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The intra- and inter-population relatedness of bovine tuberculosis-infected and -uninfected African buffaloes *(Syncerus caffer caffer)* in the Kruger National Park

by

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientae

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Declaration

I, Ingrid Rossouw, hereby declare that this dissertation submitted for the degree *Magister Scientae* at the University of Pretoria, is my own work and has not previously been submitted for a degree at this, or any other university.

Ingrid Rossouw
July 2010

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List of abbreviations

AIDS	Acquired immune deficiency syndrome
ARC-OVI	Agricultural Research Council – Onderstepoort Veterinary Institute
BCG	Bacillus Calmette-Guerin
BTB	Bovine tuberculosis
DNA	Deoxyribonucleic acid
F	Fixation index
F*is*	Inbreeding coefficient
FSTAT	F-statistics
GKNPC	Greater Kruger National Park Complex
HiP	Hluhluwe-iMfolozi Park
HIV	Human immunodeficiency virus
HWE	Hardy-Weinberg Equilibrium
IUCN	International Union for the Conservation of Nature
KNP	Kruger National Park
M. bovis	Mycobacterium bovis
Msats	Microsatellite markers
PCR	Polymerase Chain Reaction
PIC	Polymorphic information content

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Summary

The intra- and inter-population relatedness of bovine tuberculosis - infected and - uninfected African buffaloes *(Syncerus caffer caffer)* in the Kruger National Park

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The African buffalo (*Syncerus caffer*) is a member of one of Africa's most well known tourist attractions and unique grouping of mammals – the 'big five'. Historical records indicate that during the 19th century approximately 3 million African buffaloes inhabited almost the whole of sub-Saharan Africa. Several factors such as disease, habitat fragmentation, over-hunting and drought reduced the buffalo population to approximately 400 000 by 1990.

The African buffalo is host to a variety of sub-acute diseases, such as bovine tuberculosis (BTB), foot-and-mouth disease (FMD) and corridor disease (CD). Disease is an important factor which influenced African buffalo populations throughout the continent and more specifically the Kruger National Park (KNP) and is largely responsible for the fact that buffaloes are restricted to enclosed areas with strict regulations imposed on their movement. The social

organization of animals influences the distribution and spread of a disease – especially in the case of the African buffalo in the KNP.

The emergence of BTB in the largest conservation area in South Africa (the KNP), threatens wild and domestic animals and humans who are in close proximity to the Park. The potential economic losses associated with this disease are excessive.

The results presented in this thesis provide baseline information into the genetic status of sampled African buffaloes in the KNP, genetic relatedness between sampled individuals as well as BTB associations between sampled African buffaloes in the KNP, based on a limited dataset of 181 animals.

Twelve microsatellite markers were used to evaluate 181 samples which were collected from 39 locations dispersed throughout the KNP. Specific population genetic parameters revealed information based on the intra and inter - relationships at the 'per population' level as well as at the 'per prevalence group' level. Evidence indicates a medium to high level of genetic diversity, a low to medium level of inbreeding (inbreeding coefficient (*Fis)* for each group ranges between 0.143 and 0.147) and a relatively high level of migration for buffaloes associated with each prevalence group.

Pairwise relatedness estimates were determined between individuals, to reveal their level of relatedness (unrelated, full siblings, parent-offspring or half siblings), based on Queller and Goodnight's (1989) coefficient of relatedness. Relatedness was determined on different levels, intra and inter-population level, BTB infected and BTB uninfected group level as well as prevalence group levels. Evaluation of data based on these different levels and between different groups, painted an overall picture of the disease condition and genetic relatedness within and between sampled BTB infected and BTB uninfected buffaloes.

Evidence indicated that the greater majority of our sampled African buffaloes (BTB infected or uninfected), were genetically unrelated (in terms of sibling

and parent-offspring relationships), irrespective of their disease status. *M. bovis* infected buffaloes sampled and used in our study are not more closely related to each other than to uninfected buffaloes in the same population or prevalence group.

