overall loci *G*-statistic is used to classify tables and reported based on pairwise significance after standard Bonferroni corrections, with the nominal value set at 5 %. The indicative adjusted nominal level (5 %) for multiple comparisons can be set to different values.

An exact test of population differentiation, testing for hypothesis of random distribution of individuals between pairs of populations (described by Raymond & Rousset 1995) is another method to quantify population differentiation. In this method, *P*-values are calculated and compared with the significance level set at 0.05. Two populations are considered significantly different if the *P*-values were found smaller than the levels of significance. Analysis of molecular variance (AMOVA) provides a useful test for hierarchical F_{ST} analyses and is implemented in ARLEQUIN (*Version 2*; Schneider et al. 2000). This estimates population structure at different levels of specified hierarchies. This essentially determines amount of variance attributable to subpopulation substructure and yields estimations of population structure at different levels of the specified hierarchy. Significance of the different statistics for null hypothesis of no differentiation at the corresponding level can be tested using permutations.

2.7.3 Evaluating evidence from studies on inbreeding

A vast majority of controlled laboratory studies and other observations in natural populations have been an analysis of fitness levels in traits such as fecundity, fertility and zygote viability (Houle et al. 1992). Inbreeding depression was recorded in a number of controlled laboratory experiments in *Drosophila* (Miller-Philip 1994), houseflies (Bryant et al. 1986; Day et al. 2003), butterflies (Saccheri et al. 1996; 1998), beetles (Fernández et al. 1995), snails (Chen 1993), mice (Meagher et al. 2000; Leamy et al. 2001) and plants (van Treuren et al. 1993).

2.7.3.1 Laboratory experiments vs. natural observations

A review of published data on mammal and bird populations suggest that multigenerational consanguinity often significantly affects birth weight, survival, reproduction, resistance to disease, predation and environmental stress (Soule 1986; Thornhill 1993). In a series of elegant experiments in captive *Drosophila*, Miller (1994) demonstrated that the severity of inbreeding depression is increased in stressful experiments at higher levels of inbreeding. Data collated from studies on inbreeding in butterflies, birds and plants reveal that populations with reduced genetic diversity often experience reduced growth and increased extinction rates (Keller & Waller 2002).

Although numerous references have been published on inbreeding in experimental populations, extrapolating data from organisms like *Drosophila* to endangered mammal species has to be done with circumspection. Hedrick (2002b) advises caution in extrapolating data from laboratory experiments on insects that have historically large population sizes to endangered mammals with small population sizes, declining numbers and explicit social and mating structures. One should, therefore, have the effective population size from which the experimental organism is drawn, in perspective. For example, genetic drift may not play a major role in *Drosophila* but might be crucial in another species where past severe bottlenecks or founder events might have decreased effective population size.

Unlike in animal populations that have large generation intervals, inbreeding in small metapopulations such as crustaceans in rock pools, have been shown to be quite discernable. Hybrid *Daphnia* metapopulations in natural rock pools showed average fitness levels estimated to be more than 36 times than that of non-hybrids (Ebert et al. 2002). Population dynamics in populations, that undergo frequent extinction and recolonisation, is different in magnitude when compared with that in a mammal population (Keller & Waller 2002). Thus it can be misleading. It is likely, however, that the same processes that drive inbreeding in these vulnerable metapopulations, also act on other species that live in less obvious metapopulations (Ives & Whitlock 2002). Island populations, also metapopulations, run a greater risk of extinction than mainland populations (Smith et al. 1993). Fragmented and fenced in populations have very similar dynamics when compared to island metapopulations, especially if inter-population genetic transfers are negligible or if they are from the same inbred gene pool. Frankham (1997) reviewed genetic variation in mainland populations of mammals, birds, reptiles, insects and plants. He concluded that compared with island populations, there is significantly higher level of heterozygosity in the mainland populations.

2.7.3.2 Fitness consequences of inbreeding in laboratory populations

Experimentally inbred mice released into a semi-natural environment proved less fit than competing outbred controls (Meagher et al. 2000). Jiménez et al. (1994) demonstrated that inbreeding is correlated to survivorship in a population of mice that was reintroduced to a natural habitat and was significantly reduced in inbred mice. Inbred males continually lost weight while the more outbred ones regained lost weight, leading the authors to suggest that inbreeding effects are more severe in natural environments than in captivity. Saccheri et al. (1996) reported a decrease of 25 % in egg hatching rates of butterflies for each 10 % increase in inbreeding. Data from butterflies, birds and plants reveal that populations with reduced genetic diversity often experience reduced growth and increased extinction rates (Keller & Waller 2002).

2.7.4 Evidence of inbreeding depression in natural populations

2.7.4.1 Methods in published research

Inbreeding in natural populations has been studied by two methods. Long-term studies have been done that rely on extensive pedigrees of individuals, usually of wild vertebrates. These are established either by observation, or inferred by molecular markers, from which individual inbreeding coefficients (*f*) are estimated and correlated with individual measures of various fitness component or fitness-related traits (Pemberton et al. 1999; Balloux & Lugon-Moulin 2002). Such research in feral populations is, however, difficult (Slate et al. 2000). If the species is long-lived, the study has to extend decades in order to obtain good measures of fitness. Mostly, there is a lack of pedigree information extending across multiple generations. This will impede any estimate of relatedness of an individual's parents. An alternative method is the analysis of individual mean heterozygosity calculated from a number of codominant molecular markers, which is then inversely correlated with inbreeding coefficient (Thornhill 1993; Hartl & Clark 1997; Roff 1997).

The allele data collected by this method can be used to detect inbreeding levels and the fitness consequences of probable inbreeding. One downside is that the allele diversity or the number of alleles that a locus exhibits is directly correlated to the number of animals sampled for that population. If sample numbers in the individual populations vary widely, allele diversity, as calculated directly from detected alleles, would mean little. FSTAT (*Version 2.9.3.2*)

overcomes this anomaly by adapting a rarefaction index to population genetics (Petit et al. 1998). Thus FSTAT fixes 'n' as the smallest number of individuals typed for a locus in a population and adapts allele diversity by estimating the expected number of alleles in a sub-sample of 2n genes, given that 2N genes have been sampled ($N \geq n$). The probability of sampling an allele 'i' at least once in a sample of size 2n is then calculated. For allele richness at a locus for all populations considered, the same sub-sample size 'n' is kept, but 'N' is now the overall sample number of individuals genotyped at the locus under consideration.

Another less used approach involves analysing allele lengths of markers used and depends on the stepwise mutation process and coalescence of microsatellites under study (Goldstein et al. 1995). Coalescence is the point in time when two or more alleles were derived from a single ancestor. Since allele length carries historical information, an internal distance measure can be calculated that would reflect the time to coalescence for any two alleles at a locus or, averaged across loci, the mean time to coalescence for the microsatellites. The difference in repeat units between two alleles at a locus is thus related to their time since coalescence. Goldstein et al. (1995) demonstrated that the distance, when squared and averaged over many loci, is linearly correlated with the time since two populations diverged.

2.7.4.2 Fitness traits studied in inbred populations

Historically, studies in this field have concentrated on traits easily and directly correlated to fitness. Examples are reproductive traits (e.g., eggs laid, juvenile mortality) and physical traits indirectly related to fitness (e.g., height of plant, ejaculate volume). Because of the difficulties of making estimates on wild species in nature, most research has analyzed populations of domestic or captive-bred wild species (reviewed by Lacy et al. 1993). Reduced fitness is a commonly reported consequence among offspring born to closely related parents (Lynch 1993; Keller 1998). This applies particularly to stressful conditions and is a primary selective force opposing the build-up of deleterious mutations (Saccheri et al. 1996). Ralls et al. (1988) examined the zoo records of 40 captive wild animal populations belonging to 38 different species and found an average mortality rate of 33 % for inbred matings. They suggest considerably higher costs for inbred feral populations. Classic mutation accumulation studies on deleterious mutations in *Drosophila* and *Caenorhabditis elegans* indicate estimates for average homozygous effect of about 0.1 - 0.2 for inbred lines (reviewed by Wang 2000).

Estimating the cost of inbreeding depression in wild mammal populations has been an ongoing debate. There are two possible reasons for the controversy regarding the degree of

inbreeding depression in wild. Firstly, animals in the wild avoid close inbreeding (Dobson et al. 1997), and therefore do not manifest the deleterious fitness effects. Secondly, even if inbreeding does occur, organisms may deal with the deleterious genetic effects either behaviourally or physiologically before they manifest on a phenotypic level (Frankham 1997).

2.7.4.3 Results from published research

Majority of available information are from laboratory studies on animals and plants, primarily because of the lack of accurate and deep pedigree information from individuals in wild populations (Amos et al. 2001). The indigenous song sparrow population on Mandarte Island, British Columbia (Keller et al. 1994; Keller 1998) and red deer (*Cervus elaphus*) on Rum Island in British Isles (Pemberton & Albon 1992) and few other metapopulations (e.g., Saccheri et al. 1998; Ebert et al. 2002) are notable exceptions where pedigree information was available.

The sparrow population studied by Keller & co-workers (1994; 1998) was decimated as a result of a winter storm, leaving 12 surviving birds. This bottleneck resulted in mean *f* values ranging from 0.06 to 0.09. On average, the authors calculated that offspring of a full-sib mating had on average 17.5 % less likelihood of surviving a year than non-inbred birds. Inbred female sparrows also exhibited reduced lifetime reproductive success (LRS). Parental similarity was correlated to birth weight and juvenile survival in red deer (Coulson et al. 1998) and harbour seals (*Phoca vitulina*; Coltman et al. 1998). The adult reproductive success of male red deer was negatively correlated with parental similarity (Slate et al. 2000). Inbreeding depression was reported in a Speke's gazelle (*Gazella spekei*) breeding program that was established from four founder animals (Kalinowski et al. 2000). Laikre (1999) attributed a number of deleterious effects in captive Nordic carnivores, especially in bears and wolves, to inbreeding. Lack of genetic diversity in free-ranging felids has consistently been correlated to specific reproductive parameters such as semen quality (Wildt et al. 1994) and testicular morphology (Munson et al. 1996). A reduction in ejaculate quality was also reported as a consequence of inbreeding in *Gazella cuvieri* (Roldan et al. 1998).

2.7.4.4 Indirect observations from genetic rescue

The fact that inbreeding can reduce fitness of a wild population can also be indirectly inferred by heterosis effects observed after translocations. Introduction of cougars from Texas (USA) into the last remaining population of Florida panthers (*Puma concolor coryi*) significantly

reduced the incidence of detrimental traits like cowlick, kinked tail and cryptorchidism (Mansfield & Land 2001). Body size of captive-inbred Mexican wolves was significantly lower than in captive wolves with little or no inbreeding (Fredrickson & Hedrick 2002). The arrival of a single male immigrant wolf replenished population levels of the endangered Scandinavian grey wolf (*Canis lupus*), long limited in population size by lack of genetic diversity and inbreeding depression (Ingvarsson 2002). Hereditary blindness, common in inbred captive and wild Scandinavian grey wolves was significantly reduced by introduction of an immigrant wolf (Vila et al. 2003). Similar genetic rescue and restoration of fitness has been demonstrated in populations of greater prairie chickens (*Tympanuchus cupido pinnatus*; Svedarsky et al. 1998) and adders (*Vipera berus*; Madsen et al. 1999; 2004) by introducing translocated members into the populations.

2.7.4.5 Factors correlated to inbreeding

The association between cellular and humoral immunity, disease, reduced fitness and inbreeding in wild mammalian populations continues to be a matter of intense debate. A consensus on what entails definite proof of such associations is currently lacking (Acevedo-Whitehouse et al. 2003). Different factors, environmental and pathogenic, have been correlated to reduced heterozygosity, especially with respect to viral infections in populations.

2.7.4.5a Environmental stressors

Sarcoids have been reported in four populations of Cape mountain zebras, with two populations expressing a very high incidence. GDNR is outside the historic home range of the mountain zebra and the animals in BNP live under less than optimal conditions (Pierre Nel, personal communication; David Zimmermann, personal communication). There is evidence that inbreeding depression is more severe in harsher environments. This includes places with unpredictable rainfall, fluctuating temperatures and limited resources to feed young (Hoffmann & Parsons 1991, Latter et al. 1995). Populations in marginal habitats may also exhibit a high level of stress response, thus showing a high degree of variation at specific stress response loci. Such an association has been reported as crucial for selection against inbred sparrows on Mandarte Island, where the population had already suffered an intense bottleneck (Keller et al. 1994). Others have commented that exposure to environmental stress, especially competition, could translate into an increased metabolic cost for the inbred individual, which may even be lethal (Miller 1994). Keller (1998) states that the pronounced

nature of inbreeding depression under poor environmental conditions may effect the persistence time of populations.

2.7.4.5b Inbreeding and parasite resistance

In inbred Soay sheep (*Ovis aries*), allele frequencies at certain loci were distinctly associated with parasite resistance (Gulland et al. 1993; Paterson 1998). The resistance-associated *S* allele is least frequent in sheep subpopulations with the highest average faecal egg count. Conversely, a 257-base pair allele of the DRB locus in the major histocompatibility complex (MHC), associated with increased mortality and susceptibility to disease in lambs, was most frequent in the subpopulation where the faecal egg count was moderate. Coltman et al. (1999) reported on parasite-mediated selection against inbred Soay sheep and noted that parental similarity was correlated to variation in parasite load. Parasitism, reduced juvenile fitness and extreme climatic variation have been suggested as responsible for causing heavier mortality rates among populations proven to be inbred by microsatellite data, when compared to outbred populations (Coltman et al. 1999; Kalinowski et al. 2000).

2.7.4.5c Disease as a fitness trait

The role of infectious diseases as an important ecological factor in determining the selective pressure on the genomes of the surviving species is an area of emerging interest (Anderson & May 1987; Bellamy & Hill 1998; O'Brien & Evermann 1998; McClelland et al. 2003). Though most reviewers conclude that inbreeding increases susceptibility to pathogens, direct evidence regarding this conclusion has been very difficult to collect (Keller et al. 2002; Coltman et al. 1999). Current research in humans on genetic susceptibility to numerous complex diseases and various infectious diseases has started to identify various candidate genes linked with disease and inbreeding (Bellamy 2003; Rudan et al. 2003). Disease susceptibility and inbreeding has long been described in cheetahs (*Acinonyx jubatus*) (O'Brien et al. 1985; O'Brien 1998; O'Brien & Yukhi 1999). In California sea lions (*Zalophus californianus*), specific correlations have been demonstrated, with sick animals showing significantly higher than normal parental relatedness (Acevedo-Whitehouse et al. 2003). The type of sickness was correlated with degree of relatedness, with highest mean internal relatedness levels seen in individuals affected with herpesvirus-induced carcinomas, followed by those with helminth infections. The authors concluded that inbreeding could be an important factor in determining susceptibility to complex, long-lived parasitic infections. Recent data point towards similarity in the type of immune response that is crucial in both

herpesvirus-induced carcinomas and papillomavirus-induced tumours (Acevedo-Whitehouse et al. 2003). Penzhorn & Horak (1989) described CMZs as harbouring massive loads of ixodid ticks. Other researchers have commented on the high loads of specific helminth parasites in CMZs and have suggested that the large numbers noted could be a function indicating host preference for this subspecies of zebra (Krecek et al. 1994).

2.7.4.5d Disease and major histocompatibility complex (MHC) variation

Demonstrating correlation between MHC variation and resistance or susceptibility to parasites has always been considered to be a difficult experimental challenge (Garrigan & Hedrick 2003). Since a multigene family codes for the complex, it is usually difficult to separate the effects of specific alleles from background genotypes. The high variability within loci and similarity of alleles within loci frequently makes it difficult to determine the MHC sequences that are allelic from other genes (Hedrick 2002). In a study on the correlation between low MHC variation and decline of desert bighorn sheep (*Ovis canadensis*), sparce evidence could be found, with the authors reporting extensive polymorphism (Gutierrez-Espeleta et al. 2001). Hedrick (2002) suggests that temporally variable pathogens may cause an increase in polymorphism within specific alleles on MHC genes and other similar host defense loci. Due to limitations regarding experimental technique, the unknown nature of genes responsible for selective adaptation and the statistical formulae used for eventual interpretation, the implications of low genetic variation on disease should be interpreted cautiously.

2.8 Sarcoid-like tumours in Cape mountain zebras

2.8.1 Sarcoid tumour and incidence

The equine sarcoid is considered to be a virus-induced tumour, with a wide variety of clinical outcomes, manifested as a result of complex interactions between the aetiologic agent, environment, and host genome. They are neoplasms that are predominantly composed of a spindle cell population and have been extensively reported to contain bovine papillomavirus (BPV) DNA types (Lowy 2001). As far as CMZ in BNP and GDNR are concerned, as of March 2004, 53 % (n = 19) and over 22 % (n = 83) exhibited signs of the tumour (Figure. 2.1). An analysis of the histopathology has indicated a picture similar to that of the equine sarcoid (Marais et al. unpublished). Virological analysis of tumour material has confirmed the presence of BPV 1 and 2 (van Dyk et al. 2004). Ragland et al. (1966) reported the only previous record of a sarcoid epizootic, where five of 50 horses in a herd of different breeds

expressed sarcoids within a period of six weeks. The fact that these authors noted a familial pattern in this epizootic, with four of the five affected individuals being members of a highly inbred horse family is significant. The only other record of high incidence was that noted in a donkey sanctuary population in United Kingdom (Reid et al. 1994). Peak prevalence reported in this population of 4,126 mostly unrelated donkeys was 15.6 and 25.0 / 100 animals for two and five year olds respectively. The unmeasured pedigree would be a major confounder in any current or future research in this affected donkey population (Chambers et al. 2003).

Figure 2.1: **Sarcoid tumour on the abdomen of a CMZ**

Studies to date on the equine sarcoid have measured the prevalence only in horses and donkeys, seen either as disparate clinical cases in hospitals or in genetically unrelated animals kept as groups or individuals (Angelos et al. 1988; Reid et al. 1994; Broström 1995). The CMZ in BNP and GDNR can be described as the only populations where a real 'outbreak' of sarcoid tumour has been observed. It differs from the other equid populations studied so far because they are naturally breeding feral populations, highly inbred and potentially well defined.

2.8.2 Sarcoid tumours and papillomavirus infections

Over the years, research on the aetiology of the equine sarcoids has pointed towards a more definitive role played by BPV types 1 and 2 in aetiology and pathogenesis of the tumour (Amtmann et al. 1980; Angelos et al. 1991; Otten et al. 1993; Bloch et al. 1994; Reid et al. 1994; Nasir et al. 1997; 1999). Genetic susceptibility of non-host species like horses and

donkeys to bovine papillomavirus infections remains a subject that, according to an analysis of the published literature, has not been studied in detail. The relationship between published MHC subtype associations and susceptibility to tumour has not extended to studies on possible correlation with susceptibility to BPV infections in horses. No studies have been published on how inbred as compared to outbred laboratory populations would vary in their susceptibility to papillomavirus and infectivity. The best, current research reveals that susceptibility to sarcoid in the horse and donkey is associated with a multitude of risk factors, including major histocompatibility complex haplotypes, age and sex (Chambers et al. 2003).

2.8.2.1 Genetic susceptibility to sarcoid in horses

Breed susceptibility of horses to sarcoids has been reported, showing differing susceptibility levels among breeds. Serologically, associations with the class I allele (A3, A3W13, and A1) and MHC class II (DW13) have been reported (Broström et al. 1988; Lazary et al. 1994). A deficiency in the immune response that occurs during sarcoid formation, with involvement of certain MHC class II alleles, was recently suggested as the key to the development of lesions subsequent to BPV infection (Chambers et al. 2003). In ELA DW13 heterozygous stallions, there was a strong association between inherited DW13 antigen and susceptibility to sarcoid, indicating Mendelian segregation in diseased half siblings (Lazary et al. 1994). The disease thus appears to be in linkage disequilibrium with certain serologically defined locus I and II ELAs, which are gene products of the MHC or some other susceptibility genes. These ELA specificities represent allelic gene products, but, whether these are coded for by single locus or multi loci and the exact location of these loci on the equine genome or MHC is yet to be determined. The codominant expression of ELA in horse and its inheritance as simple Mendelian traits, coupled with the observation that sarcoids among offspring are significantly associated with one of the parental haplotypes, strongly suggest that predisposition for sarcoids in horses is probably due to an autosomal, dominant, ELA-linked gene with incomplete penetrance (Meredith et al. 1986; Broström et al. 1988). Conclusive proof on whether these genes are sublethal, incompletely penetrating or both and whether intense inbreeding in a population can expose these susceptibility genes to cause disease at statistically significant rates, is still wanting.