Keywords: African buffalo; Kruger National Park; bovine tuberculosis; microsatellite markers; population genetic parameters; relatedness; BTB transmission

Disclaimer

This thesis consists of a series of research chapters that have been prepared as stand-alone manuscripts for publication purposes. Some unavoidable repetition may therefore occur between chapters.

Chapter 1

General introduction

"Maar vir my gee U krag soos die van 'n buffel"

Psalm 92 verse 11

1. Introduction

Population genetics is the study of genetic variation among populations, which therefore, predicts diversity within a population. It is based on Mendel's laws and other genetic principles as they relate to entire populations and involves several fields of study including wildlife management and conservation, animal breeding, ecology, systematics, computational and evolutionary biology (Hartl and Clark, 2007). Conservation biology is the study of the earth's biodiversity and aims to preserve species, their habitats and ecosystem for future generations (Frankham *et al.*, 2002). Conservation genetics aims to preserve biodiversity and species and reduce the risk of extinction based on genetic principles.

Our environment is constantly placed under stress, which results in the rapid decline of our natural resources. The primary cause for this rapid decline is an increased human population, development and habitation. Several external factors regulate wild animal populations, some of which include food supply, water quality and availability, climate, predation and disease (Van Hooft *et al.*, 2002).

Several factors combine to support conservation and assist in reversing the rate of extinction, such as politics, science, economics, biomedical and genetic technologies. Species conservation strategies require multidisciplinary baseline information used to devise actions to determine and minimize threats to animal survival. Genetic microsatellite marker technologies have contributed to the management and conservation of several species, ranging

from reptiles to mammals. Microsatellites have become popular markers and have been used for parentage evaluation, gene mapping, population genetic studies, linkage studies and animal genetic forensics.

Population and conservation genetics were incorporated in this study.

African buffaloes are large herbivores and form an important component of the ecosystems and the plant communities they depend upon. These large herbivores shape the plant communities they graze, thereby influencing the quality, quantity and structure of the available forage and indirectly influencing the distribution of other grazing species (Winnie *et al.*, 2008). The African buffalo can, therefore, be regarded as a keystone species in certain areas, as they shape and influence their surrounding ecosystems.

2. Background information

2.1 The African buffalo *(Syncerus caffer)*

The African buffalo is recognized as Africa's only wild bovine species. Three distinct sub-species are recognized, with distinct phenotypic differences related to their horn configuration, coat colour and size. The two types of savannah sub-species include the Cape buffalo, *Syncerus caffer caffer* and the West African buffalo, *Syncerus caffer brachyceros*. The third sub-species is the smaller red or forest buffalo, *Syncerus caffer nanus* and is mainly distributed across the rain forests of western and central Africa. The Cape buffalo is widely distributed throughout sub-Saharan Africa, predominantly in the savannah biomes of eastern and southern Africa. Together with elephants (*Loxodonta africana*) and wildebeest (*Connochaetes taurinus*), buffaloes form the bulk of the large herbivore biomass in Africa (Van Hooft *et al.*, 2002). Food and water supply, predation and disease are the main regulators of these wild animal populations.

The African buffalo is a large herbivore found in herds that vary in size from 50 to 1000 individuals, with an average size of 250 individuals (Ryan *et al.*, 2006). The average herd size may, however, vary considerably depending on

the vegetation density and food supply of a particular area (Van Hooft *et al*., 2003). The buffalo herd is a mixed unit that typically consists of adult cows and bulls, sub-adult cows and bulls, juveniles and calves. African buffaloes exhibit a seasonal social ecology, where they form large, mixed herds during the breeding season and divide into smaller, mixed herds and bachelor groups for the remainder of the year. In addition to their seasonal social organization, African buffaloes may also engage in local and long-distance dispersal, where individuals exchange between groups throughout the year (Ryan *et al*., 2006). Within these mixed buffalo herds, cows rarely move out of their own (native) herds (Prins, 1996). Young female buffaloes (heifers) move between herds *via* splinter groups while adult cows seem to remain with the breeding herd of choice and exhibit strong philopatric behaviour. An exception to strong female philopatric behaviour was however, observed in Botswana, where frequent herd-switching among adult female buffaloes took place (Van Hooft *et al*., 2003).

In principle, males are regarded as the dispersing sex (Greyling, 2007). Adult bulls often move between mixed herds and bachelor groups and are consequently less tightly clustered than cows and individuals of different age groups (Cross *et al*., 2005). An example of male dispersal can be observed at Lake Manyara National Park, where males can migrate up to 50 km across the Park's border (Van Hooft *et al*., 2003). Male bachelor groups usually form during the non-breeding season, while during the breeding season they are present in mixed herds and compete for mating (Ryan *et al*., 2006). A recent retrospective study looking at African buffaloes that escaped from the KNP (between 1998 and 2007) indicated that adult bulls dispersed at the highest rate of the age/gender classes, followed by adult females (Van Schalkwyk, L. July 2010, personal communication). The herding behaviour of bulls is of importance as it plays an important role in inter-herd disease transmission (Greyling, 2007). From an epidemiological point of view, dispersal (male or female) is of great importance, especially since spread of disease is related to animal movement and their consequent dispersal characteristics (Greyling, 2007).

African buffaloes breed throughout the year in some areas and are seasonal breeders in other parts, their reproduction peaks are associated with seasonal rainfall (Prins, 1996). Births, however, tend to be seasonal where rainfall is a limiting factor. Females cycle every 23 days and are in oestrus for three to five days. The mean gestation period is approximately 343 days, after which a single calf is born. An inter-birth interval of around two years is expected (Prins, 1996). Within the KNP, the calving season falls between January and April, with calving peaks during January and February. This time period coincides with optimal grass growth, production and quality within the KNP (Greyling, 2007). A buffalo's sex can easily be determined when the animal is approximately three years of age by evaluation of the shape of the animals' head and horns (larger horns that can grow up to 160 cm can be observed in males). Buffalo cows are characterized as adult, sub-adult or juvenile. Adult cows are six years and older, sub-adult cows are between three and six years and juveniles are generally older than calves (newborn to three years) and younger than sub-adults. Cows and bulls are fully grown between six and seven years of age, their development, does however continue (Prins, 1996).

2.1.1 African buffalo status

According to the International Union for the Conservation of Nature (IUCN 2004), the African buffalo, *Syncerus caffer* is a lower risk conservation dependant species. The total African buffalo population is approximately 900 000 across the continent, most of which are Cape buffalo (*Syncerus caffer caffer),* due to the difficulty to census forest buffaloes (*Syncerus caffer nanus*). An approximate forest buffalo population estimate is 60 000. Details on the African buffalo status can be viewed at the International Union for Conservation of Nature website at <http://www.iucnredlist.org/details/21251>.

The African buffalo is affected by environmental factors such as drought, habitat fragmentation and loss, hunting pressure and disease. Habitat fragmentation has increased and persisted since the end of the 19th century as a result of increased human population growth, habitation and cultivation (Van Hooft *et al*., 2002). In Kenya between 1977 and 1994, habitat

fragmentation resulted in a 15 % decline in the African buffalo population and in southern Africa between 1991 and 1996 a 50 % decline in the African buffalo population was observed due to habitat fragmentation (Van Hooft *et al*., 2002). Historically rinderpest also proved to be a major threat to the African buffaloes, as an epidemic of the 1890's virtually destroyed the southern African population (Van Hooft *et al*., 2002).

Buffaloes were hunted for trophies and as a food source. Even today, the African buffalo is a highly sought after trophy animal and is therefore of high economic value and importance. The market value for live buffaloes varies, depending on whether the animal is disease-infected or disease-free. Disease-infected animals are of much lower economic value (referring specifically to diseases like foot-and-mouth disease (FMD), corridor disease (CD) and bovine tuberculosis (BTB)).

2.1.2 Buffalo phylogeny

Phylogeny is the summary of an organism's history and its evolutionary relationships as they change over time. Connections between all groups of life as derived from their ancestor relationships can thus be established. Table 1.1 describes the taxonomic classification of the African buffalo.