2.8.2.2 Papillomavirus infections and genetic susceptibility

Cottontail rabbit papillomavirus (CRPV) induced infections are more common in rabbits with unique MHC class II haplotypes (Han et al. 1992; 1994). Studies on CRPV infection in

rabbits also point toward class II alleles playing a major role in the immune response to HPV infection (Favre et al. 1997). Furthermore, gene mutations that potentially predispose humans to a number of papillomavirus mediated skin diseases/conditions have also been studied (Majewski 1997). Recent research on oncogenic human papillomaviruses and skin conditions attributed to these viruses has revealed abnormal susceptibility to infection to a group of HPV genotypes, including the oncogenic HPV5. An example is skin carcinomas developing in patients with non-melanoma skin cancer (Harwood & Proby 2002) and epidermodysplasia verruciformis (Ramoz et al. 1999), familial psoriasis (Nair et al. 1997), autoimmune bullous disorders and even burns (Favre et al. 2000). Evidence exists that human leukocyte antigen (HLA) polymorphisms, (especially certain class II alleles) may predispose individuals to the development of specific papillomavirus diseases like cervical cancer and recurrent respiratory papillomatosis (Breitburd et al. 1996; Gelder et al. 2003). These reports suggest a definite relationship between expression of certain specific but yet unknown genes and susceptibility to papillomavirus infections in multiple numbers of species

2.8.2.3 Papillomavirus and immunosupression

Lowy (2001) stated that cancers attributable to PV infections typically do not occur until many years after the initial infection. The reason for this long delay is considered to be factors such as impaired cellular immune function and exposure to co-carcinogens. Skin lesions such as squamous cell carcinomas caused by papillomaviruses have been reported in older domestic cats with waning immunity and in immunocompromised cats affected with FIV (Carney et al. 1990; Egberink et al. 1992; Tachezy et al. 2002). Recently, a *Felis domesticus* papillomavirus was isolated and cloned from an immunosupressed cat with an inherited immunodefiency (Tachezy et al. 2002). The possibility that papillomaviruses pre-exist as non-pathogenic or latent in host tissue, but become oncogenic when the local environment is sufficiently modified for their entry and proliferation, has been suggested (Kidney et al. 2001).

Reduction in major histocompatibility complex diversity due to genetic bottlenecks and subsequent inbreeding may contribute to uniform population sensitivity to emerging infectious pathogens, and been extensively reviewed in the *Felidae* (Yukhi & O'Brien 1990; O'Brien & Yuhki 1999). A general scarcity of records to prove that papillomaviruses have emerged and spread in populations that underwent historic demographic genetic reduction exists. An epidemic of an oral focal epithelial hyperplasia (FEH)-like disease in an inbred pygmy chimpanzee (*Pan paniscus*) colony was reported (Van Ranst et al. 1991). A novel

papillomavirus genome was cloned in this study, with the genomes of PCPV-1 and HPV-13 showing extensive sequence homology. FEH, a rare disease, was also encountered in historically isolated Navajo Indians and Greenlandic Inuit populations and HPV-13 isolated (Van Ranst et al. 1992).

2.8.2.4 Cape mountain zebra populations and immune status

Anecdotal evidence points to impaired immune function in CMZ populations. There are three necropsy reports of CMZ originating from GDNR that died of a disease symptomatic of African Horse Sickness (AHS) (Deon Scaap & Ian Espie personal communication). Though the putative virus was not isolated, the apparent susceptibility of these animals to a disease, symptomatic either of AHS or equine encephalosis is unique in itself, as zebras in general have been reported to be resistant to both (Lord et al. 1997). Reports of high mortality in CMZ foals during heavy snowfalls have been recorded in South Africa (Penhorn 1984; Lloyd & Rasa 1989). Tumour affected CMZ at GDNR exhibit higher mortality rates than non-affected due to reasons that seem unrelated to any apparent climatic variations (Pierre Nel personal communication). Other than the general observation that these animals generally harbour high tick loads (Young & Zumpt 1973; Penzhorn 1984; Penzhorn & Horak 1989) no comparative studies have been done on parasitic loads between sarcoid affected and non-affected zebras. Young & Zumpt (1973) also commented on the high incidence of subclinical equine babesiosis in CMZ. A number of references indicate that comparative analyses of parasitic loads, internal and external, in affected and non-affected animals, could be an indirect way of determining whether there is evident immunosupression (Coltman et al. 1999; Slate et al. 2000; Krebs et al. 2002).

Chapter III

Application of *Equus caballus* microsatellites for genotyping endangered mountain zebra populations

Abstract

Cape mountain zebra (CMZ; *Equus zebra zebra*) populations in South Africa have recovered from near extinction, to reach to the current population of approximately 1600 animals. Except three, all extant CMZ populations originated from a small number of founder animals conserved within the Mountain Zebra National Park. The Namibian Hartmann's mountain zebra subspecies (HMZ; *Equus zebra hartmannae*), on the other hand, has a history of being migratory and outbred, with the current population numbering over 25000 animals. Four Cape mountain zebra populations, Bontebok and Karoo National Parks and Gariep Dam and Karoo Nature Reserves and the Namibian Hartmann's mountain zebra population, were genotyped using 16 microsatellite genetic markers. These markers, originally isolated and cloned from the domestic horse (*Equus caballus*) genome, were applied to amplify similar DNA within mountain zebra genomes. DNA archiving and extraction and was done using proprietary FTA® technology. Microsatellite primers were successfully multiplexed for high throughput and polymerase chain reactions were carried out on the archived samples. Final products were visualised fluorescently using an automated genetic analyser and subsequent marker data generated was analysed using open-source software. Domestic horse microsatellites proved to be successful for genotyping individual mountain zebras.

3.1 Introduction

Mountain zebras (*Equus zebra zebra and Equus zebra hartmannae*) are categorised in the World Conservation Union Red List as vulnerable (*Version 3.1*) and endangered (*Version 2*), respectively (Friedman & Daly 2004). While the Cape mountain zebra (CMZ) is listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora Appendix I, the Hartmann's mountain zebra (HMZ) is listed in Appendix II. Over-exploitation and other human excesses had depleted the Cape subspecies to few dozen surviving animals in the 1930s. The majority of the current CMZ population is derived from the few surviving animals that thrived after the establishment of Mountain Zebra National Park (MZNP) in 1937 (Bigalke 1952). These animals are managed in fragmented populations, including 6 national parks, 10 provincial and 17 private reserves (Novellie et al. 1996; 2002). In contrast, Hartmann's zebras are still found all along its historical range in Namibia, albeit in decreasing numbers.

Very few genetic studies among zebra populations have been undertaken (Bowland et al. 2001; Moodley 2002). Bowland et al. (2001) utilised allozyme genetic markers to characterise genetic diversity within *Equus quagga* populations. Protein markers, however, have low variability and are less effective for the characterisation of genetic variation in response to recent range fragmentation and population bottlenecks (Goldstein 1999). Moodley (2002) utilised radionucleotide-labelled genetic markers isolated from the domestic horse (*Equus caballus*) to compare genetic diversity between mountain and plains (*Equus burchellii*) zebra populations. Application of microsatellites originally isolated and cloned from one species to study another closely related species is common, especially within the field of conservation genetics (Breen et al. 1994; Slate et al. 1998).

Establishment of a simple but rapid, inexpensive and contemporary method for determination of genetic variation among different CMZ populations assumes importance with the outbreak of tumour-like growths called 'sarcoids' in certain populations. Studies have highlighted the important role played by genetics in expression of such tumours in the domestic horse (Lazary et al. 1994; Chambers et al. 2003). It is possible that inbreeding within CMZ populations is related to the increased incidence of this virus-induced tumour. Appropriate genetic tests are required that can determine heterozygosity levels in the individual CMZ to identify more outbred and genetically different CMZ for translocations. Such tests should preferably be run using an internationally acceptable protocol, with high throughputs, and be reproducible in genetic laboratories worldwide. This study utilised International Society of Animal Genetics

(ISAG) recommended microsatellites, proven to be highly polymorphic in the horse, for genetic characterisation of selected mountain zebra populations. The primers of the selected microsatellites were fluorescent-dye labelled and amplified in polymerase chain reactions as multiplexes. This enables much higher throughputs. An automated genetic analyser was used for data collection, which processed the raw DNA data and specialised computer software subsequently enabled visualisation. Cape and Hartmann's mountain zebra subspecies are karyotypically similar (chromosome number = 32; Heinichen 1969), with little phenotypical variation between them. In the frame of this study, we analysed populations from both subspecies of mountain zebra, with the aim of establishing an effective genotyping technique for these two subspecies and thus provide the basis for use of genetic markers as an adjunct for effective conservation programs.

3.2 Methods

Figure 3.1: **Map of southern Africa indicating areas from where the samples originated**
Hartmann's zebra samples: AUB - Auasberg, GMB - Gamsberg, KHL - Khomas Hochland, NKL - Naukluft, NKD - Northern Kamanjab District and OMU - Omaruru
Cape mountain zebra samples: BNP - Bontebok National Park, GDNR - Gariep Dam Nature Reserve, KNP - Karoo National Park and KNR - Karoo Nature Reserve
The probably distinct CMZ populations are MZNP - Mountain Zebra National Park, DHNR - De Hoop Nature Reserve, KMNR - Kamanassie Nature Reserve, and GMNR - Gamka Mountain Nature Reserve

3.2.1 Animals

In a previous study on population genetics of mountain and plain zebras, Moodley (2002) collected and extracted DNA from Cape and Hartmann's mountain zebra samples. The

extracts were kindly made vailable for this project. The HMZ samples (n = 84) came from populations in and around Gamsberg, Auasberg, Omaruru, Naukluft, Kamanjab District (north) and Khomas Hochland areas in Namibia (Figure 3.1). These consisted of dried tannery, museum and field-dried skin samples and blood samples collected after immobilisation. Our samples were obtained from CMZ in the Bontebok National Park (n = 12) and Gariep Dam Nature Reserve (n = 17). These zebras were immobilised by the wildlife veterinarians of South African National Parks and Free State Department of Environmental Affairs and Tourism. Once immobilised, 10 ml blood was collected from the jugular vein in barcode-labelled EDTA BD vacutainer™ tubes. These were then refrigerated (4 ºC) until processed in the laboratory. Another 24 extracted CMZ-DNA samples, collected by Moodley (2002) from Karoo National Park (n = 12) and Karoo Nature Reserve (n = 12), were also made available to us (Figure 3.1).

3.2.2 DNA extractions and archiving

3.2.2.1 Standard extraction protocol

The extracted DNA samples, donated by Moodley (2002), were processed following the standard SDS-Proteinase K/phenol-chloroform protocol (Sambrook et al. 1989). Skin and tissue samples were treated with STE buffer and extracted with the standard protocol. Proteinase K digests proteins and SDS disrupts the cell membranes, releasing DNA from the nucleus. Blood in EDTA BD vacutainer™ tubes was lysed using erythrocyte lysis buffer (0.32 M sucrose, 10 mM Tris (pH 7.6), 5mM $MgCl_2$ and 1% [v/v] Triton X100). Nucleated cells were pelleted and resuspended in sodium chloride-Tris-EDTA (STE) isotonic lysis buffer (0.15 M NaCl, 1mM EDTA, 50 mM Tris (pH 8.0). DNA was further purified with phenol and subjected to chloroform/isoamyl alcohol extractions. The extracted DNA was precipitated with sodium acetate and isopropanol. The precipitated DNA was centrifuged and washed in ethanol to remove traces of isopropanol. The pellet was then air-dried and subsequently mixed with 50 - 500 µl Tris-EDTA buffer [10 mM Tris (pH 8), 1 mM EDTA (pH 8)] and left to dissolve for at least 12 hours at 55 °C. Isolated DNA was then analysed for quality and yield using UV spectrophotometry before polymerase chain reactions were conducted (Moodley 2002).

3.2.2.2 DNA storage using FTA® paper technology

This study used proprietary FTA® (Whatman Bioscience, USA) technology for extraction and storage of DNA from blood samples collected from zebras. This is a simplified method for

archiving of samples for future reference and subsequent purification and analysis of pure DNA (Whatman Bioscience 1999). The FTA® paper is cut and mounted in a 35 mm slide frame (Figure 3.2), without glass backing. The filter paper matrix is impregnated with a combination of protein denaturants, a chelating agent and a free-radical trap designed to protect and entrap nucleic acids. When blood containing nucleated cells dries on the matrix, cell membranes and organelles are lysed and nucleic acids released, causing both RNA and DNA to become entrapped within the fibres of the matrix. The integrity of DNA is preserved and since nucleic acids remain immobilised within the matrix, there is little possibility of cross-contamination between samples. Antimicrobial agents within the matrix allow for storage of samples without microbial or environmental degradation at room temperature for decades. It thus offers a simple and compact archival system for biological samples and eliminates the need for expensive and space consuming cold-storage facilities.

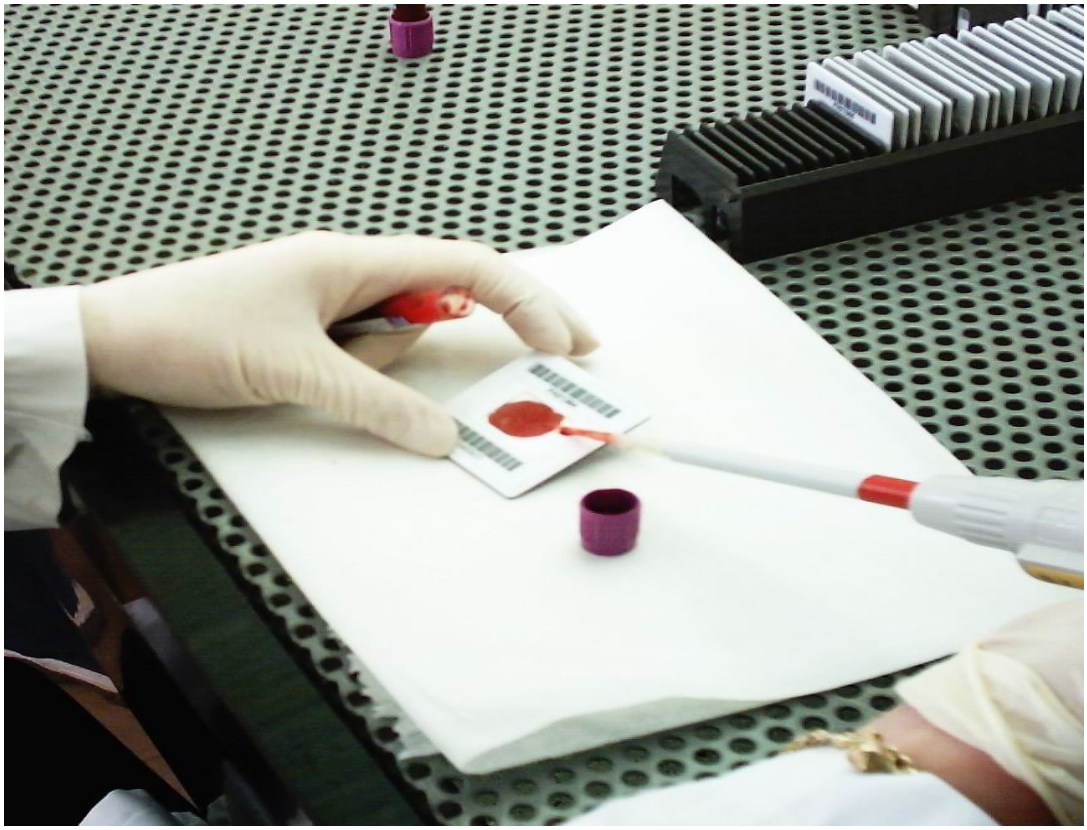


Figure 3.2: **Spotting blood from EDTA vacutainer™ tubes into a barcoded FTA® paper slide under a fume- hood**

Blood from EDTA tubes was spotted on to the exposed surface of the FTA® paper with a 100-µl pipette and put aside for a few minutes to dry under laminar airflow. The dried FTA® paper was then packed into slide-cassettes (shown in background in Figure 3.2) and stored in filing cabinets in a temperature-controlled room maintained at between 15 - 20 °C. Each slide was labelled with two barcodes (Figure 3.2). The primary barcode corresponded to the one on the EDTA blood tube. This was the sample number. A second barcode (standard species-specific

laboratory number) was used for databasing all collected samples using ACCESS© software (Microsoft Corporation™, USA). All information regarding each sample is recorded in this database and data backed up in an independent server.

3.2.2.3 Purification of DNA trapped in FTA® paper

A 2 mm circular paper piece was punched out of each DNA-impregnated matrix card using the Harris Micro Punch™ tool. The construction of the matrix allows for direct PCR analysis on the punched-out piece. This eliminates shearing forces associated with normal purification protocols using organic solvents. The punch was then transferred to a PCR amplification tube and 200 µl of FTA® purification reagent added to each tube. This proprietary purification reagent allows haeme and other PCR inhibitors to be washed out during the five-minute incubation period during which the tube is agitated at room temperature. The FTA® punch was washed three times, thus ensuring the maximal removal of the purification reagent during each pipetting procedure. TE buffer (200µl: 10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0) was then added to the tube and agitated. After incubation for 5 minutes at room temperature, the buffer was drawn off. This step was repeated two times. The FTA® punch was then air-dried and followed immediately by PCR amplification.

3.2.3 Microsatellites

Microsatellites originally isolated from the domestic horse (*Equus caballus*) were tested for amplification on the zebra samples. Sixteen of these markers were initially analysed in the veterinary genetics laboratory's library of Cape mountain zebra samples before being screened for polymorphism in the populations of interest (Table 3.1).

***Table 3.1*: Number and origin of samples and success of amplication**

Species	Number of samples	Origin of samples	Microsatellites amplified/population
Equus zebra hartmannae	84	Gamsberg Auasberg Omaruru Naukluft Kamanjab District (North) Khomas Hochland	15
Equus zebra zebra	53	Bontebok National Park	16
		Gariep Dam Nature Reserve	16
		Karoo National Park Karoo Nature Reserve	9

Individual microsatellite primer sequences and references are detailed in *Appendix 1I*.

3.2.3.1 Primer end-labelling

Equus caballus microsatellite primers were ordered from Applied Biosystems SA (Pty) commercial oligonucleotide synthesiser. Forward primers were end-labelled with flourophores, by stating the dye label required for each (FAM®, NED®, VIC® and PET®) and the sequences of the primers.

3.2.3.2 Multiplex PCR settings and conditions

Amplification of several microsatellite markers was attempted in a single PCR multiplex. Primer combinations were chosen based on the capacity of each marker to co-amplify in a PCR with similar annealing temperatures. The absence of overlapping of allelic size-range in the same set was also tested. Multiplex group A consisted of a panel of seven microsatellite markers and were labelled with a different flourochromes (Table 3.2).

***Table 3.2*: Microsatellites amplified and fluorochrome per multiplex**

Locus	Fluorescence
Multiplex A- ROX400 size standard	
ASB17	VIC-green
VHL20	FAM-blue
AHT4	FAM-blue
HMS6	VIC-green
ASB23	VIC-green
HTG4	FAM-blue
AHT5	VIC-green
Multiplex B- ROX400 size standard	
HTG10	NED-black
HMS3	NED-black
LEX33	NED-black
ASB2	FAM-blue
LEX3	FAM-blue
Multiplex C- LIZ500 size standard	
LEX52	FAM-blue
UMO11	VIC-green
HMS42	NED-black
LEX64	PET-red

Multiplex group B consisted of a panel of five microsatellite markers. The fluorescent dyes, each possessing a distinct emission spectrum, were chosen so that microsatellites of non-overlapping allele size range could be labelled with a single colour. Multiplex C with four markers was PCRed separately as these have different annealing temperatures. The primers for each locus consisted of one primer labelled with a fluorescent dye and an unlabelled primer.

3.2.3.3 Polymerase chain reaction primer concentrations

Multiplex PCR amplifications were carried out in a 10 µl reaction using either a *Geneamp*® PCR system 9700 or 2700 thermocycler (Applied Biosystems Inc., Foster City, CA). PCR amplification mix was added to the PCR tube containing the dried FTA® paper segment. Approximately 20 ng of genomic DNA, trapped within the 2 mm punched-out segment, was used as template. The final working concentrations for multiplexes A, B and C are detailed in Table 3.3 below.

***Table 3.3*: Primer mix for PCR multiplexing (All volumes in µl)**

Primer mix for PCR for multiplex-A (100 reactions)				
Primer	**Primer concentration**	**Concentration**	**Forward primer volume**	**Reverse primer volume**
VHL20	20 nM	0.20	10	10
HTG4	20 nM	0.07	3.5	3.5
HMS6	20 nM	0.60	30	30
ASB23	20 nM	0.20	10	10
ASB17	20 nM	0.11	5.5	5.5
AHT5	20 nM	0.20	10	10
AHT4	20 nM	0.08	4	4
Total Primer Volumes			73	73
			Primer Volume	**146**
			10XPCR Buffer	**30**
			Water	**124**
			Total Volume	**300**
Primer mix for PCR for multiplex-B (100 reactions)				
LEX33	10 nM (forward) 20 nM (reverse)	1.00	100	50
HTG10	20 nM	0.20	10	10
ASB2	20 nM	0.15	7.5	7.5
HMS3	20 nM	0.16	8	8
LEX3	20 nM	0.12	6	6
Total Primer Volumes			131.5	81.5
			Primer Volume	**213**
			10XPCR Buffer	**30**
			Water	**57**
			Total Volume	**300**
Primer mix for PCR for multiplex-C (100 reactions)				
			Combined Forward and Reverse Primer Volumes	
LEX52	4 nM	0.10	25	
UMO11	4 nM	0.04	10	
HMS42	4 nM	0.04	10	
LEX64	4 nM	0.10	25	
Total Primer Volume			70	
			Primer Volume	**70**
			10XPCR Buffer	**30**
			Water	**200**
			Total Volume	**300**

Each polymerase chain reaction was carried out in 10 µl reactions and the reaction mastermix for each multiplex was made up for 100 reactions of 10 µl (Table 3.4).

***Table 3.4*: PCR mastermix combination for multiplexes A, B and C (All volumes in µl)**

PCR Mastermix (100 reactions)		
Order	**Component**	**Volume (µl)**
1	Primer mix	280
2	Water	432
3	10XPCR buffer	100
4	25 mM $MgCl_2$	100
5	10 mM dNTP mix	80
6	Amplitaq Gold®	8
	Total volume	**1000**

Each mastermix contained 280 µl primer mix consisting of unlabelled reverse primers and fluorescently labelled forward primers, 432 µl water, 100 µl of 10X PCR buffer, 100 µl of 25 mM $MgCl_2$, 80 µl of 10 mM dNTP and 8 µl of 0.1 units Ampli*Taq Gold*® DNA polymerase (Applied Biosystems Inc., Foster City, CA). The total reaction volume (10 µl / well, for 100 reactions) amounted to 1 ml for each multiplex master mix. Of this, 10 µl was added to each well, along with template DNA (20 ng each), before initiating PCR.

3.2.3.4 Polymerase chain reaction temperature profiles

All PCR cycles were preceded by an initial step of Ampli*TaqGold*® DNA polymerase (Applied Biosystems Inc., Foster City, CA) activation for 10 minutes at 94 °C. PCR reactions were run for 30 cycles of 94 °C for 60 seconds for denaturation, followed by annealing at 60 °C for multiplex A and C and 56 °C for multiplex B for a total of 30 seconds and a final extension step at 74 °C for 45 seconds. The cycles were terminated with incubation at 74 °C for 10 minutes and machine-controlled snap cooling to 4 °C. The amplification products were retained within the PCR machine till ready for loading.

Sample loading into the genetic sequencer was done by mixing 1 µl of PCR product with a solution made up of mixing 25 µl each of internal lane size standards; Genescan Rox400™ for multiplex A and B and Liz500™ for multiplex C, along with 1 ml of deionised Hi Di formamide. The three multiplexes were loaded separately into the genetic sequencer.

3.2.4 Genotype determination

Electrophoresis was carried out in an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA). The internal size standard, included with each sample, allow for the automatic sizing of alleles. The machine was automated to proceed with electrokinetic injections (5s, 15 kV) and electrophoresis of PCR products at 15 kV in Performance Optimised Polymer 4 (POP-4™) (Applied Biosystems). Ensuing data was automatically

recorded by ABI PRISM 310 Collection Software application, *Version 3.0.0* (Applied Biosystems, Foster City, CA).

3.2.4.1 Allele visualisation and readout

STRand software (*Version 2.2.224*) (Board of Regents, University of California, Davis) was used to analyse data recorded from the genetic analyser. Allele sizes were determined by the Local Southern method. They were assigned with alphabetical symbols, in order from smallest to the largest. This is based on a middle-sized allele assigned as M. *STRand* software was developed at University of California, Davis. This automates and speeds up analysis of DNA fragment length polymorphism samples run on fluorescence-based gels. The advantage of using *STRand* over the proprietary *GENESCAN*© software (Applied Biosystems, Inc.) is that it is an open-source software and hence economical and can be easily adapted to integrate with existing systems. It allows for problem-free and easy reading and subsequent assigning of alleles, with gel reading times decreasing by as much as 50 – 80 %. Unlike *GENESCAN*©, this software also has the advantage that it can be manipulated by assigning specific range values for particular markers used and according to each species studied.

3.3 Results

All sixteen microsatellites, originally designed for amplification in domestic horses, were amplified successfully in CMZ from BNP and GDNR. In Hartmann's zebras, fifteen microsatellites amplified successfully. Because locus LEX33 failed to amplify in all Hartmann's zebra samples, it was excluded from further comparative analyses.

The linkage relationships of loci selected for this study are currently unknown for mountain zebras. In domestic horse, microsatellite LEX3 is an X-linked locus (Chowdhary et al. 2003) and it is possible that it might be sex-linked in mountain zebras too. This microsatellite was thus excluded from further genetic analyses. Comparitive population genetic studies between Hartmann's and affected CMZ populations were thus confined to allele data obtained from 14 microsatellites that excluded LEX33 and LEX3.

A graphical comparison of the alleles found in each subspecies (Figure 3.3) indicates that HMZ populations have retained a greater number of alleles than the Cape subspecies. Except in the case of LEX33, HMZ showed consistently higher diversities in every microsatellite locus of the panel used by us (Appendix III). The allele heterozygosities of the mountain

zebra were compared to those of domestic horse breeds (Table 3.6). Although the allele numbers differed from those reported in domestic horse breeds, they were higher in Hartmann's zebras than in CMZ.

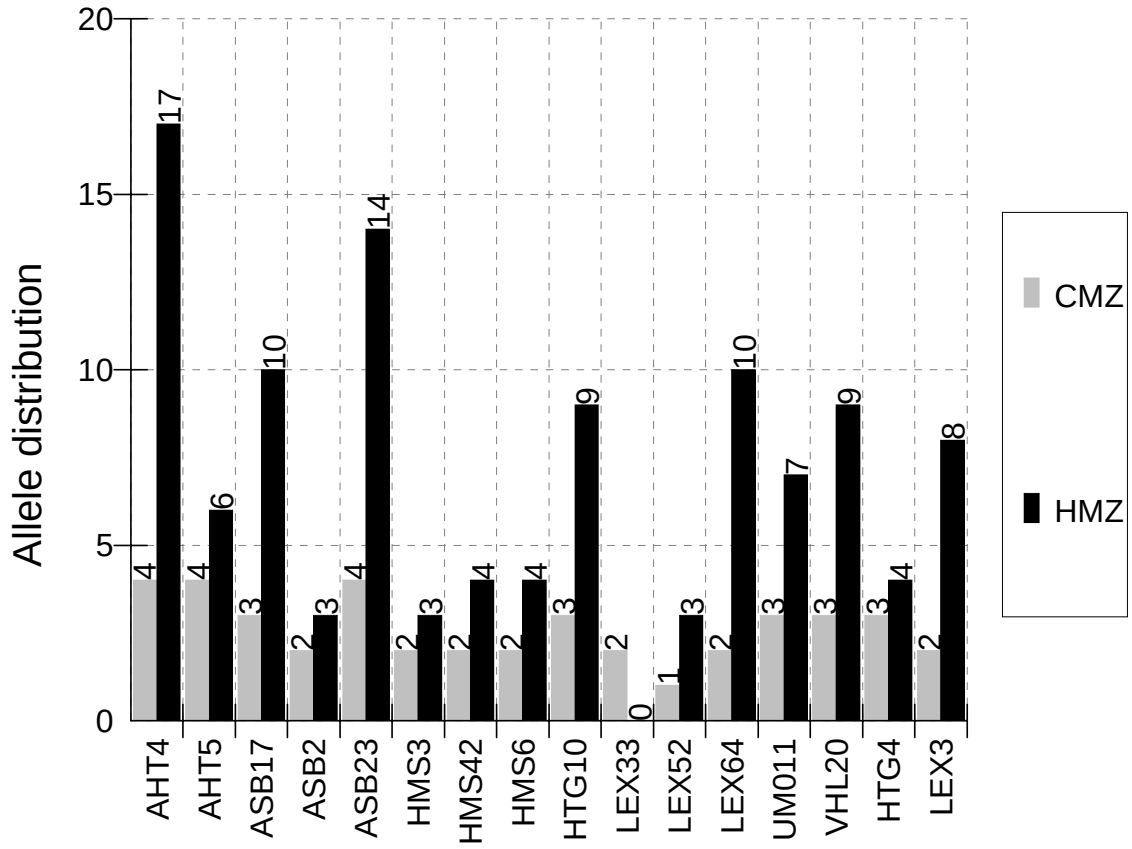


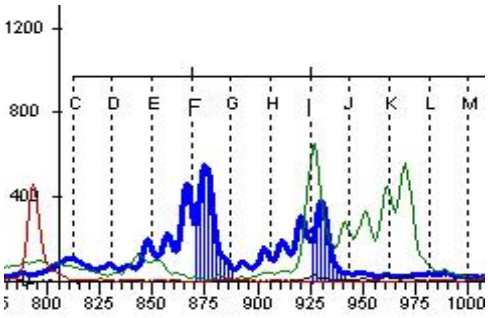
***Figure 3.3*: Comparative allele distribution in CMZ and HMZ populations**

Selected electropherograms of fluorescent-labelled alleles produced by the software *STRand* corresponding to different microsatellites are shown in Figure 3.4. Allele recognition was consistent and enabled easy determination of individual genotypes.

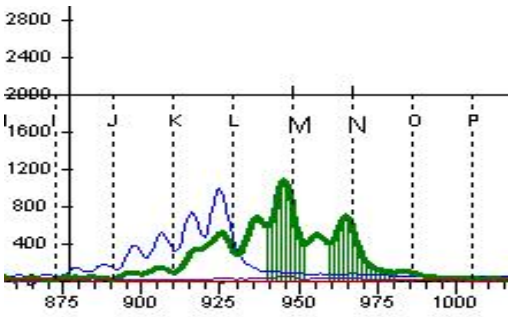
Table 3.5 **Comparative heterozygosities between CMZ, HMZ and domestic horse breeds**

Microsatellites	Horse Chromosome	Annealing Temperature (ºC)	Cape mountain zebra			Hartmann's mountain zebra			Domestic horse breeds^			VGL Thoroughbred Data+		
			N	Alleles	H_E	N	Alleles	H_E	N*	Alleles	H_E	N	Alleles	H_E
AHT4	24	60	51	4	0.591	84	17	0.817	50000	11	0.809	16499	11	0.732
AHT5	8	60	50	4	0.591	84	6	0.536	50000	11	0.809	16405	9	0.704
ASB2	15	56	29	1	0	26	3	0.521	50000	14	0.847	16490	13	0.826
ASB17	2	60	52	3	0.536	84	10	0.769	50000	22	0.871	16354	15	0.773
ASB23	3	60	51	4	0.427	82	14	0.898	80	6	0.625	16470	10	0.786
HMS3	9	56	52	2	0.503	74	3	0.239	50000	11	0.822	16503	8	0.683
HMS6	4	60	51	2	0.239	80	4	0.515	50000	8	0.759	16431	9	0.608
HMS42	20	60	28	2	0.07	71	4	0.242	NA	NA	NA	NA	NA	NA
HTG4	9	60	47	3	0.55	81	4	0.589	50000	8	0.687	16513	6	0.537
HTG10	21	56	52	3	0.633	74	9	0.695	50000	12	0.845	16167	12	0.784
LEX3	X	56	29	2	0.508	63	8	0.781	50000	14	0.859	NA	NA	NA
LEX33	4	56	29	2	0.407	0	0	0	50000	12	0.834	6278	11	0.709
LEX52	18	60	28	1	0	69	3	0.124	28	7	0.71	NA	NA	NA
LEX64	20	60	27	2	0.409	66	10	0.776	28	5	0.54	NA	NA	NA
UMO11	20	60	28	3	0.658	71	7	0.354	12-36	8	0.7	NA	NA	NA
VHL20	30	60	52	3	0.363	83	9	0.446	50000	10	0.83	16513	10	0.751

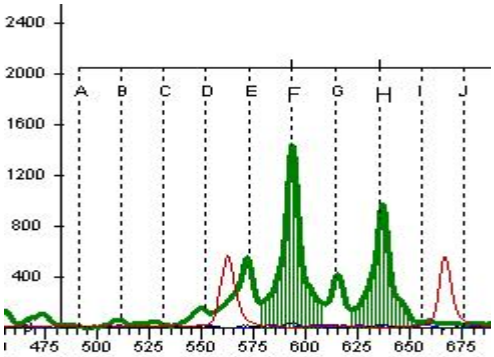
H = Expected heterozygosity
*50000 from Bowling & Ruvinsky, 2000.
^Data for marker ASB23, Irvin et al. 1998; LEX52, Coogle & Bailey 1997; LEX64, Coogle & Bailey 1999; UMO11, Meyer et al. 1997.
+Veterinary Genetics Laboratory Thoroughbred Database (Faculty of Veterinary Science, Onderstepoort; accessed on 22nd November, 2004)
NA indicates that the data is unavailable.



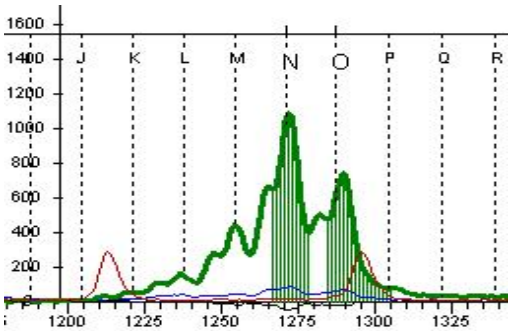
AHT4 in HMZ: F.I (128. 134)



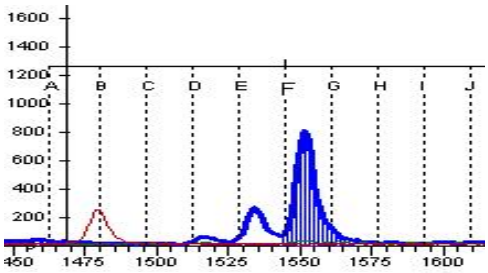
AHT5 in HMZ: M. N (137. 139)



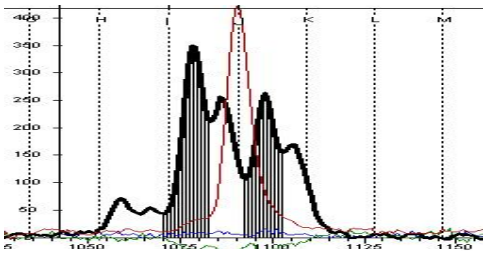
ASB17 in HMZ: F. H (93. 97)



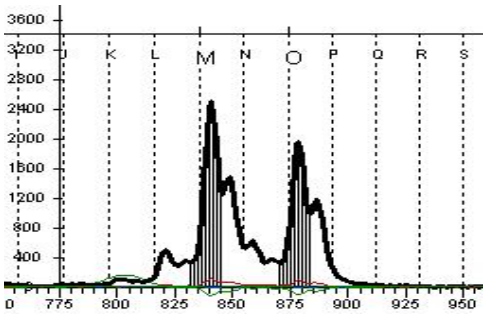
ASB23 in CMZ: N. O (197. 199)



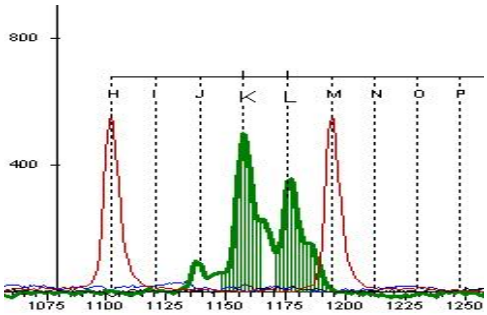
ASB2 in CMZ: F (228)



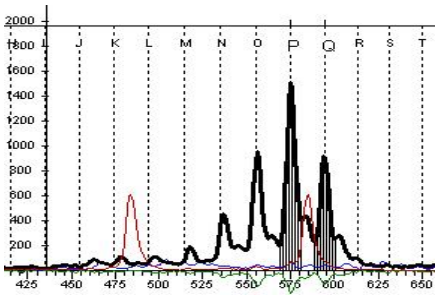
HMS3 in HMZ: I. J (148. 150)



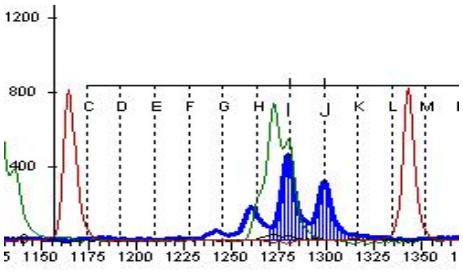
HMS42 in HMZ: M. O (120. 124)



HMS6 in CMZ: K. L (156. 158)



HTG10 in HMZ: P. O (99. 101)



HTG4 in CMZ: I. J (173. 175)

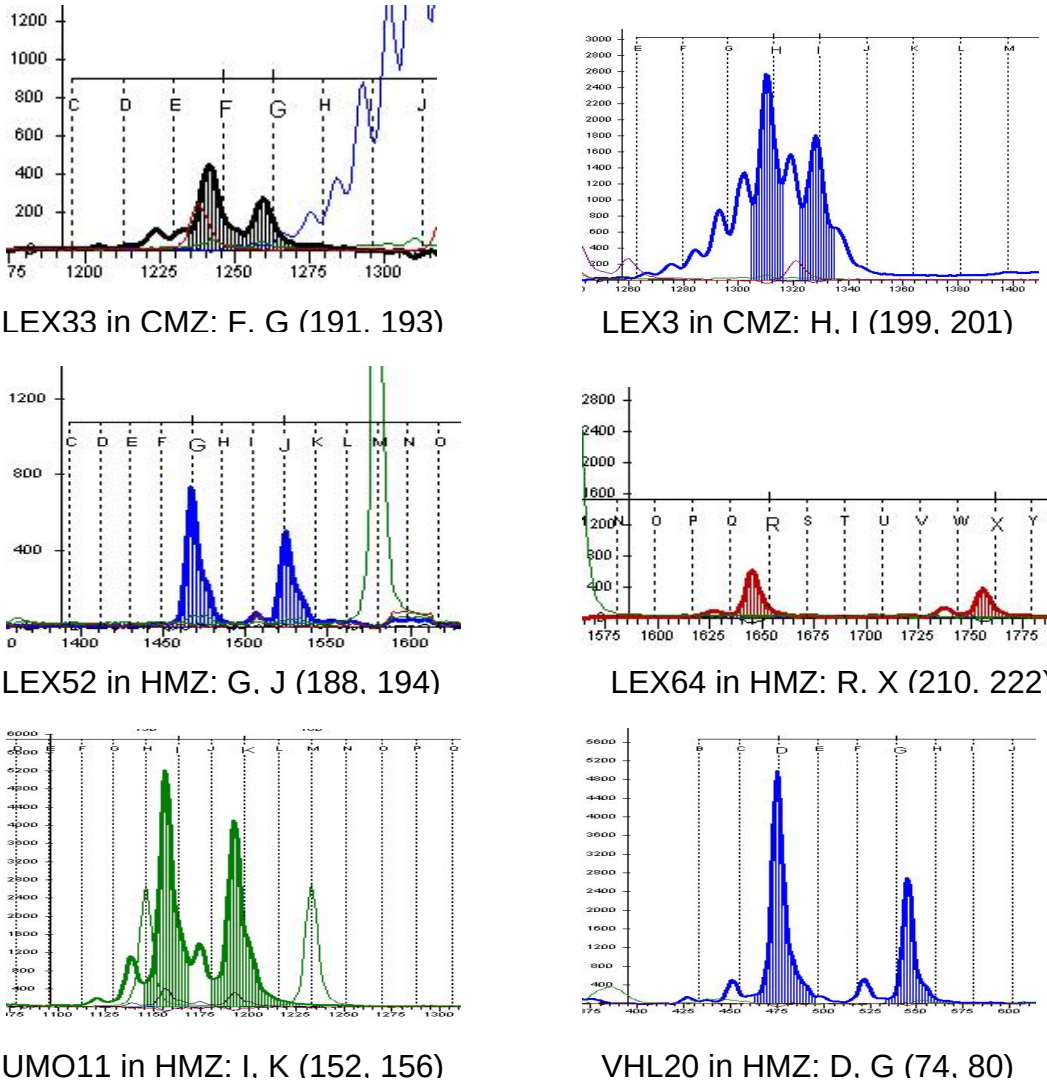


Figure 3.4: **Electropherograms representing fluorescent-labelled microsatellite alleles (shaded peaks) in some representative mountain zebras, sized from left to right according to an internal size standard (*STRand*). Microsatellite marker name and subspecies of amplification, alphabetical allele denomination and allele sizes (bp) as recorded by *STRand* are indicated**

3.4 Discussion

In general, domestic horse microsatellite markers were successfully applied for genotyping of mountain zebras. PCR amplification produced specific products for each of the 16 microsatellites studied in the CMZ populations in BNP and GDNR. Microsatellite data were verified for absence of mistyping and typographic errors, checked for large allele drop-outs (Wattier et al. 1998), scoring errors due to stuttering and for possible presence of null alleles. Stutter bands were easly recognised and allele types consistently amplified. Excepting for LEX33 in HMZ, PCR artefacts such as null alleles were not obvious in this study. LEX33 may have failed to amplify in Hartmann's zebras for a number of reasons. The most likely cause was that the primer-binding region of this locus underwent mutation and subsequent

non-detection within the software reading ranges resulted. Whether it is indicative of deeper genetic differences between the two subspecies needs to be investigated.