Table1.1 Taxonomic classification of the African buffalo *(Syncerus caffer)*

Kingdom	Animalia
Phylum	Chordata
Class	Mammalia
Order	Cetariodactyla
Sub-order	Ruminantia
Family	Bovidae
Tribe	Bovini
Genus	*Syncerus*
Species	*caffer*

The Bovidae is the most diverse artiodactyl family, consisting of 137 extant species representing five sub-families and 13 tribes. Fossil records indicate that the earliest Bovini roamed the earth some six million years ago (Pitra *et al*., 1997). Today, this monophyletic Bovini tribe includes the largest grazing ruminants, which include bison, yak and buffalo species. Their phylogenies were resolved and inferred using DNA sequencing techniques, which allowed quantitative estimates of the genetic relationships among the Bovini tribe to be established (Pitra *et al*., 1997).

2.1.3 The distribution and habitat of African buffalo

Historical records indicate that African buffaloes have inhabited almost the whole of sub-Saharan Africa, constituting 35 % of the large herbivore biomass (Prins, 1996). At the end of the 19th century, the rinderpest epidemic swept across the African continent, severely reducing the African buffalo population to very small numbers. It was estimated that for every 1 buffalo that survived, 10 000 died (Simonsen *et al*., 1998). The rinderpest epidemic together with habitat fragmentation contributed to the population decline, from the 3 million individuals that roamed the continent during the 19th century, to only 400 000 in 1990. This decline in population size did, however, not cause a significant decline in the genetic diversity of the species (Van Hooft *et al*., 2002).

The African buffalo occupies and disperses through a wide range of habitats and is found in most major vegetation types and biomes of sub-Saharan Africa, in the presence of a permanent water source (Simonsen *et al*., 1998). Their habitat varies between dense lowland tropical forests to savannah grasslands, tropical savannah woodland to dry bush. Essential habitat requirements include a good grass cover, water and shade. Due to their poor ability to regulate body temperature, the African buffalo usually feeds at night.

2.2 The Kruger National Park (KNP)

The Sabie Game Reserve, which included the present day Skukuza, Malelane and Pretoriuskop camps, was the precursor of the Kruger National Park.

Colonel James Stevenson - Hamilton, a Scottish cavalry officer who fought during the war of the British Empire against the Boer republics in 1902, was appointed as the first warden of the Sabie Game Reserve and played a key role in creating the KNP as we know it today (Scholes and Dennis, 1995). In 1926 the Sabie Game Reserve, Shingwedzi Game Reserve and adjacent farms were combined to form the KNP which was opened to visitors in 1927. Most of the KNP has therefore been under formal protection for the past 80 years.

The KNP is one of the largest formally-conserved areas in Africa and comprises approximately 2 million ha. The Park is situated in the lowveld, in the north-eastern region of South Africa (Mabunda *et al*., 2003). The KNP is bordered to the north by Zimbabwe, to the east by Mozambique, to the south and south west by the Mpumalanga province and to the west by the Limpopo province. The Park's western boundary ranges from a high-density communal area to private and provincial game reserves (Mabunda *et al*., 2003). These private and provincial game reserves have been incorporated to form part of the Greater Kruger National Park Complex (GKNPC). The KNP extends 350 km from north to south and an average width of approximately 60 km from east to west (Mabunda *et al*., 2003).

The KNP boasts with a vast array of species, which include 147 mammalian species, 505 birds, 119 reptiles, 49 fishes, 34 amphibians. It is home to approximately 28 500 buffaloes. Details of the various animal species within the KNP can be viewed at the South African National Parks website at <http://www.sanparks.org/parks/kruger/>.

Two climatic zones stretch across the KNP, to the north, the tropical and sub-tropical zones and to the south the temperate zone. The KNP is situated in a summer rainfall area, with the rainy season from October to March, and the dry season between April and September. Details of the climatic zones associated with the KNP can be viewed at the South African National Parks website at <http://www.sanparks.org/parks/kruger/>.