The amplification of LEX3 in samples with known sex suggested a pattern fitting a sex-linked locus. All known males were found hemizygous whereas females either homo- or heterozygous for this locus. Conclusive proof of the sex-linked nature of this locus, however, remains to be determined. If LEX3 is indeed linked to the X-chromosome in the mountain zebra, it would be a valuable addition to the microsatellite panel, enabling easy sex-determination. The microsatellite polymorphisms were found to generate acceptable exclusionary powers for parentage analysis in both subspecies, since the numbers of stallions siring progeny are higher than in domestic horses. The total two parent exclusionary power values obtained from the full panel of 16 microsatellites were 97.83 % for CMZ and 99.97 % for HMZ (excluding LEX33) and would be adequate for paternity testing in both subspecies.

Erratic amplification of microsatellite alleles was noted in a few CMZ samples that were collected and extracted as part of a previous study (Moodley 2002). This may have been due to the condition of the original samples (weathered field and museum skin samples) from which DNA was later extracted. Error-free and full-panel amplication was achieved for the remaining samples. It underlines the importance of proper processing and storage of DNA samples for the replication of previous studies. All 15 microsatellites amplified to produce identifiable products in both subspecies (Appendix III). This validates the reliability of the FTA® paper storage and archiving system and the procedures for locus amplification and visualisation in mountain zebras.

The methods used by us detected greater polymorphism. Moodley (2002) analysed 200 HMZ samples using 15 variable microsatellites and reported a lower average expected heterozygosity (0.511) compared to this study (0.54; 84 zebras and 14 variable loci). Using 15 microsatellites on CMZ samples, this study also indicated higher heterozygosity levels for the BNP and GDNR populations: 0.314 vs. 0.232 and 0.321 vs. 0.159, respectively. Nevertheless, the higher levels of polymorphism could also have been a function of the inherent variation in the microsatellites and primers used. Detection of polymorphism, however, has been reported to be superior using a computer software-controlled fluorescent-labelled allele detection system, run in a DNA sequencer (Jordana et al. 1999; 2001).

The study shows that the methods employed for storing and processing DNA in FTA® paper and for microsatellite analyses are quick and easy to perform. The use of different fluorescent

markers gives the flexibility to use microsatellites with size ranges that overlap, enabling simultaneous analyses. Unlike allozymes (Bowland et al. 2001), microsatellites are neutral genetic markers and are not constantly under selection. This enables a more reliable determination of population parameters among the animals investigated. Automated software-controlled *STRand* software (*Version 2.2.224*; Board of Regents, University of California, Davis) readout of genetic data is comparatively more error-free and easier than interpreting gel readouts from X-ray films. Furthermore, it provides a higher degree of automation in handling and processing DNA samples, enabling more animals to be genotyped in comparatively less time. By validating the application of commercial horse microsatellites in both mountain zebra subspecies, it is hoped that inclusion of genetic data will be a feature in future decision-making for conservation of these subspecies.

Chapter IV

Population genetics of sarcoid tumour-affected and non-affected South African *Equus zebra zebra* and Namibian *Equus zebra hartmannae* populations

Abstract

Outbreaks of sarcoid tumours have been reported in a few endangered and isolated Cape mountain zebra (CMZ; *Equus zebra zebra*) populations in South Africa. Sarcoid tumour and other papillomaviral diseases in animals and humans have been correlated to genetic factors coded by genes within the major histocompatibility complex and other regions. This study aimed at determining the levels of heterozygosity and inbreeding in the sarcoid tumour-affected and comparing them to unaffected mountain zebra populations. The diseased CMZ populations investigated were in the Bontebok National Park (BNP) and Gariep Dam Nature Reserve (GDNR). The non-diseased populations were CMZ in the Karoo National Park (KaNP) and the Karoo Nature Reserve (KaNR) of South Africa and the outbred Hartmann's mountain zebras (HMZ; *Equus zebra hartmannae*) in Namibia. Samples collected from these populations were analysed using domestic horse microsatellites to obtain allelic information. The results were subjected to genetic analyses using appropriate statistical techniques. Tumour-affected populations had the lowest levels of heterozygosity (0.386 vs. 0.427 for tumour-free CMZ and 0.607 for HMZ) and polymorphism. Wright's F_{IS} values indicated an overall deficit of heterozygotes in the affected and non-affected CMZ populations. On the other hand, the Namibian subspecies was relatively outbred (0.171 and 0.179 for Wright's F_{IS} and Nei's G_{IS}, respectively). Considerable population substructuring, as indicated by F_{ST} values, was revealed for all CMZ populations. Tumour-affected populations were genetically different (27.87 %) from non-affected CMZ, as revealed by hierarchical *F*-statistics. With the outbreak of sarcoid tumours, the genetic management of the Cape subspecies now assumes special importance. An imaginative and long-term conservation policy to increase levels of genetic diversity in Cradock-derived populations is thus warranted.

4.1 Introduction

Appearance of fibropapillomatous growths have been documented in various species (Schulman et al. 2001; 2003) and extensively studied within the *Equidae* (Reid et al. 1994b; Chambers et al. 2003), where the condition is referred to as equine sarcoids. This papillomavirus-induced condition is highly correlated with certain serological and genetic factors coded by genes suspected to lie within the major histocompatibility complex region in the domestic horse (*Equus caballus*; Marti et al. 1993; Lazary et al. 1994). The emergence of papillomavirus-induced disease is associated with lowered immunity and presence of environmental or genetic cofactors (Campo 2003). Ragland et al. (1966) reported an outbreak of sarcoids in horses and noted a familial pattern in the epizootic, with affected animals originating from a highly inbred family. Epidemiological studies have also been reported in horses (Angelos et al. 1988; Broström, 1995) and donkeys (Reid et al. 1994a), albeit from disparate clinical cases or from unrelated groups of animals where the unmeasured pedigree would have been a major confounder in any research undertaken (Chambers et al. 2003).

Sarcoid disease and correlations with Equine Leukocyte Antigens (ELA) Loci I and II serological factors, possibly representing gene products of the major histocompatibility complex (MHC) or some other susceptibility genes, have been reported (Broström et al. 1988). The expression of an autosomal, dominant and MHC-linked gene/s with incomplete penetration (Meredith et al. 1986) probably contributes to the predisposition of horses to sarcoids and papillomavirus activation. Current research indicates that susceptibility to sarcoid in the horse and donkey is associated with a multitude of risk factors, including major histocompatibility complex haplotypes, age and sex (Chambers et al. 2003). Genetic susceptibility to cottontail rabbit papillomavirus has been documented in rabbits (Favre et al. 1997). A similar susceptibility is seen in humans (Lowy 2001) and other animals (Kidney et al. 2001; Tachezy et al. 2002). Inbreeding has been correlated to disease susceptibility in cheetahs (*Acinonyx jubatus*; O'Brien & Yukhi 1999), California sea lions (*Zalophus californianus*; Acevedo-Whitehouse et al. 2003) and humans (Rudan et al. 2003). Increased parental similarity has been correlated to higher internal parasite loads in Soay sheep (*Ovis aries*; Coltman et al. 1999), decreased birth weight, juvenile survival (Coulson et al. 1998) and lowered adult reproductive success in red deer (*Cervus elaphus*; Slate et al. 2000) and increased mortality in harbour seals (*Phoca vitulina*; Coltman et al. 1998). Historical cosanguinity also increased mortality from parasitism and extreme climatic variation (Keller et al. 1994; Coltman et al. 1999; Kalinowski et al. 2000). Inbreeding depression was manifested in *Gazella cuvieri* (Roldan et al. 1998), Speke's gazelle (*Gazella spekei*;

Kalinowski et al. 2000), captive Nordic carnivores (Laikre 1999) and free-ranging felids (Wildt et al. 1994; Munson et al. 1996).

Cape mountain zebra (CMZ) populations in South Africa have multiplied from the brink of extinction in the 1930s (Bigalke 1952) to reach current levels of around 1600 animals (Friedman & Daly 2004). Most extant stock was derived from the few animals that survived and thrived in Mountain Zebra National Park (MZNP). They were later translocated to form new populations. Bontebok National Park (BNP) and Gariep Dam Nature Reserve (GDNR), established in 1985 and 1986, respectively, are two examples. These populations have recently expressed a high incidence of equine sarcoid-like tumours, with 53 % of BNP and 22 % of GDNR CMZ currently visibly diseased. The CMZ populations in the Karoo National Park (KaNP) and Karoo Nature Reserve (KaNR) are currently tumour-free and were also established by founder animals translocated from MZNP. Previously, we have validated the use of fluorescent-labelled horse-microsatellite genetic markers in multiplex panels, for mountain zebras (Chapter III). In this study, samples from four CMZ populations, two non-affected and two tumour-affected populations, were genetically profiled and compared with Namibian Hartmann's mountain zebra (HMZ) populations.

4.2 Methods

4.2.1 Animal origin

Samples were obtained from mountain zebra populations in Namibia and South Africa (see Chapter III). The Namibian and two tumour-free South African populations were sampled as part of a previous study on population genetics of zebras (Moodley 2002) and made available for this project.

4.2.2 DNA extraction and genotyping

Samples were processed and DNA extracted by the standard SDS-Proteinase K / phenol-chloroform protocol (Moodley 2002) or FTA® paper (Whatman Bioscience, USA) technology. Sixteen domestic horse microsatellite primers were end-labelled with flourophores and were assigned to three multiplex panels (detailed in Chapter III). The primer concentrations and PCR profiles were as previously described. Electrophoresis on the PCR products was carried out in an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA). Allele assignment was done using the ABI PRISM 310 Collection Software

application (*Version 3.0.0*; Applied Biosystems, Foster City, CA) and *STRand* software (*Version 2.2.224*; Board of Regents, University of California, Davis).

4.2.3 Population genetics

Observed and expected mean heterozygosities, polymorphic information content (PIC) for each microsatellite loci (Botstein et al. 1980) and two paternity average exclusion powers (probability of exclusion, as described by Jamieson 1994) were calculated using CERVUS 2.0 (Marshall et al. 1998).

Microsatellite loci were tested to estimate whether the populations analysed conform to Hardy-Weinberg equilibrium with GENEPOP software package, (*Version 3.3*; Raymond & Rousset 1995). A Markov chain method was used to calculate exact probabilities, to estimate without bias, the exact *P*-value (at 1000 dememorisation steps, 1000 batches and 10,000 iterations per batch). The significance of resulting *P*-values from multiple tests were assessed for significance using sequential Bonferroni correction (Rice 1989). Two population groupings, the pooled CMZ and HMZ population, were analysed for heterozygote deficit or excess for the nine common loci. Hardy-Weinberg exact tests for up to four alleles were then carried out with similar Markov chain parameters for all tests. Genotype disequilibrium was tested using Markov chain and Fisher's exact procedures by GENEPOP software. The settings for the exact probabilities were 5000 dememorisation steps, 1000 batches and 10,000 iterations per batch.

Nei's diversity values for each locus and overall (Nei 1978) and Nei's (1972) genetic standard distances were calculated using FSTAT (*Version 2.9.3.2*; Goudet 1995). The 'within population' inbreeding statistic (F_{IS}) for each locus was calculated using FSTAT (*Version 2.9.3.2*). Comparative allele richness values were calculated for CMZ and HMZ populations as implemented in FSTAT, adapting a rarefaction index (Petit et al. 1998). Permutation tests with sequential Bonferroni procedure determined the significance levels and F_{IS} values were obtained by jackknifing over loci. Global tests for population differentiation by pairwise tests were carried out using FSTAT (*Version 2.9.3.2*). Single locus values between mountain zebra populations were estimated and populations tested for significant departure from zero by permutation (1000 replicates) of individual genotypes between samples. The overall loci G-statistic was used to classify contingency tables and reported based on pairwise significance after standard Bonferroni corrections, with the nominal value set at 5 %. The indicative adjusted nominal level (5 %) for multiple comparisons was set at 0.005. Overall *F*-statistic values were calculated for the following population groupings: Group A consisting of pooled

samples from the two sarcoid affected populations, Group B consisting of tumour-free CMZ populations and Group C, the pooled sample of Hartmann's zebras. Wright's *Fst* (θ) (Weir & Cockerham 1984) and 95 % confidence intervals of θ were calculated by bootstrapping (1000 replicates). Overall relatedness, measured between all the CMZ populations as the average relatedness of individuals within samples when compared to the whole (Queller & Goodnight 1989), was measured.

ARLEQUIN (*Version. 2*; Schneider et al. 2000) was used to estimate exact tests of population differentiation (10,000 Markov chain and 5000 dememorisation steps). *P*-values were calculated and compared with the significance level set at 0.05. A hierarchical F_{ST} analysis was carried out using analysis of molecular variance (AMOVA), as implemented in ARLEQUIN (Schneider et al. 2000), in order to estimate population structure at different levels of the specified hierarchy. Differentiation was analysed between tumour-affected and non-affected Cape mountain zebra subpopulations. The null hypothesis of no differentiation at the corresponding level was tested at 20,000 permutations.

4.3 Results

4.3.1 Microsatellite typing and amplification

DNA deterioration was noted in samples collected from CMZ museum specimens and from field specimens from KaNP and KaNR. This probably contributed to the non-amplification, high rate of typing failure and deficit of heterozygotes noted for five microsatellites; ASB2, HMS42, LEX52, LEX64 and UMO11. As a result, these five microsatellites were not used for subsequent comparative analysis between CMZ populations. LEX33 failed to amplify in all the HMZ tested. LEX3 was excluded from comparative analyses of population genetic parameters since it was found to be sex-linked in the horse (Chowdhary et al. 2003) and the chromosome linkage status of this locus is yet to be determined for zebras. Hence, nime microsatellites were used for all comparative analyses that included the two populations, KaNP and KaNR (Table 4.1).

4.3.2 Heterozygosity values in mountain zebra populations

Sarcoid tumour-affected populations had lower mean heterozygosity levels, when compared with tumour-free CMZ populations (0.386 vs. 0.427; Tables 4.1 & 4.2).

***Table 4.1*: Combined population data from tumour-affected populations (BNP and GDNR)**

Locus	Population data from tumour-affected subpopulations with nine informative loci					
	Alleles	Animals typed	H_O	H_E	PIC	PE
AHT4	3	29	0.552	0.586	0.508	0.306
AHT5	3	29	0.138	0.133	0.127	0.066
ASB17	3	29	0.552	0.446	0.356	0.186
ASB23	2	29	0.414	0.436	0.336	0.168
HMS3	2	29	0.517	0.506	0.374	0.187
HMS6	2	29	0.103	0.16	0.145	0.073
HTG10	2	29	0.552	0.508	0.375	0.187
VHL20	3	29	0.414	0.431	0.348	0.182
HTG4	2	29	0.172	0.267	0.228	0.114

Mean alleles per locus = 2.44
Mean heterozygosity = 0.386
Mean PIC = 0.311
Cumulative exclusion probability = 0.805081
H_O: Observed heterozygosity; H_E: Expected heterozygosity; PIC: Polymorphic information content; PE: Probability of exclusion

The mean numbers of alleles and the mean polymorphic content detected for the nine common informative loci are represented in Figure 4.1. The mean allele diversities detected (excluding LEX3) in mountain zebra populations are represented in Figure 4.2.

***Table 4.2*: Combined population data from tumour-free populations (KaNP and KaNR)**

Locus	Population data from tumour-free subpopulations with nine informative loci					
	Alleles	Animals typed	H_O	H_E	PIC	PE
AHT4	4	22	0.5	0.576	0.576	0.327
AHT5	3	21	0.238	0.39	0.39	0.185
ASB17	3	23	0.739	0.627	0.627	0.328
ASB23	4	22	0.364	0.411	0.411	0.207
HMS3	2	23	0.261	0.487	0.487	0.181
HMS6	2	22	0.409	0.333	0.333	0.136
HTG10	2	23	1	0.511	0.511	0.188
VHL20	2	23	0.304	0.264	0.264	0.112
HTG4	2	18	0.278	0.246	0.246	0.105

Mean alleles per locus = 2.67
Mean heterozygosity = 0.427
Mean PIC = 0.355
Cumulative exclusion probability = 0.866555
H_O: Observed heterozygosity; H_E: Expected heterozygosity; PIC: Polymorphic information content; PE: Probability of exclusion

The expected heterozygosity values in HMZ were comparatively higher for each microsatellite analysed (Table 4.3).

The lack of allele diversity for CMZ microsatellites that amplified, compared to the outbred HMZ populations, is outlined in more detail in Appendix 1.