The two major types of geological substrate found within the KNP, includes the more fertile granite substrates in the western part of the Park and the less fertile basalt substrate which underlies the eastern part of the Park. The geological substrates influence the plant community heterogeneity as well as the quality and quantity of available forage for grazers and browsers and therefore, indirectly influence animal behaviour and distribution (Winnie *et al.*, 2008). Herbivores such as the African buffalo can influence the soil nutrient distribution and cycling rates, which in turn influences the quality and quantity of available forage, plant distribution and plant growth rates (Winnie *et al.*, 2008).

The KNP is characterized by its vast botanic diversity, which includes approximately 1980 different plant species. The Parks flora is divided into 16 different eco-zones, which depend on the location, rainfall and animal distribution within a particular region. The Parks vegetation is classified into sub-arid and wooded savannah regions, excluding the wetter areas within the Park. Details of the botanic diversity within the KNP can be viewed at the South African National Parks website at <http://www.sanparks.org/parks/kruger/>.

In South Africa, the two largest, free-ranging African buffalo populations are confined within the Hluhluwe-iMfolozi Park (HiP) and the KNP. Within the KNP approximately 28 500 African buffaloes are distributed across 100 herds, with an average herds size of approximately 244 individuals. The average herd size may, however, vary depending on the available water and food source availability (Greyling, 2007). African buffalo dynamics within the KNP have changed and fluctuated considerably over the past 100 years, mostly as a result of climatic changes and disease within the area (Greyling, 2007).

2.3 Bovine Tuberculosis (BTB)

Bovine Tuberculosis (BTB) is a chronic, progressive bacterial disease caused by *Mycobacterium bovis*. BTB is an airborne, multi-host pathogen, which is currently re-emerging worldwide, in both wildlife and livestock. BTB can affect

all mammalian species, which differ in their susceptibility. The number of wild African species affected by BTB is currently on the rise (Renwick *et al*., 2006). Detection and evaluation of a chronic disease (such as BTB) is hampered by several factors, some of which include the test accuracy, changing disease status as well as animal and population migration (Cross *et al*., 2009).

Using molecular typing techniques and a combination of markers (IS6*110*, PGRS RFLP, spoligotyping), Michel and co-workers genetically characterized and compared different *M. bovis* isolates from various wildlife species within the Greater Kruger National Park Complex (GKNPC) (Michel *et al*., 2009). Their work revealed the existence of two strain types of BTB within the KNP, which includes the C8 strain and C8v strains. The C8v strains are derived from C8 strains by mutations and are, therefore, genetically closely related to C8 strains. The C8v strains represent an indicator for the evolution of *M. bovis* within the KNP. The dominant C8 strains are more prevalent in the southern regions of the Park, while variant C8v strains are more commonly found in the central and northern regions of the Park. The dominant C8 strain has been found in 73 % of *M. bovis* isolates with the remaining 27 % present as variants of the dominant strain (Michel *et al*., 2009).

2.3.1 Disease history

BTB is an exotic disease in South Africa, believed to have originated in the Mediterranean, before classical times. The disease was introduced locally by livestock of the British and Dutch settlers, who colonized the region during the 19th century (Renwick *et al*., 2006). BTB was first diagnosed in cattle in South Africa in 1880 and in wildlife in 1928. The greater kudu (*Tragelaphus strepsiceros*) in the Eastern Cape region, was the first reported wildlife species to be infected with the disease (Renwick *et al*., 2006). It has been shown that BTB was transmitted by domestic cattle to African buffaloes during the 1950's in the south eastern corner of the KNP before fencing of the southern boundary of the reserve had been completed. The disease was, however, only discovered in the KNP in 1990 (Michel *et al*., 2009). In 1992, the initial disease prevalence was 4.4 % in the central region and 27.1 % in

the southern region. By 1998 these values had increased to 16 % and 38.2 % in the central and southern regions respectively and had spread to several other wildlife species. The disease thus spread in a northerly direction, with a high prevalence in the southern region (Michel *et al*., 2006).