***Table 4.3*: Population data from Namibian Hartmann's mountain zebras**

Locus	Population data from Hartmann's zebras with nine informative loci					
	Alleles	Animals typed	H_O	H_E	PIC	PE
AHT4	17	83	0.759	0.814	0.788	0.641
AHT5	5	83	0.518	0.525	0.438	0.25
ASB17	10	83	0.627	0.765	0.73	0.561
ASB23	14	82	0.841	0.898	0.882	0.782
HMS3	3	74	0.108	0.239	0.215	0.111
HMS6	4	79	0.316	0.507	0.454	0.272
HTG10	9	74	0.622	0.695	0.668	0.5
VHL20	9	83	0.446	0.446	0.425	0.271
HTG4	4	81	0.309	0.589	0.499	0.296

Mean alleles per locus = 8.33
Mean heterozygosity = 0.609
Mean PIC = 0.566
Cumulative exclusion probability = 0.99571
H_O: Observed heterozygosity; H_E: Expected heterozygosity; PIC: Polymorphic information content; PE: Probability of exclusion

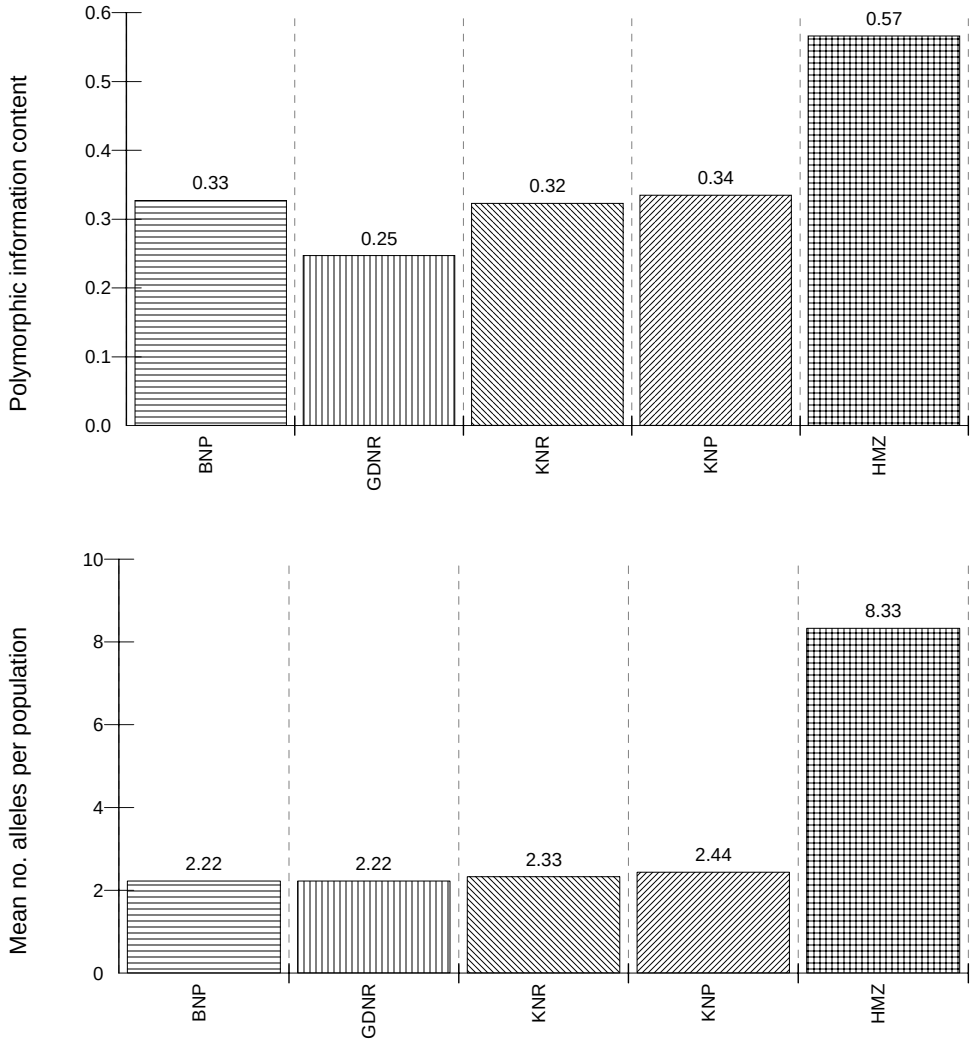


***Figure 4.1:* Mean number of alleles and mean polymorphic information content per population**

The microsatellites, ASB2, HMS42 and LEX52, were homozygous in all the BNP-CMZ genotyped. Mountain zebra from GDNR exhibited lower heterozygosity values than BNP. One microsatellite locus, LEX52, was found homozygous in all GDNR animals typed. CMZ from KaNP also had low levels of polymorphism and exclusion probability values. However, higher heterozygosity values were recorded when compared to BNP and GDNR. CMZ in KaNR had a similar heterozygosity value and polymorphism compared to the population in KaNP.

When data of the 14 informative loci of the two sarcoid-affected CMZ populations (n = 29) was combined, the mean heterozygosity decreased from 0.386 to 0.334. The mean polymorphic information content detected within tumour-affected populations was 0.311, compared to 0.355 for the tumour-free CMZ and 0.566 for Hartmann's zebra populations.

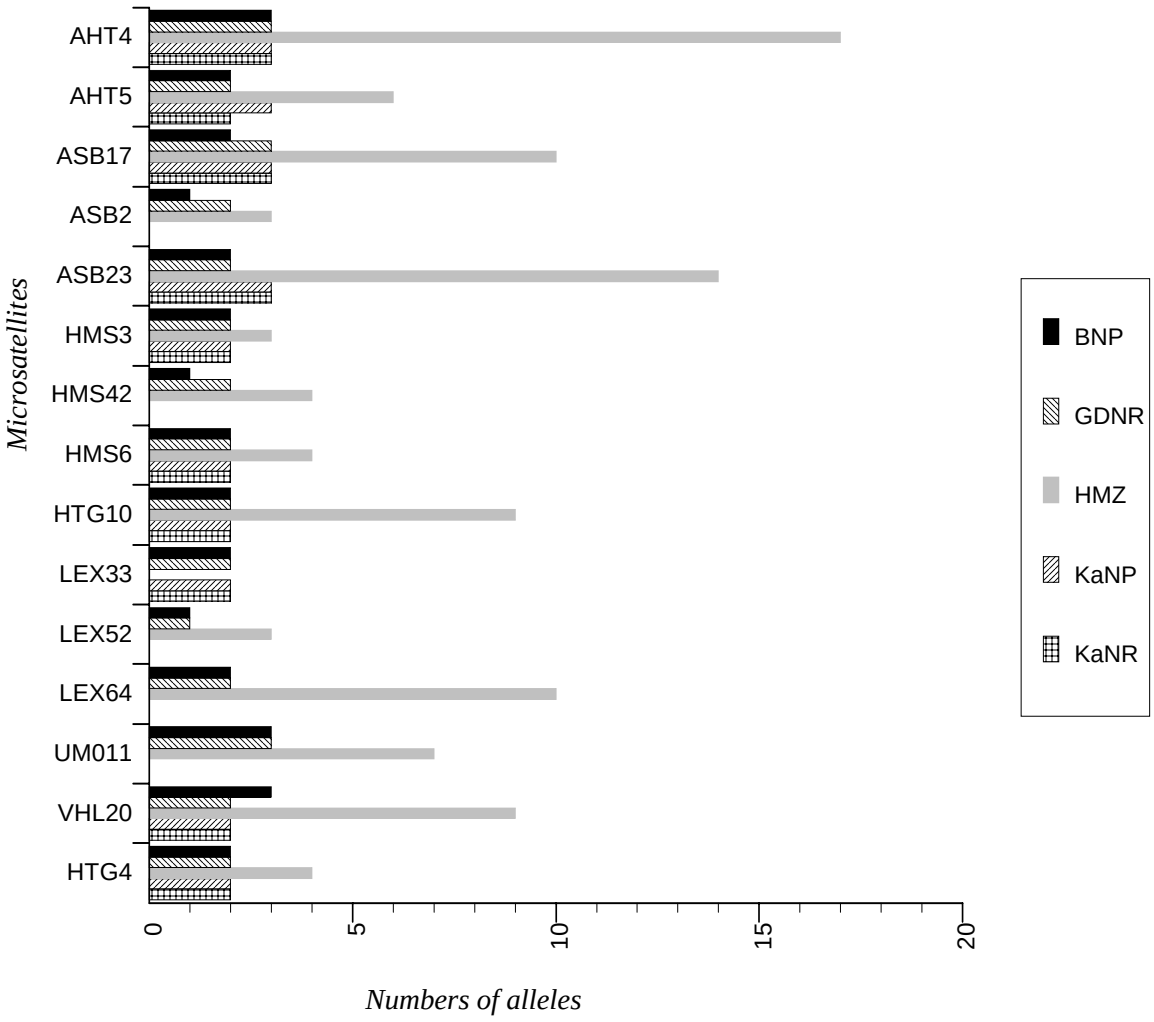


Fig 4.2: **Total allele diversity per locus per population**

Appendix III details the allele distributions for individual microsatellite loci for each subspecies.

4.3.3 Population differentiation in mountain zebra populations

4.3.3.1 Gene diversity and allele richness

Gene diversity values for all nine microsatellites were consistently lower in all CMZ populations when compared to Hartmann's zebras (Table 4.4). Allele richness values were in accordance with the heterozygosity values determined earlier, with the tumour-affected and non-affected CMZ populations exhibiting lower values than HMZ (Table 4.5).

Table 4.4: **Gene diversity across populations**

Locus	Gene diversity per locus and sample		
	Tumour-affected	Tumour-free	Hartmann's zebra population
AHT4	0.587	0.578	0.814
AHT5	0.133	0.394	0.525
ASB17	0.444	0.625	0.766
ASB23	0.436	0.412	0.898
HMS3	0.506	0.492	0.24
HMS6	0.161	0.331	0.508
HTG10	0.507	0.5	0.695
VHL20	0.432	0.263	0.446
HTG4	0.268	0.245	0.591
AVERAGE	**0.386**	**0.427**	**0.609**

Tumour-affected populations = Bontebok National Park and Gariep Dam Nature Reserve populations
Tumour-free populations = Karoo National Park and Karoo Nature Reserve populations

Table 4.5: **Comparative allele richness across all populations**

Locus	Allele richness in mountain zebra populations		
	Tumour-affected	Tumour-free	Hartmann's zebra population
AHT4	3	3.97	9.3
AHT5	2.721	2.983	3.551
ASB17	2.621	3	7.326
ASB23	2	3.789	10.984
HMS3	2	2	2.424
HMS6	1.994	2	3.226
HTG10	2	2	7.522
VHL20	2.621	2	5.52
HTG4	2	2	3.208
AVERAGE	**2.329**	**2.638**	**5.896**

Tumour-affected populations = Bontebok National Park and Gariep Dam Nature Reserve populations
Tumour-free populations = Karoo National Park and Karoo Nature Reserve populations

4.3.3.2 Tests for Hardy-Weinberg equilibrium and linkage disequilibrium

In CMZ, three microsatellite loci indicated rejection of the null hypothesis of random association of alleles, that is Hardy-Weinberg equilibrium, from among the nine microsatellite markers (Table 4.6). In HMZ, *P*-values indicative of deviation from HWE after multiple tests were seen for HMS3, HMS6 and HTG4. Taking all possible locus pairs into account across all populations, genotypic disequilibrium calculations indicated significant linkage between two locus pairs, AHT4 and AHT5 ($\chi^2 = 33.54$; D.F. = 10, $P = 0.00022$) and ASB23 and HTG10 ($\chi^2 = 34.83$; D.F. = 6, $P = 0.0000$).

Significant heterozygote deficit was observed for two alleles, AHT5 and HTG4, among the CMZ populations analysed (Table 4.7). Five alleles exhibited significant heterozygote deficit within Hartmann's zebra population, with HMS6 exhibiting the greatest deficit.

Table 4.6: **Hardy-Weinberg probability values (*P*) and standard error (*S.E*) values of nine loci**

Locus	**Cape mountain zebra**		**Hartmann's mountain zebra**	
	P-values	S.E.	*P*-values	S.E.
AHT4	0.5199	/	0.0461	0.0023
AHT5	0.0000	/	0.0689	0.0007
ASB17	0.4127	/	0.0094	0.0004
ASB23	0.6410	/	0.0159	0.0006
HMS3	0.1728	/	0.0000	/
HMS6	1.0000	/	0.0001	/
HTG10	0.0000	/	0.1512	0.0020
VHL20	0.2986	/	0.2043	0.0036
HTG4	0.0000	/	0.0000	/

Probability (*P*) values indicate the probability of rejecting Hardy-Weinberg equilibrium; the null hypothesis cannot be rejected at $p > 0.01$ (low level of significance). The null hypothesis can be rejected for $p < 0.01$.

4.3.3.3 Estimating levels of population differentiation

Nei's unbiased estimates of average heterozygosity and genetic distance values were compared for tumour-affected and tumour-free CMZ and HMZ populations (Table 4.8).

Gene diversity values were lowest for tumour-affected populations (39.5%) and highest for HMZ (52.3%). The overall value for Nei's population differentiation parameter (*Gst*'), also an indicator of heterozygote deficit, illustrated that tumour-affected CMZ populations have a greater degree of differentiation (0.156) and deficit of heterozygotes than tumour-free CMZ (0.082) and Hartmann's zebras (0.08).

Table 4.7: **Heterozygote deficit and excess and *F*-values across nine loci**

	Heterozygote deficit		Heterozygote excess		F_{IS}	
Populations	*P*-value	S.E.	*P*-value	S.E.	W & C	R & H
MICROSATELLITE AHT4						
CMZ	0.0564	-	0.9469	-	0.105	0.138
HMZ	0.0751	0.003	0.923	0.003	0.068	0.027
MICROSATELLITE AHT5						
CMZ	0.0001	-	1	-	0.697	0.431
HMZ	0.0077	0.0002	0.993	0.0002	0.013	0.238
MICROSATELLITE ASB17						
CMZ	0.9608	-	0.0554	-	-0.186	-0.144
HMZ	0.0014	0.0001	0.9985	0.0001	0.182	0.127
MICROSATELLITE ASB23						
CMZ	0.3126	-	0.7895	-	0.081	0.026
HMZ	0.0147	0.0007	0.9851	0.0006	0.063	0.068
MICROSATELLITE HMS3						
CMZ	0.122	-	0.9581	-	0.199	0.201
HMZ	0	-	1	-	0.549	0.758
MICROSATELLITE HMS6						
CMZ	0.6438	-	0.7827	-	0.016	0.017
HMZ	0.0001	-	1	-	0.377	0.225
MICROSATELLITE HTG10						
CMZ	0.9247	-	0.0753	-	-0.186	-0.141
HMZ	0.0011	0.0001	0.999	0.0001	0.106	0.172
MICROSATELLITE VHL20						
CMZ	0.5399	-	0.5323	-	-0.007	-0.016
HMZ	0.373	0.0034	0.6643	0.0034	0	0.007
MICROSATELLITE HTG4						
CMZ	0.0044	-	0.9956	-	0.616	0.338
HMZ	0	-	1	-	0.478	0.286

F_{IS}: The 'within population' inbreeding estimate; W&C: Weir and Cockerham's (1984) estimate of F_{IS}; R & H: Robertson and Hill's (1984) estimate of F_{IS}

Table 4.8: **Nei's diversity values for Cape and Hartmann's populations sampled**

Locus	*Ht*			*Gst*			*Gst'*			*Gis*		
	DCMZ	DFCMZ	HMZ	DCMZ	DFCMZ	HMZ	DCMZ	DFCMZ	HMZ	DCMZ	DFCMZ	HMZ
AHT4	0.605	0.586	0.809	0.051	0.089	0.007	0.097	0.163	0.014	0.01	0.016	0.017
AHT5	0.137	0.391	0.521	0.003	-0.028	-0.005	0.006	-0.058	-0.011	-0.042	0.412	-0.02
ASB17	0.45	0.625	0.748	0.005	0.011	0.009	0.009	0.023	0.018	-0.242	-0.201	0.171
ASB23	0.406	0.402	0.902	0.172	0.016	0.005	0.293	0.032	0.01	-0.123	0.094	0.061
HMS3	0.506	0.485	0.242	0.158	0.141	-0.011	0.273	0.247	-0.023	-0.238	0.363	0.467
HMS6	0.181	0.344	0.506	0.029	0.091	-0.001	0.056	0.166	-0.002	0.359	-0.386	0.35
HTG10	0.508	0.5	0.655	-0.006	0	0.016	-0.013	0	0.031	-0.088	-1	0.04
VHL20	0.46	0.256	0.442	0.166	0.066	0.003	0.284	0.124	0.005	-0.174	-0.237	-0.02
HTG4	0.303	0.243	0.577	0.133	-0.025	0	0.235	-0.05	0	0.253	-0.102	0.529
Overall	**0.395**	**0.426**	**0.523**	**0.085**	**0.043**	**0.042**	**0.156**	**0.082**	**0.08**	**-0.072**	**-0.127**	**0.179**

DCMZ: Bontebok National Park and Gariep Dam Nature Reserve populations; DFCMZ: Karoo National Park and Karoo Nature Reserve populations; HMZ: Hartmann's mountain zebra

Ht: The overall gene diversity

Gst: Nei's analogue of the parameter F_{ST}, denoting the heterozygote deficit within populations or measure of population differentiation

Gst': The equivalent estimator to F_{ST} but independent of the number of samples

Gis: Nei's analogue to F_{IS}, the 'within population' inbreeding estimate

Wright's (1965; 1978) F_{IS} statistic for each locus was measured and 'within population' inbreeding values for individual populations were obtained using nine microsatellites (Table 4.9).

***Table 4.9*: Within population inbreeding values for mountain zebra populations**

Locus	F_{IS} values of the different populations				
	BNP	GDNR	KaNP	KaNR	HMZ
AHT4	0.028*	-0.012	0.416**	-0.252	0.068***
AHT5	-0.048	-0.032	0.416**	0.407*	0.013***
ASB17	-0.203	-0.286	-0.128	-0.268	0.182***
ASB23	-0.048	-0.151	0.185*	-0.08	0.063***
HMS3	-0.375	-0.103	0.681**	-0.176	0.549***
HMS6	0.436**	NA	-0.048	-0.5	0.377***
HTG10	-0.132	-0.043	-1	-1	0.106***
VHL20	-0.197	-0.103	-0.294	NA	NA
HTG4	0.29*	NA	-0.077	-0.125	0.478***
All	-0.044	-0.106	0.024**	-0.287	0.171***

F_{IS} : The 'within population' inbreeding estimate ($f \cong F_{IS}$ or value of deficit of heterozygotes
*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$
BNP: Bontebok National Park, GDNR: Gariep Dam Nature Reserve, KaNP: Karoo National Park, KaNR: Karoo Nature Reserve; HMZP: Hartmann's mountain zebras

The results of the group differentiation tests, to determine differences between 'disease-affected' and 'unaffected' groups and HMZ, as a single out-group, is given in Table 4.10. The tumour-affected group exhibited the highest level of relatedness (0.29) and heterozygote deficit (0.16). Overall relatedness, for all the CMZ populations combined was as high as 48 % ($P = 0.0651$).

***Table 4.10*: Group comparison table for different populations**

Group comparison tables with three classifications			
Group	Observed heterozygosity	Gene diversity	Relatedness
A	0.352	0.352	0.293
B	0.455	0.408	0.168
C	0.505	0.607	NA

Group A: Tumour affected CMZ populations; Group B: Unaffected CMZ populations and Group C: *Equus zebra hartmannae* population

***Table 4.11*: Population differentiation at a set level of significance**

	GDNR	KaNP	KaNR	HMZ
BNP	*	*	*	*
GDNR		*	*	*
KaNP			NS	*
KaNR				*

*5% nominal level (Under Bonferroni correction, the actual P value for differentiation corresponds to the nominal level [0.05] divided by the number of tests [200 permutations] = 0.00025; Goudet, 1995)
BNP: Bontebok National Park; GDNR: Gariep Dam Nature Reserve; KaNP: Karoo National Park; KaNR: Karoo Nature Reserve and HMZP: Hartmann's mountain zebra population

Pairwise tests of population differentiation between the four CMZ populations reached levels of significance for all population pairs except between KaNP and KaNR (Table 4.11). Exact global tests for differentiation among samples, using the Fisher exact test, revealed non-significant levels of population differentiation between Bontebok and Gariep populations at $P = 0.0573 \pm 0.01$. The Gariep population showed an exact P-value of differentiation of 0.0661 (± 0.009) and 0.06775 (± 0.009) between KaNP and KaNR, respectively.

A hierarchical F_{ST} analysis using analysis of molecular variance (AMOVA) after setting two subdivision levels (between affected and non-affected CMZ populations and between two subspecies) and variance components were calculated (Table 4.12). It revealed a higher level of differentiation between the tumour affected and non-affected CMZ populations, than among all the CMZ subpopulation and within subpopulations; 0.27873 vs. 0.12029, and 0.1801, respectively. The differentiation between affected and non-affected CMZ populations (27.87 %) was greater than when calculated without using a hierarchical analysis among all CMZ populations (23.24 %).

Table 4.12: **Population structuring between Cape mountain zebra population groups**

Sources of variation	Variance component	Percentage of variation	Fixation indices (%)
Among groups (Between sarcoid-affected and non-affected CMZ populations)	0.38571	18.01	27.873
Among all CMZ populations	0.21122	9.86	12.029
Within CMZ populations	1.54471	72.13	18.01

Genetic substructuring in CMZ, as determined by F-statistics, was further analysed through a pair-wise analysis of different populations (Table 4.13).

Table 4.13: **Genetic structure of mountain zebras through an analysis of their subpopulations^**

Population	$F_{IS} = f$	$F_{IT} = F$	$F_{ST} = \theta$
BNP-GDNR (Tumour affected CMZ)	-0.077 (0.050)	0.096 (0.062)	0.161 (0.048)
KaNP-KaNR (Unaffected CMZ)	-0.115 (0.164)	-0.024 (0.170)	0.082 (0.038)
Hartmann's zebra population	0.168 (0.056)***	0.181 (0.053)	--

^ From nine informative loci
f: within population inbreeding estimate; F: total inbreeding estimate; θ: measure of population differentiation
Standard deviation in parenthesis estimated from jackknife over loci
***$P < 0.001$, from permutation tests in FSTAT programme

The 'within population' inbreeding estimates ($f = F_{IS}$) indicated an overall deficit of heterozygotes in the affected and non-affected Cape mountain zebra populations. The required level of significance was only reached in the HMZ. Total inbreeding estimates (F_{IT}) for HMZ (0.181) indicated a relatively outbred population.

4.4 Discussion

Inbreeding and its effects in populations have been well documented (Ralls 1988, Lacy 1993, Hedrick 2000, Ives 2002, Charlesworth 2003) and correlations with disease susceptibility reported (Coltman et al. 1999, Acevedo-Whitehouse et al. 2003, Altizer et al. 2003, Rudan et al. 2003). Appearance of sarcoid tumours in equids has been highly correlated with specific MHC haplotypes (Marti et al. 1993, Broström et al. 1995, Chambers 2003). Compelling evidence of the involvement of BPV in the formation of sarcoid tumour in equids has been published (Campo 2003, Chambers 2003). BPV oncoprotein E5 has been demonstrated to downregulate MHC expression *in vitro* (Ashrafi et al. 2002) and E5 expression in sarcoid tumours confirmed (Carr et al. 2001). Tumour samples from GDNR and BNP have been analysed and found to contain BPV 1 and 2 DNA (van Dyk et al. 2004). Preliminary studies indicate similarity in histopathology between CMZ and horse sarcoids (Marais et al. unpublished). This study compared genetic parameters between tumour-affected and non-affected CMZ and HMZ populations.

The mean number of alleles was lowest in the diseased populations (BNP and GDNR; 2.22), with GDNR having the lowest polymorphic information content (Figure 4.1). Mean heterozygosity values were lowest for the tumour-affected populations (0.386) compared to tumour-free populations (0.427) and HMZ (0.609; Tables 4.1 & 4.3). The diseased population had lower comparative allele richness values (2.33 vs. 2.64 for the tumour-free animals and 5.896 for HMZ; Table 4.5), calculated by adapting a rarefaction index (Petit et al. 1998). This corrects for the differences in sample numbers that might be encountered in each population compared. Observed heterozygosity and gene diversity values were found to be lowest in the tumour-affected populations (Table 4.10).

Six microsatellite loci (AHT5, HTG10 and HTG4 in CMZ and HMS3, HMS6 and HTG4 in HMZ) showed consistent departure from Hardy-Weinberg equilibrium (Table 4.6), indicating that the null hypothesis of 'random union of gametes' is not validated. Due to insufficient number of alleles present at each locus (four or less) to run the Markov chain method, the standard error values were not obtained for any loci in CMZ and three in HMZ (HMS3, HTG6 and HTG4) (Table 4.6). The observed deviations from Hardy-Weinberg equilibrium in CMZ and HMZ populations were due to a high heterozygote deficit (see Appendix III).

Tests for heterozygote deficit indicate the lack of genetic diversity for all microsatellites analysed within CMZ populations. Recent co-sanguinity might be the biggest contributor for the lack of allele diversity in Cape mountain zebra populations. Genetic drift and fixation of

private alleles is suspected to have accelerated this differentiation between different CMZ populations. This argument is bolstered by the moderately high F_{ST} values within CMZ populations, confirming the presence of a significant subpopulation structure (Wahlund effect due to high genetic drift). Nei's population differentiation ($G_{ST'}$) values, corrected for sample numbers, indicate the presence of considerable genetic structure in tumour-affected CMZ populations (0.156 vs. 0.08 for disease-free and HMZ, respectively; Table 4.8). FSTAT (*Version 2.9.3.2*) implements Nei's formulae for obtaining unbiased estimates of average heterozygosity and genetic distance when the number of individuals sampled is small. Most loci analysed in both diseased and non-diseased CMZ populations reflects the effect of high heterozygote deficits. This is suspected to have contributed to the appearance of negative values when calculations for 'within population' inbreeding estimates were conducted. Similar calculations for HMZ populations indicated a significantly outbred population (0.179; Table 4.8).

The level of relatedness was high between all CMZ populations, with the diseased population showing comparatively higher values than tumour-free animals (0.29 vs. 0.17; Table 4.10). The Namibian subspecies, on the other hand, was found relatively outbred, with high levels of allele polymorphism and moderately low level of inbreeding. Weir & Cockerham's (1984) F_{IS} value of 0.171 ($P < 0.001$; Table 4.9) and a Nei's (1978) G_{IS} value of 0.179 (Table 4.8) for this subspecies indicate this. As expected, the lack of heterozygotes within CMZ resulted in very few loci yielding significant results for each of the four populations. Only three loci (AHT4, HMS6 and HTG4) in BNP, five in KaNP (AHT4, AHT5, ASB23 and HMS3) and one in KaNR (AHT5) reached levels of significance in 'within population' inbreeding estimations in CMZ populations (Table 4.9). In the case of HMZ, however, all but one loci (VHL20), showed highly significant values ($P < 0.0001$). The overall inbreeding estimate in HMZ (0.171) indicates a relatively outbred population. Cornuet & Luikart (1996) state that where populations are deviating from mutation drift equilibrium, allelic numbers initially reduce more rapidly than heterozygosity levels, so that the number of alleles observed is usually less than expected from the observed heterozygosity. Population bottlenecks of short duration severely reduce the number of alleles present in the population but have relatively little effect on heterozygosity. In the case of most CMZ populations investigated (see Appendix 1-A to 1-D), the observed heterozygosity for multiple microsatellites was higher than expected heterozygosity, causing classical *F*-statistics values (Wright 1978; Weir & Cockerham 1984) for individual loci to be negative (Table 4.7 & Table 4.9).

The presence of a 'reproductive substructure' may well have accelerated the genetic drift, which was driven by long serving stallions maintaining multigenerational harems. The hypothesis of random distribution of individuals between pairs of CMZ populations was tested by exact tests of population differentiation and carried out by not assuming Hardy-Weinberg equilibrium. *G*-statistics, as implemented in Goudet (1995), was used to classify the resulting contingency tables. It revealed significant level of differentiation between all four CMZ populations, excepting between Karoo National Park and Karoo Nature Reserve. The comparable exact tests of population differentiation in ARLEQUIN (*Version 2*), with the dememorisation phase for the Markov chain set at 5000, did not produce significant levels of population differentiation. The reason may be that ARLEQUIN uses Fisher's exact test on a 2x2 contigency table, where each *P*-value has the same weight, whereas FSTAT implements Goudet's *G*-statistic, where *P*-values for very polymorphic loci are weighted more than those for nearly monomorphic loci (Goudet, 1995).

Two locus pairs (AHT4 - AHT5 and ASB23 - HTG10) were found to exhibit significant linkage among all the possible locus pairs at a global level. Of these alleles, AHT4 also showed a significant heterozygote deficit in all CMZ populations. Removing these loci for purposes of calculating inbreeding levels, however, did not change the values obtained by any significant manner. Although population differentiation tests have revealed significant differences between CMZ populations and between diseased and non-diseased populations, the two tumour-affected populations may have a deeper level of similarity due to factors that remain to be determined. The AMOVA results showed a higher level of population differentiation between affected and non-affected CMZ (27.87 %) than between all CMZ populations (Table 4.12), which is consistent with this hypothesis. More detailed studies, using greater numbers of markers spanning the length of the CMZ genome, might clarify the presence or absence of loci linked with susceptibility.

The Cape subspecies of mountain zebra suffered extreme population decimation towards the middle of the 19th century (Penzhorn 1988). The few dozen mountain zebras that remained in the Mountain Zebra National Park formed the origin of most CMZ populations. CMZ within BNP and GDNR have been afflicted with a condition similar to equine sarcoid, a virus-induced tumour that is highly correlated to certain genetic factors (Chambers et al. 2003). This investigation revealed that the two diseased populations have the least genetic variation of the four CMZ populations investigated and are representative of most extant CMZ populations. Apart from being highly inbred, these populations have high level of genetic substructuring. It could thus be demonstrated that CMZ, after surviving the historically

documented genetic bottleneck (Penzhorn 1984), have very little genetic variation left. Furthermore, most of the extant CMZ populations, derived from MZNP, are probably genetically similar. Genetic drift and fixation of alleles are reinforced in these isolated populations, where conditions are not conducive for natural inbreeding-avoidance behaviours and where the inherent social behaviour of CMZ accelerates the process of substructuring. Stripe-pattern recognition mechanisms and other natural adaptations are possibly less functional under intensively managed and artificially fenced-in conditions (Penzhorn & Novellie 1991).

This study does not purport to draw direct conclusions regarding the inbred nature of these two populations and expression of sarcoid tumours. The results however are in line with other recent studies where associations were found between diseases and inbreeding (O'Brien & Yukhi 1999; Acevedo-Whitehouse et al. 2003). More than 30 years back, Young & Zumpt (1973) studying the parasites and diseases of the CMZ in MZNP, made the following statement: "*Inbreeding could already have reduced the inherent resistance of these animals to diseases and parasites by now and may even become a bigger problem in the future if the necessary provision is not made for the introduction of sufficient new genetic material*." Comparative immunological studies in healthy and diseased CMZ populations may shed more light on the cellular immunity status of these inbred populations. Epidemiological studies would clarify the role of vectors, if any, in transmission of the virus responsible and the relatedness of the causative BPV types with those isolated from nearby farms. The diseased CMZ populations offer substantial opportunities for researching genetic factors that apparently regulate the appearance of the sarcoid tumour in equids.

Chapter V

General conclusions

5.1 Introduction

An extensive horse (*Equus caballus*) microsatellites database has been developed as part of the equine genome project. The INRA* and ArkDB** horsemap databases (accessed, 17th November, 2004), currently report 1097 and 966 microsatellites, respectively. It is estimated that the number of available markers for the horse genome will exceed 3000 within few years. Apart from development of such markers, laboratories across the world have been using horse microsatellites for parentage verification and pedigree analysis, for commercial and research purposes, on a routine basis. Before being accepted as a marker for inclusion in a panel, these microsatellites are carefully selected by an international panel of equine geneticists (International Society for Animal Genetics) and extensively tested for polymorphism and reliability in laboratories worldwide. A panel of horse microsatellites, with proven polymorphism and recommended by ISAG, was applied to two closely related subspecies of mountain zebra.

Karyotype comparisons between equids and zebras have been numerous (Santani et al. 2002; Chowdhary et al. 2003; Yang et al. 2003) but to date, few genetic studies on zebra populations have been published (Bowland et al. 2001; Moodley 2002). To our knowledge, this is the only study in zebras, where multiplexed microsatellite markers with fluorescent labels were genotyped for alleles in an automated genetic sequencer. Multiplexing microsatellite allows the use of multiple numbers of markers and enables flexibility in using microsatellites with size ranges that overlap. This technique enables simultaneous analyses of the whole panel of markers in a single step and speeds up genotyping procedures. The results of this study validate that heterologous PCR primer pairs isolated from the horse genome can be used to amplify homologous products in a related species, the Cape and Hartmann's mountain zebra. All the horse primers used were successful in amplifying a reproducible and specific product in the mountain zebra genome. Allele sizes were not used to calculate genetic parameters since sizes calculated with the in-lane size standard would be different from the actual sizes determined when cloned in a vector (Tozaki et al. 2001). True allele sizes can nevertheless be determined by comparing sizes of cloned microsatellites to sample allele sizes

*http://locus.jouy.inra.fr/cgi-bin/lgbc/mapping/common/summary.operl?BASE=horse

**http://www.thearkdb.org/browser?species=horse&objtype=stats

as determined by *STRand*.

The study has assumed that the microsatellites evolve under the stepwise mutation model (SMM; Ellegren 2002). The lower degree of genetic variability that may result from using microsatellites that have been originally isolated from a different species, could however cause distortions in resulting analyses. The Cape and Hartmann's mountain zebra subspecies are karyotypically inseparable (Heinichen 1969) and morphologically so similar that phenotypical dissimilarity between them is disputed. It is assumed that ancestral populations of these two subspecies would have exhibited a similar degree of polymorphism if the same domestic horse microsatellites had been used. This could avoid any bias in microsatellite polymorphism levels because of preferential inter-specific or subspecific amplification. Since outbred and historically free-ranging CMZ populations are no longer available to determine baseline polymorphism levels for microsatellites, it is predicted that the levels of polymorphism detected within the Namibian populations would be a true reflection of the original genetic diversity, had CMZ populations not been driven to near extinction.

5.2 Genotyping using horse microsatellites

The methods described in Chapter III demonstrate a quick and simple procedure for genetic analysis in the mountain zebra, by using FTA® paper based DNA extraction and multiplex PCR using fluorescent-labelled polymorphic markers. With the outbreak of sarcoid tumour-like growths in CMZ, the availability of an inexpensive and contemporary method for determination of genetic variation could be an effective conservation tool for this subspecies. With regard to parentage analysis, the International Stud Book Committee (ISBC) recommends a high probability of exclusion (PE) value of over 99.95 % (0.9995). However, any probable paternity evaluations attempted for mountain zebras only require a lower PE value. The reason for this is that the numbers of stallions siring foals in mountain zebra populations is likely to be much higher than in Thoroughbred or other domestic horse populations, on which the ISBC values were computed. Therefore, the values for the full panel of 16 microsatellites, 97.83 % for CMZ and 99.97 % for HMZ (excluding LEX33), would be quite adequate for paternity analysis. The microsatellite polymorphisms were found to generate acceptable exclusionary power for parentage analysis. This panel of markers has the potential for acting as the standard reference for a myriad of purposes, commercial and research, by the accredited international laboratories that routinely run the standard horse panel.

LEX3 was included in the multiplex panel to evaluate if the locus is X-linked as it is in domestic horses (Chowdhary et al. 2003). The locus exhibited single allele types in all male mountain zebras tested and did not exclude any known males. More tests are required to clarify linkage at this marker. Considering that the locus amplified well in both subspecies, it would be potentially useful for sex-typing and parentage analysis.

5.3 Comparative diversities

Hunting and farming drove CMZ populations close to extinction by the middle of 19th century (Bigalke 1954). This historical reduction in numbers has in all probability resulted in the loss of a considerable number of alleles by the time MZNP was established. Genetic drift and unsustainable translocations probably contributed to accelerate this loss. Current management practices of maintaining small populations in confined parks probably decrease the natural inbreeding-avoidance behaviours. This study revealed that the mean number of alleles was lowest in the two tumour-affected populations (GDNR and BNP: 2.22) followed by KaNR (2.33) and KaNP (2.44; Figure 4.1). Overall, Hartmann's mountain zebras were found to have the highest mean number of alleles per locus (8.44).

Genetic diversity studies in the family Equidae have reported a range of values, depending on the nature of genetic markers used. This is very evident from studies on estimates of expected heterozygosity (H_E) in horses. For example Bowling and Ruvinsky (2000) analysed 38 loci (including 22 blood-groups and 16 microsatellite loci) in different breeds (n = 50,000) and found values ranging from 0.461 for Thoroughbreds to 0.478 for Arabs. Others (Cothran & van Dyk 1998) using ten biochemical and seven blood-group loci reported lower values for Thoroughbreds (0.325) and Arabs (0.304). Cunningham et al. (2001) reported a higher expected heterozygosity level (0.646), from 211 Thoroughbreds and using 13 polymorphic loci. The Thoroughbred genetic database of the Veterinary Genetics Laboratory, University of Pretoria (16513 Thoroughbred horses- 12 microsatellites, November 2003) found a mean expected heterozygosity value of 0.724, which was higher than all others reported so far.

High heterozygosity levels are common amongst feral populations of equidae. In donkeys, Bellone et al. (1998) reported H_E of 0.623 in Baudet du Poitou donkey populations. Jordana et al. (2001) reported an H_E of 0.712 in Catalonian and Ivankovic et al. (2002) reported an H_E of 0.68 - 0.70 in Croatian donkeys populations. Allozyme analysis for diversity levels in plains zebra (*Equus quagga*) populations (Bowland et al. 2001) reported levels ranging from 0.121 to 0.129. More recent work by Moodley (2002) revealed heterozygosity levels ranging from

0.519 to 0.795 for the different plains zebra populations investigated. A lower average expected heterozygosity (0.511) was reported for Hartmann's zebras (200 zebras and 15 microsatellite loci) compared to this study (0.54; 84 zebras and 14 microsatellite loci). The same study found much lower heterozygosity values for Bontebok and Gariep populations compared to ours (0.232 vs. 0.325 and 0.159 vs. 0.309, respectively). These discrepancies may be attributed to the differences in methodology and genetic markers used in each study. Detection of polymorphism, however, is superior using a computer software-controlled fluorescent-labelled allele detection system, run in a DNA sequencer. A literature review revealed a similar report on variation in heterozygosity levels in two different studies in Catalonian donkeys (Jordana et al. 1999; 2001). The first study (Jordana et al. 1999) utilised 10 % polyacrylamide gel electrophoresis and ethidium bromide staining for visualisation. The same population was studied using fluorescent dye-labelled primers and the amplified products visualised on an Applied Biosystems 310 DNA Sequencer with GENESCAN Analysis software (Jordana et al. 2001). The authors report that the average number of alleles detected per locus increased from 2.7 ± 0.7 to 7.7 ± 1.0. As a result, the average expected heterozygosity (H_E) increased from 0.546 (± 0.049) to 0.712 (± 0.038), causing PIC and PE values also to increase significantly.

Cape mountain zebra populations exhibited lower levels of expected heterozygosity in all the populations analysed (H_E = 0.295 – 0.425; Appendix I), with the sarcoid tumour affected populations exhibiting lower levels than unaffected (0.386 vs. 0.427) populations. Hartmann's zebra populations showed heterozygosity levels that can be regarded as normal for outbred wild equid populations (H_E = 0.54 – 0.57). Low numbers of alleles were detected in the case of the Przewalskii's horse (*Equus przewalskii*; Bowling & Ruvinsky 2000). Heterozygosity levels however were similar to domestic horse breeds (0.474; SD ± 0.044). Other historically bottlenecked and inbred mammalian populations have also revealed low heterozygosity levels. Examples include the cheetah (*Acinonyx jubatus*) (heterozygosity: 0.39; Menotti-Raymond & O'Brien 1995), Ethopian wolf (*Canis simensis*) (0.21 – 0.36; Gottelli et al. 1994), the northern hairy-nosed wombat (*Lasiorhinus krefftii*) (0.27; Taylor et al. 1994), koala (*Phascolarctos cinereus*) (0.33; Houlden et al. 1996) and spectacled bear (*Tremarctos ornatus*) (0.38; Ruiz-Garcia 2003).

5.4 Population differentiation

In mountain zebra, where there is a definite social structure, demographic and social factors contribute to maintaining genetic polymorphism. The stallion actively herds females and

maintains a breeding herd. It is usual for such harems to be bred for long periods by a single stallion. Breeding herds are strictly segregated from non-breeding and bachelor herds (Rasa & Lloyd 1994). Presence of socially isolated groups can promote fixation of rare alleles and prevent their extinction due to localised fixation events (Bowland et al. 2001). It is quite probable that a 'reproductive substructure' within subpopulations of CMZ, aided by the selective translocation and seeding of stallions from MZNP, exists. It is also probable that such males were already homozygous at multiple loci prior to translocation. Following translocation, a few stallions would have sired the majority of the foals, with the other males forming bachelor herds and not contributing to the gene pool. The propensity of CMZ stallions to lead breeding herds over long durations could have exacerbated such reproductive sub-structuring, contributing to the high deficiency of heterozygotes and fixation of alleles within a subpopulation. The accelerated onset of Wahlund effects (population sub-structuring) would explain the exaggerated heterozygote deficiency detected and partly explain deviations from Hardy-Weinberg equilibrium.