2.3.2 Aetiology and Pathogenesis of BTB

Mycobacterium bovis (M. bovis) is a bacterial pathogen of the *Mycobacterium tuberculosis* complex (Michel, 2002). Other members of this closely related mycobacteria complex include *M. tuberculosis, M. africanum and M. canettii. M. microti* and *M. pinnipedii* (Ayele *et al*., 2003). *M. bovis* causes a chronic infection in a wide range of mammalian host species, including humans (Jolles *et al*., 2005).

Bovine tuberculosis has been diagnosed in several domesticated cattle breeds throughout the majority of African countries, the number of outbreaks, cases and deaths do, however, vary between different regions. The incidence of *M. bovis* in both humans and cattle in industrialized countries has decreased dramatically as a result of proper animal tuberculosis control and elimination programmes and the pasteurization of milk (Cosivi *et al*., 1998). In developing countries, however, *M. bovis* infection in humans and cattle is widely distributed due to poor control measures and sporadic pasteurization of milk. The majority of people affected by BTB are young children and people with weakened immune systems (Cosivi *et al*., 1998). The disease has spread to wild bovids in Africa, with the African buffalo regarded as the main reservoir throughout the continent (Renwick *et al*., 2006).

Disease transmission between domestic animals and susceptible wildlife, and *vice versa*, is the highest when pasture or territory is communally shared (Renwick *et al*., 2006). The two main transmission routes of BTB include the alimentary and respiratory pathways. Respiratory transmission is the major cause of tuberculosis transmission in both human and cattle populations (Renwick *et al*., 2006).

Alimentary transmission may occur in one of three ways. Firstly, BTB transmission may take place *via* the excretion of mycobacteria in sputum, draining sinuses, ingestion of infected milk, faeces or urine of an infected animal, and the subsequent consumption of these contaminated materials by other animals. This route of transmission is common in grazing animals that share water points, salt supplementary licks, or animals that congregate at night for protection against predators. Secondly, alimentary transmission of BTB is associated with prey to predator transmissions. This route of infection involves the consumption of primary infected material by a susceptible animal. This route of transmission is especially important in the conservation of Africa's high profile carnivores (lion) that share their territory with infected prey species. Buffaloes are regarded as one of four preferential prey species of lions within the KNP. Frequent exposure of BTB infected buffaloes, consequently lead to the spatial spread of the disease within lion prides, especially in areas of high BTB prevalence in buffalo populations (Michel *et al*., 2006). Spread of the disease can also occur between lions within and among prides. The third pathway is where transmission takes place *via* the percutaneous route. This mode of infection has been suggested in the kudu, where contaminated thorns have damaged or scratched their delicate ear or facial skin (Renwick *et al*., 2006).

The spread of *M. bovis* within ruminants and large carnivores is a relatively slow process and clinical signs may take years to appear. Most infected animals are sub-clinically affected and only once advanced stages of the disease have been reached, disseminated lesions will appear. The severity of the disease is associated with the infectious dose, route of infection and immune robustness of the individual (Renwick *et al*., 2006). Clinical manifestations of BTB are similar in cattle and buffalo (De Vos *et al*., 2001). In bovines, lesions are found as tubercles, which vary in appearance, size, distribution and severity. Tubercles vary in size from microscopic to several centimetres in diameter. These tubercles may partially or fully replace the functional tissue of the affected organ. Spread of BTB in the African buffalo, mainly takes place *via* the aerosol route. Most buffaloes develop lesions within three to six months of infection. These lesions are mainly located in the

lymph nodes of the head, tonsils, lungs and thoracic regions and may spread to more distal sites. Lesions may further develop into necrosis, followed by cavitations and liquefaction. BTB is regarded as a slow, progressive disease of African buffaloes, mortality of adult buffaloes may, therefore, only occur three to five years after infection (Renwick *et al*., 2006).