The possibility of presence of 'null alleles' (non-amplifying alleles) that could lead to false observations of excess homozygotes, causing some heterozygous subjects to be falsely genotyped, was investigated. Null alleles are primarily caused by a mutation in the primer-binding site (Pemberton et al. 1995). The allele does not to amplify in such cases and if scored as monomorphic, can lower estimates of heterozygosity. False characterisation of a polymorphic locus is only likely in cases where all subpopulations uniformly exhibit homozygote excess at this locus, under similar amplification conditions. Thus, it was crucial that allele size ranges of the microsatellites used in this study were similar in CMZ and HMZ. The basis for analysing populations of two closely related subspecies with markers from a different species is the probability that genetic conservation of base pairs coding for similar loci across subspecies exists. On amplification, there was no evidence that any locus had changed size range in the two subspecies. Similarly, all loci that were homozygous in the CMZ were found to have multiple numbers of alleles in HMZ. An exception to this was LEX33, which did not amplify in all HMZ samples analysed. The failure of this locus to amplify was probably due to a fixed mutation in the primer-binding region, resulting in non-amplification.

It can be concluded that the successful amplification and demonstrable polymorphism of identical microsatellites in the outbred HMZ, with similarity in allele-amplification size ranges, reduces the probability that null alleles are responsible for the homozygosity observed. Further studies on the Kamanassie or De Hoop populations, suspected to be

different from MZNP genetic stock (Moodley 2002; Novellie et al. 2002) would clarify this matter. Conclusive proof of the pervasiveness of such alleles, if any, can only be detected through finding mismatches between known mother-offspring pairs.

5.4.1 Hardy-Weinberg equilibrium

Deviation from HWE was noted for three microsatellite loci within each subspecies (AHT5, HTG10 and HTG4 in CMZ and HMS3, HMS6 and HTG4 in HMZ). Again, the extreme population fragmentation may have lead to prominent Wahlund effects, causing sub-structuring and the observed distribution of loci. These heterozygote deficiencies are suspected to cause deviations from Hardy-Weinberg proportions for the CMZ populations analysed. Variation in allele frequency due to sampling error in small populations can also occur, and may lead to deviations from the Hardy-Weinberg principle (Hartl & Clark 1989). All the samples from GDNR originated from adult zebras aged over four years. It is probable that the vast majority of CMZ samples originated from captured adult zebras intended for translocation, and would then represent a limited number of generations. Therefore, sampling error cannot be discounted as a possible cause for the deviation detected. Furthermore, factors that are basic to an assumption of HWE would be violated during translocation from or seeding into, a population of zebras. These sub-populations of CMZ did, in all probability, never did mate randomly for the assumption of 'random union of gametes' to have been fulfilled. The limited number of animals sampled from each CMZ population might also have contributed to the deviations (Feulner et al. 2004). Five of the loci investigated showed a comparative lack of heterozygotes within HMZ. This may have been partly due to the presence of null alleles, especially for ASB2 and HMS3. Other factors that may cause a lack of heterozygotes still need to be investigated.

5.4.2 *F*-statistics and population structuring

F-statistics measure inbreeding as a probability of autozygosity relative to an ancestral population. In defining an ancestral population while calculating *F*, one assumes that all alleles present in the ancestral population are not identical by descent. The applicability of this assumption to the sampled CMZ populations is suspect due to reasons explained previously.

Mitochondrial DNA evidence points to Hartmann's and Cape mountain zebras as having close similarities as far the structure of their respective ancestral populations are concerned

(Moodley 2002). While the Cape subspecies suffered heavy population decimation, Hartmann's zebra populations continued to flourish and maintained high diversity levels in feral populations. As described earlier, the CMZ gene pool in the MZNP (that was probably already inbred to a degree when founded) was the source of the majority of herds that were established elsewhere in South Africa. Although the bottleneck probably resulted in a serious loss of heterozygosity, the severity of lack of allele diversity in populations investigated might have originated from the seeding of these subpopulations from a relatively new population (MZNP) and other subpopulations (e.g., Karoo National Park). This might have exacerbated the accompanying random genetic drift, causing an extreme founder effect that is seldom seen at such levels in natural mammal populations. A close comparison can be made with the Przewalskii's horses, where the current population (n ~ 2000) originated from a captive-bred population of 12 founder animals (Bowling & Ruvinsky 2000). Inbreeding and genetic drift in early generations accounted for a loss of 60 – 70 % of the original alleles and low levels of MHC diversity (Hedrick et al. 1999). Although the level of heterozygosity in the Przewalskii's horse was comparable to that in domestic horses (0.474; SD ± 0.044) (Bowling & Ruvinsky 2000), the average number of alleles detected was much lower.

The translocations of CMZ to other parks may have contributed to a reduction in the effective population size and increased inbreeding levels. It could have effectively contributed to reduction in allele diversity, at the same time causing the persistence of rare alleles driven by the reproductive dominance of few stallions. In the loci investigated in this study, there was very little evidence of the existence of private alleles. This may have been due to the similarity and very recent origins of the original gene pool from which all the sampled CMZ animals originated.

Nevertheless, this study demonstrated significant genetic differentiation between the various CMZ populations. The extreme population fragmentation and fixation of certain alleles in seeded populations is a very feasible scenario that may have contributed to the detectable genetic differentiation. Nei's sample number corrected G_{ST} values (0.127 for CMZ and 0.08 for HMZ) indicate such a population differentiation.

In contrast, the 'within population' inbreeding estimate, indicating a value for deficit of heterozygotes or F_{IS} values, was predictably high. Negative values were the norm, excepting for the Karoo National Park population (0.024, $P < 0.01$). The HMZ exhibited a F_{IS} value of 0.171 ($P < 0.001$) and a comparable Nei's G_{IS} value of 0.179, indicating the outbred nature of the population.

The negative results obtained in estimating F_{IS} in the small CMZ subpopulations were possibly due to the overall lack of information regarding the founder population. Such information is essential in order to obtain a good estimate of the true level of homozygosity, and generally involves genotyping of animals of different generations. The inbreeding coefficient estimated for the CMZ would thus be an estimate of the increase in homozygosity as compared to a poorly defined base population (current CMZ population samples arising probably from a single generation). Under such conditions, the observed heterozygosity is usually slightly higher than the expected one. This results in negative values for the average level of marker inbreeding in the reference population (Baumung & Sölkner 2003). This was exactly the case with the majority of loci investigated in CMZ, with observed heterozygosity values being generally higher than expected values (Appendix 1-A to 1-D). The Karoo National Park population, where the majority of alleles had a higher expected heterozygosity value than observed, thus probably contributing to the only significant 'within population' inbreeding estimate (F_{IS}) value, was the exception. It is worth considering the fact that the Karoo National Park population was established earlier (1978) than the populations at BNP (1986), GDNR (1985) and KaNR (1981). This may have played a role in the observed distribution of alleles, possibly due to availability of multigenerational samples.

Genetic linkage between two locus pairs: AHT4 - AHT5 and ASB23 - HTG10 was detected at significant levels. Removing these from calculations on population differentiation and inbreeding, however, did not significantly change the results. Since the locations of these loci within the 32 chromosomes in the mountain zebra have yet to be mapped, the linkage detected cannot be confirmed as spurious or real at this stage.

Group analysis between populations revealed that sarcoid tumour affected populations had the lowest levels of allelic richness, observed heterozygosity values and gene diversity levels. Allele richness levels reflected the comparative heterozygosity values, with Hartmann's zebra populations exhibiting high values for all the loci investigated. Tumour affected populations also exhibited the highest levels of heterozygote deficit and relatedness. The relatedness values between Bontebok and Gariep Dam populations indicated a high percentage of animals (almost 30 %) sharing similar alleles for the loci sampled. Pairwise population differentiation tests revealed that there was a significant level of differentiation between all the CMZ populations (except between Karoo National Park and Karoo Nature Reserve) even after conservative Bonferroni corrections. We attribute this to the genetic drift and allele fixation

after translocation, coupled with reproductive sub-structuring within confined CMZ populations.

Hierarchical analysis from *AMOVA* indicate that sarcoid tumour affected populations (BNP and GDNR) are sub-structured. The genetic differentiation between all the analysed CMZ subpopulations is lower than that between affected and non-affected CMZ populations (12 % vs. 27.9 %). This is intriguing, since all the extant CMZ populations analysed arose from a recent single gene pool. It may indicate a deeper pattern of specific similarities within disease-affected populations that is undetectable with the current tools at our disposal. Further comparative investigation with at least 22 polymorphic markers, sampling the majority of the affected population and comparing these to multigenerational samples obtained from MZNP would shed more light into this variation.

5.4.3 MHC associations

A strong possibility exists that the *Equus caballus* MHC region and the markers close to the coding regions are conserved in mountain zebras. A recent study looked at three microsatellite markers (UM-011, HTG-05 and HMS-42) located on the horse chromosome containing MHC and found UM-011 to be significantly associated with mould allergens (Curik et al. 2003). The authors suggested that such an association might be due to this marker being closely linked to the horse MHC class II *DRB* locus, which is important in host defense against pathogens. Assuming these markers are conserved in the mountain zebra, this study did not find any significant association between these markers and sarcoid tumour affected populations, with UMO11, HTG5 and HMS42 exhibiting only 1 - 3 alleles between them. No significant levels of linkage were detected in genotypic disequilibrium tests, as would have been the case if these loci were indeed closely linked in mountain zebra as they are in domestic horses. The possible presence of such a ‘genetic hitchhiking effect’, as reported by Curik et al. (2003), could not be verified in this study.

5.5 Sarcoid tumours and Cape mountain zebras

Small isolated populations like CMZ, which in all probability had a high genetic load within its ancestral population, are bound to experience considerable genetic drift, even in the short term. Vila et al. (2003) report genetic rescue, whereby a lone migrant male wolf increased the average heterozygosity, caused the rapid spread of new alleles and contributed to significant inbreeding avoidance within a pack of wolves. This might not occur with CMZ, where current

translocations are made from similar genetic stock (MZNP) and social behaviour tends to prevent new stallions from breeding (Penzhorn & Novellie 1991). Penzhorn (1984) noted the displacement of six mountain zebra herd stallions during a 3-year period in MZNP. Genetic diversity is promoted by such social behaviour and might be diminished in small and fenced-in populations. Lynch et al. (1995) suggest that such populations are at a high risk of accumulating new mutations via mutational meltdowns, over a period of time. Hedrick and Kalinowski (2000) comment that the expected effect of inbreeding on fitness could possibly vary among different species and is potentially unpredictable in an unexamined endangered species. As reviewed earlier in Chapter II (section 2.7.4.5), there is mounting evidence for a definite correlation between reduced heterozygosity and disease in mammalian populations. Genetic associations between sarcoid tumours and particular *Equus caballus* serotypes, and between papillomavirus infections, immunity and genetic susceptibility have been published (Chapter II, section 2.8.2). A large number of publications also indicate possible associations between inbreeding and disease. Nevertheless, information regarding specific correlations between a virus-induced condition and increased homozygosity is generally lacking (Acevedo-Whitehouse et al. 2003). Although inbreeding has been proven to decrease fitness traits, and has included research into correlations between disease and pathogen emergence, sparse evidence exists for specific pathogen-induced diseases that developed as a result of multigenerational consanguinity.

As part of ongoing studies in BNP, there is evidence of higher tick burden in sarcoid affected than non-affected zebras. Sarcoid-affected zebras in GDNR have higher mortality rates due to reasons yet to be determined. We hope that this study has added more evidence regarding the emergence of a virus-induced disease in an inbred population. The sarcoid tumour in equids is virus-induced, generally non-metastasising and is due the confluence of certain poorly defined factors, one of which is certainly genetic. This research prompts the question whether or not the intense inbreeding that these CMZ subpopulations have undergone, has produced the essential genetic predisposition or immunosupression, for emergence of disease at an epizootic level.

One of the assumptions in this study is that mean heterozygosity reflects inbreeding and the heterozygosity at marker loci reflects heterozygosity at yet undefined and unlinked trait loci (Balloux et al. 2004; Slate et al. 2004). The possibility that marker loci have direct effects on fitness is largely ruled out by using non-functional microsatellite genetic markers. There was no evidence for significant linkage disequilibrium, which would have indicated that markers used in this study are in physical linkage, and thus play a role.

5.6 Establishing a genetic database for the Cape mountain zebra

The panel of 16 microsatellites tested here, can be used to genotype individual mountain zebras and aid in parentage verification or for building a genetic database of the species. The case for a CMZ genetic database is urgent, considering the emergence of diseases like sarcoids, linked closely to consanguinity. Current CMZ populations in Kamanassie and Gamka Mountain Nature Reserves are too fragile and small to be considered as new genetic material for translocations. Thus, identification of non-Cradock genetic lineages for preferential translocation, to form new populations or supplement existing populations, is important. Translocations from the MZNP are currently made without reference to any genetic database or pedigree records. There is a high probability that sufficient genetic variation within the 500 odd CMZ of the MZNP still exists. Records show that the only non-Cradock genetic contribution to the current MZNP population was a stallion called '*Tom*', translocated from Kamanassie Nature Reserve in 1970 (Penzhorn 1985). This zebra stallion was a prolific breeder and maintained a long-standing harem. The lineage contributed by this animal needs to be identified and conserved for future translocations. Identification of allele patterns specific to Kamanassie mountain zebras and identifying similar private alleles within current herd stallions and mares in MZNP and De Hoop Nature Reserves can help to determine and isolate this lineage before it gets diluted and lost forever. The genetic archival, genotype testing and identification method validated in this study has the potential to contribute towards this endeavour. The eventual establishment of such a database will also enable genetic differentiation of more outbred populations and even specific herds.

5.7 Conclusion

The availability of commercial horse primers with fluorescent labels and use of Applied Biosystems 310 DNA Sequencer with *STRand* analysis software enabled us to detect the presence and absence of genetic variability in different mountain zebra populations accurately. This study sets the stage for more extensive studies to be carried out on sarcoid affected populations using more specific multiplexes and primers that amplify specific regions of interest. Using a standardised technique also allows easy comparisons to be made between laboratories.

Comparison of heterozygosity between mountain zebra populations is currently restricted due to the difficulty of obtaining samples from historically different but small and highly vulnerable populations such as Kamannassie (n = 31) and Gamka Mountain (n = 28) Nature

Reserves (Novellie et al. 2002). Karyotypically similar, the outbred Hartmann's populations are an excellent substitute for comparative reasons. These analyses showed amplification of 15 microsatellite loci within set size ranges for both subspecies. The main cause of heterozygote deficiency detected within all CMZ populations studied can be attributed to inbreeding. The Wahlund effect due to extreme population sub-structuring and random genetic drift would only have contributed and accelerated homozygosity levels.

A recent study analysed the mitochondrial control region in southern African zebras and suggested augmenting the genetically depauperate CMZ stock with the outbred Hartmann's zebra gene pool (Moodley 2002). The current policies in South Africa, however, encourage conservation managers and those providing animals for reintroductions, to maintain genetic separation between populations of CMZ and HMZ. Along similar lines, the IUCN equid specialist group (Oakenfull & Ryder 2002) notes that the primary threat facing the mountain zebra is that the two subspecies may interbreed, with the loss of pure stock. Groves & Bell (2004) did multivariate analyses on phenotypical (pelagic and craniometric) parameters between CMZ and HMZ. These authors state that the two mountain zebra subspecies are absolutely different and even propose that they be regarded as separate species. We agree with the IUCN recommendation that conservation policies and determining evolutionary significant populations of equids should be based not only on genetic studies, but with information incorporated from studies on morphology, behaviour and habitat of the populations in question. The genetic differences or similarities between the two subspecies needs to be explored thoroughly before one can realistically propose a change in policy and suggest interbreeding the two subspecies of mountain zebra populations.

Hedrick & Kalinowski (2000), drawing on their experience from the Speke's gazelle captive breeding program, comment that populations exhibiting signs of low fitness because of past fixation from genetic drift have the potential to improve. The affected CMZ populations are currently under investigation for possible therapeutic consideration and genetic enrichment from more outbred CMZ populations. This work has made the process of identifying more heterozygous zebras for translocation possible. It now only requires a few millilitres of blood, skin or hair samples for analysis, which can be carried out quickly. Regarding the problem of equine sarcoids in CMZ, further research on these populations is warranted. A comparative investigation on the immunological status of the different CMZ populations and epidemiological studies would possibly shed more light on equine sarcoids.

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Appendix I- Tabulated genetic data from individual mountain zebra populations (H_O: Observed heterozygosity; H_E: Expected heterozygosity; PIC: Polymorphic information content; PE: probability of exclusion)

Appendix I-A

Locus	Population data from Bontebok National Park with 14 informative loci (n=12)					
	Alleles	Animals typed	H_O	H_E	PIC	PE
AHT4	3	12	0.667	0.685	0.582	0.362
AHT5	2	12	0.167	0.159	0.141	0.071
ASB17	2	12	0.583	0.489	0.359	0.179
ASB2	1	12	0	0	0	0
ASB23	2	12	0.167	0.159	0.141	0.071
HMS3	2	12	0.583	0.431	0.328	0.164
HMS42	1	12	0	0	0	0
HMS6	2	12	0.167	0.29	0.239	0.12
HTG10	2	12	0.583	0.518	0.373	0.187
LEX52	1	12	0	0	0	0
LEX64	2	12	0.333	0.29	0.239	0.12
UM011	3	12	1	0.67	0.566	0.349
VHL20	3	12	0.667	0.562	0.432	0.237
HTG4	2	12	0.333	0.464	0.346	0.173

Mean alleles per locus = 2
Mean heterozygosity = 0.337
Cumulative exclusion probability = 0.9020

Appendix I-B

Locus	Population data from Gariep Dam Nature Reserve with 14 informative loci (n=17)					
	Alleles	Animals typed	H_O	H_E	PIC	PE
AHT4	3	17	0.471	0.465	0.401	0.23
AHT5	2	17	0.118	0.114	0.105	0.052
ASB17	3	17	0.529	0.415	0.342	0.183
ASB2	2	17	0	0.114	0.105	0.052
ASB23	2	17	0.588	0.513	0.374	0.187
HMS3	2	17	0.471	0.428	0.329	0.165
HMS42	2	16	0	0.121	0.11	0.055
HMS6	2	17	0.059	0.059	0.055	0.028
HTG10	2	17	0.529	0.508	0.372	0.186
LEX52	1	16	0	0	0	0
LEX64	2	15	0.467	0.48	0.357	0.178
UM011	3	16	0.688	0.643	0.552	0.34
VHL20	2	17	0.235	0.214	0.186	0.093
HTG4	2	17	0.059	0.059	0.055	0.028

Mean alleles per locus = 2.14
Mean heterozygosity = 0.295
Cumulative exclusion probability = 0.8627

Appendix I-C

Locus	Population data from Karoo National Park with 9 informative loci (n=12)					
	Alleles	Animals typed	H_O	H_E	PIC	PE
AHT4	3	12	0.25	0.42	0.363	0.207
AHT5	3	12	0.25	0.42	0.363	0.207
ASB17	3	12	0.667	0.594	0.477	0.276
ASB23	3	12	0.417	0.507	0.424	0.243
HMS3	2	12	0.167	0.507	0.368	0.184
HMS6	2	12	0.167	0.159	0.141	0.071
HTG10	2	12	1	0.522	0.375	0.188
LEX33	2	7	0.429	0.495	0.354	0.177
VHL20	2	12	0.5	0.391	0.305	0.152
HTG4	2	8	0.25	0.233	0.195	0.097

Mean alleles per locus = 2.4
Mean heterozygosity = 0.425
Cumulative exclusion probability = 0.8661

Appendix I-D

Locus	Population data from Karoo Nature Reserve with 9 informative loci (n=12)					
	Alleles	Animals typed	H_O	H_E	PIC	PE
AHT4	3	11	0.727	0.628	0.519	0.312
AHT5	2	10	0.2	0.337	0.269	0.134
ASB17	3	12	0.833	0.652	0.555	0.342
ASB23	3	11	0.273	0.385	0.326	0.178
HMS3	2	12	0.333	0.391	0.305	0.152
HMS6	2	11	0.636	0.455	0.34	0.17
HTG10	2	12	1	0.522	0.375	0.188
LEX33	2	9	0.667	0.523	0.372	0.186
VHL20	2	12	0.083	0.083	0.077	0.038
HTG4	2	11	0.273	0.247	0.208	0.104

Mean alleles per locus = 2.3
Mean heterozygosity = 0.422
Cumulative exclusion probability = 0.8708

Appendix I-E

Locus	Population data from Hartmann's zebras with 14 informative loci (n=84)					
	Alleles	Animals typed	H_O	H_E	PIC	PE
AHT4	17	84	0.762	0.817	0.792	0.647
AHT5	6	84	0.524	0.536	0.452	0.264
ASB17	10	84	0.619	0.769	0.735	0.567
ASB2	3	26	0.115	0.521	0.427	0.239
ASB23	14	82	0.841	0.898	0.882	0.782
HMS3	3	74	0.108	0.239	0.215	0.111
HMS42	4	71	0.113	0.242	0.225	0.122
HMS6	4	80	0.325	0.515	0.461	0.277
HTG10	9	74	0.622	0.695	0.668	0.5
LEX52	3	69	0.101	0.124	0.119	0.061
LEX64	10	66	0.727	0.776	0.75	0.597
UM011	7	71	0.155	0.354	0.333	0.197
VHL20	9	83	0.446	0.446	0.425	0.271
HTG4	4	81	0.309	0.589	0.499	0.296

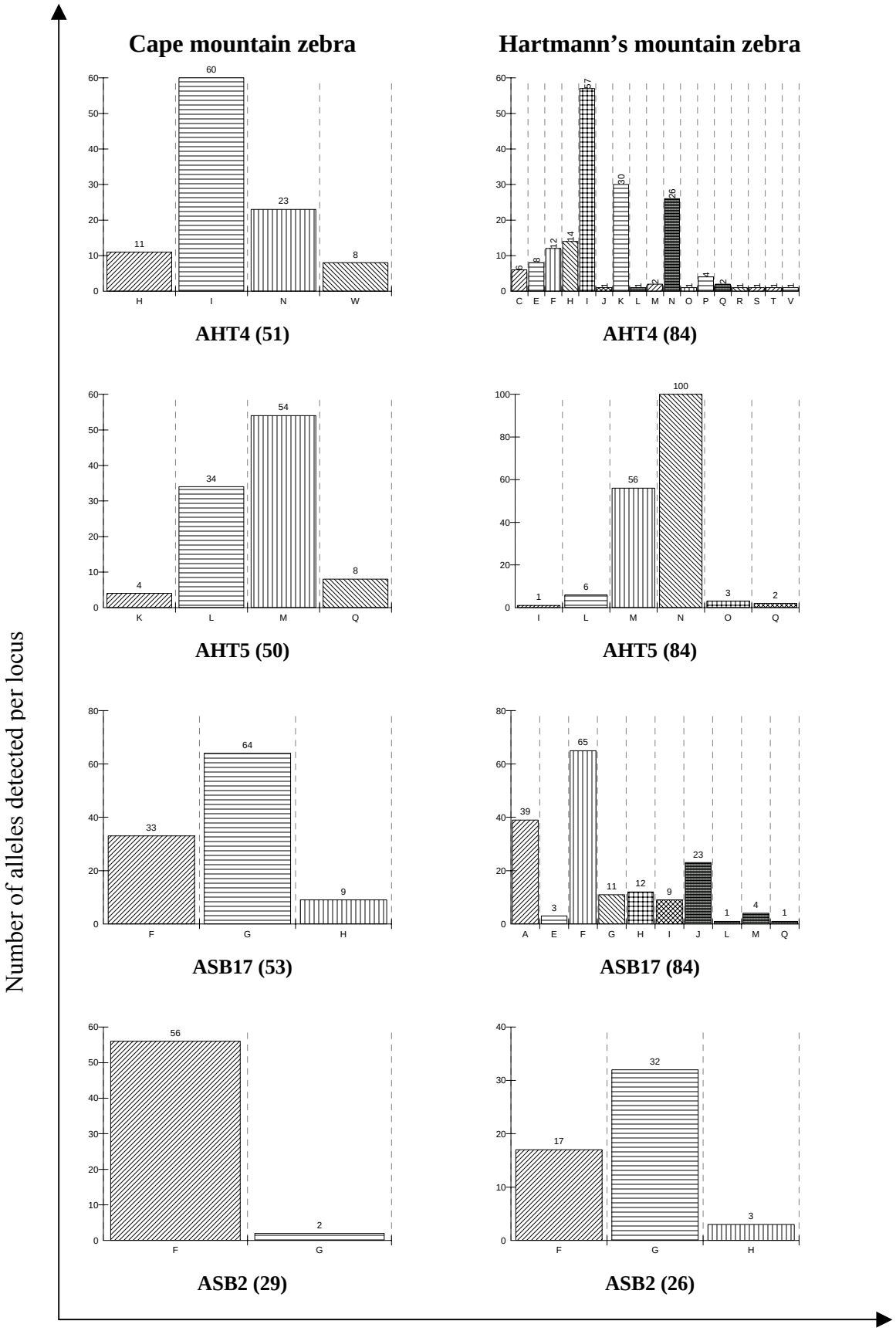
Mean alleles per locus = 7.36
Mean heterozygosity = 0.537
Cumulative exclusion probability = 0.99918

Appendix II **- Microsatellite primer sequences and references**

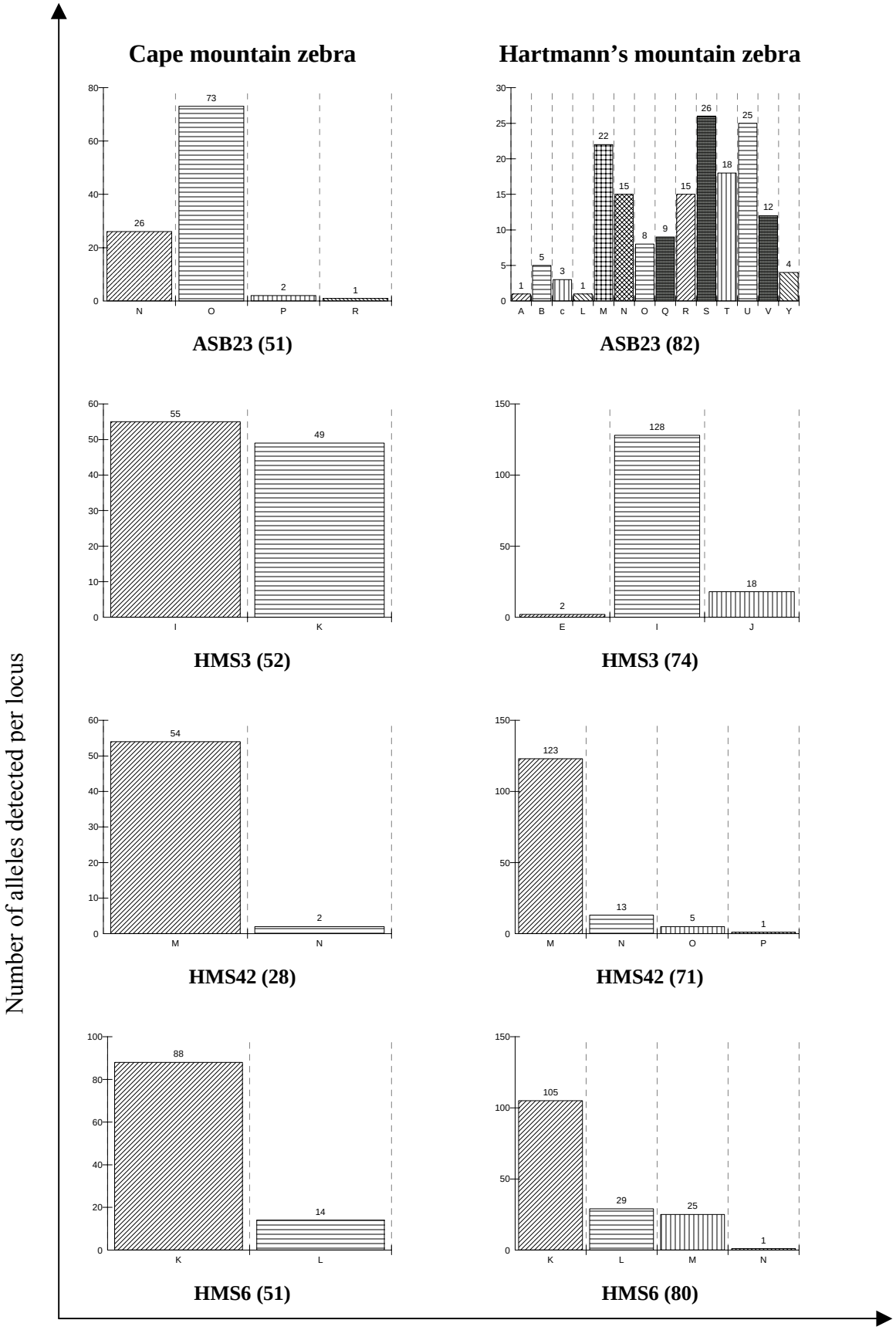
Locus	Direction	Primer sequence (5'- 3')	Reference
AHT4	forward reverse	AACCGCCTGAGCAAGGAAGT GCTCCCAGAGAGTTTACCCT	Binns *et al*. (1995)
AHT5	forward reverse	ACGGACACATCCCTGCCTGC GCAGGCTAAGGAGGCTCAGC	Binns *et al*. (1995)
ASB2	forward reverse	CCTTCCGTAGTTTAAGCTTCTG CACAACTGAGTTCTCTGATAGG	Breen *et al*. (1995d)
ASB17	forward reverse	GAGGGCGGTACCTTTGTACC ACCAGTCAGGATCTCCACCG	Breen *et al*. (1995c)
ASB23	forward reverse	GAGGTTTGTAATTGGAATG GAGAAGTCATTTTTAACACCT	Breen *et al*. (1995b)
HMS3	forward reverse	CCAACTCTTTGTCACATAACAAGA CAATCCTCACTTTTTCACTTTGTT	Guérin *et al*. (1994)
HMS6	forward reverse	GAAGCTGCCAGTATTCAACCATTG CTCCATCTTGTGAAGTGTAACTCA	Guérin *et al*. (1994)
HMS42	forward reverse	TAGATTTCTTAAGTGCAAATAGTGG GAACTGCTATAGATATACCTAATCC	Godard *et al*. (1998)
HTG4	forward reverse	CTATCTCAGTCTTCATTGCAGGAC CTCCCTCCCTCCCTCTGTTCTC	Ellegren *et al*. (1992)
HTG10	forward reverse	CAATTCCCGCCCCACCCCCGGCA TTTTTATTCTGATCTGTCACATTT	Marklund *et al*. (1994)
LEX3	forward reverse	AACATCTAACCAGTGCTGAGACT AAGAACTAGAACCTACAACTAGG	Coogle *et al*. (1996)
LEX33	forward reverse	TTTAATCAAAGGATTCAGTTG TTTCTCTTCAGGTGTCCTC	Coogle and Bailey (1996)
LEX52	forward reverse	GGAACGGAAGAGTGTAGTTTT CATTTATTCATCAGCGATTTG	Coogle and Bailey (1997)
LEX64	forward reverse	ACCCTTTCCGCAGACAA CACATCAGAGCCCATCTTCTC	Coogle and Bailey (1997)
UM011	forward reverse	TGAAAGTAGAAAGGGATGTGG TCTCAGAGCAGAAGTCCCTG	Mickelson *et al*. (1999)
VHL20	forward reverse	CAAGTCCTCTTACTTGAAGACTAG AACTCAGGGAGAATCTTCCTCAG	Haeringen *et al*. (1994)

Appendix III

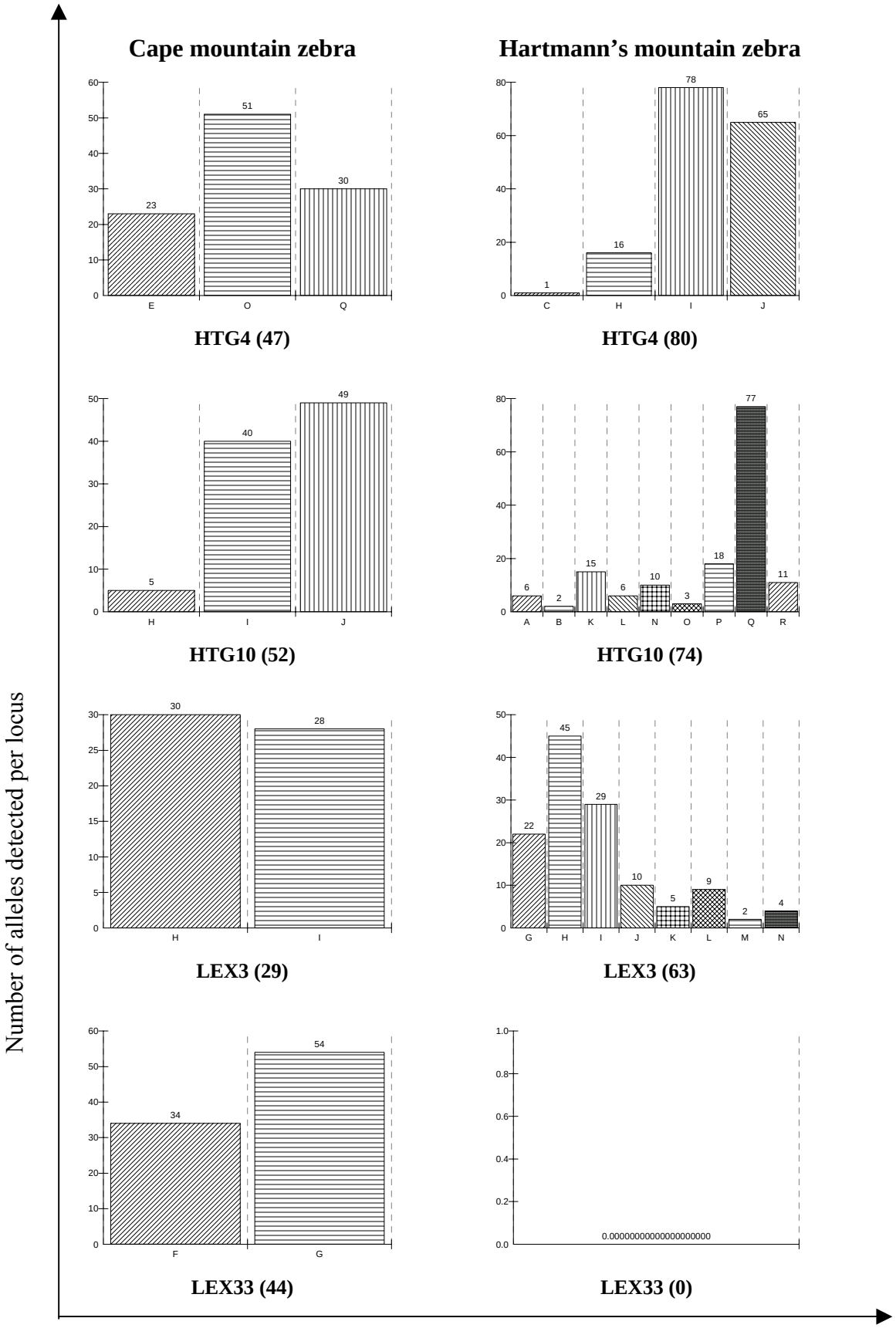
Allele distributions for individual microsatellite loci in CMZ and HMZ

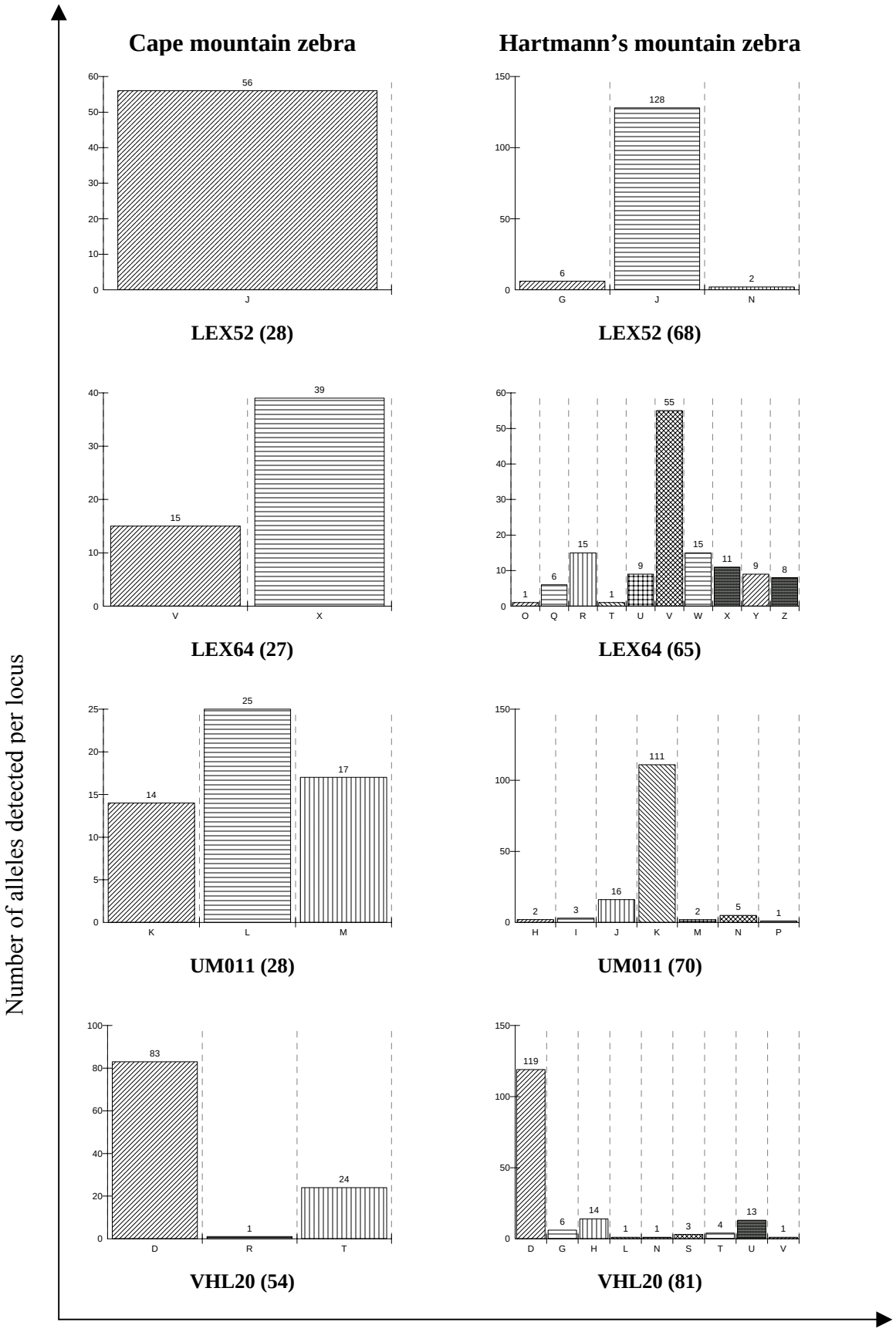


Allele types per locus (Numbers in paranthesis indicate the number of zebra typed/locus)



Allele types per locus (Numbers in paranthesis indicate the number of zebra typed/locus)


Cape mountain zebra
Hartmann's mountain zebra
HTG4 (47)
HTG4 (80)
HTG10 (52)
HTG10 (74)
LEX3 (29)
LEX3 (63)
LEX33 (44)
LEX33 (0)
Number of alleles detected per locus
Allele types per locus (Numbers in paranthesis indicate the number of zebra typed/locus)



Allele types per locus (Numbers in paranthesis indicate the number of zebra typed/locus)