2.3.3 Importance of BTB

BTB is an important zoonotic disease, with the ability to spread to humans *via* aerosol and/or the ingestion of raw, infected milk (Thoen *et al*., 2006). *Mycobacterium bovis* is classified as a Risk Group 3 pathogen (agents associated with serious human disease for which preventative or therapeutic interventions may be available) for public health. Details of Risk Group 3 pathogens can be viewed at Berkley lab, Lawrence Berkley National Laboratory website at <http://www.lbl.gov/ehs/biosafety/manual/html/AppxB.shtml>. Prevalence of the disease has been reduced in developed countries, but the complete eradication thereof has been hampered by disease reservoirs in wildlife species. The importance of tuberculosis in wildlife has increased, not only as a potential reservoir of infection for domestic animals, but also as a threat to important wildlife species (De Lisle *et al*., 2002). Once contact between infected wildlife species and domestic cattle have been made, infection with *M. bovis* may arise, such occurrences have been observed in North America, Great Britain and New Zealand (Michel, 2008). National and international trade is severely influenced by the diagnosis of BTB in a game species, due to animal movement restrictions and subsequent loss of revenue.

Individuals frequently exposed to either un-pasteurized milk or dairy products, or individuals frequently exposed to BTB infected livestock, are considered to be at greater risk of contracting the disease. This risk increases if individuals are placed under immunological stress, induced by an HIV infection (Michel *et al*., 2006). In 2003 in South Africa, an estimated 5.3 million individuals were living with HIV. Currently in South Africa, approximately 50 % of new human tuberculosis cases can be ascribed to prior infection with Human

immunodeficiency virus (HIV). With the current HIV - acquired immune deficiency syndrome (AIDS) epidemic, one should consider zoonotic tuberculosis a potential health risk in immuno-compromised people (Michel *et al*., 2006).

2.3.4 BTB control

South Africa has established BTB eradication programmes for cattle, while BTB control measures for wildlife is still under debate (Renwick *et al*., 2006). Ethical and ecological factors need to be taken into account in order to establish ideal control measures of BTB in free-ranging wildlife species. The choice of BTB control measure depends on the primary objectives of a particular ecosystem. In the KNP, for example, an obligation is made towards the protection of the host, pathogen infected species as well as to control the pathogen (Michel *et al*., 2006). No active BTB control measures have, however, been implemented in the KNP, but the disease is closely monitored with research conducted on the epidemiological determinants of the disease (Michel *et al*., 2006). For control programmes to be effective, a thorough understanding of the epidemiology of the disease in wildlife is required (De Lisle *et al*., 2002).

Possible containment, control and eradication strategies have been evaluated since the early 1990's. Along the western and southern boundary of KNP an electrified perimeter fence was originally erected to prevent animals with foot-and-mouth disease (FMD), to cross the border and infect livestock of the adjacent farming communities (Renwick *et al*., 2006). In HiP a capture-test and-slaughter scheme has been developed and applied to BTB infected African buffalo. This scheme can, however, not be applied in the KNP, due to the size of the individual buffalo herds, their home ranges and certain environmental characteristics associated with the KNP. A disease-free African buffalo breeding programme was established and has since ended in the KNP (Renwick *et al*., 2006).

Vaccination programmes in wildlife were initially implemented to decrease the rate of spread of BTB to domestic animals. Vaccination programmes have since been implemented on an experimental basis to protect valuable or endangered wildlife species and is regarded as the most common control measure applied to wildlife reservoirs. (De Lisle *et al*., 2002). The successful vaccination of wildlife species against BTB requires a safe, cost effective vaccine that is easy to deliver and requires a single administration (De Lisle *et al*., 2002). The only vaccine currently available for wildlife vaccination is the bacillus Calmette-Guerin (BCG) vaccine. BCG provides protection to cattle, deer, badgers and ferrets (Cross and Getz, 2006). A study to test the effectiveness of BCG on African buffalo populations was conducted recently. Vaccination trials revealed that the BCG vaccine did not provide sufficient protection (de Klerk *et al.,* 2010).

2.3.5 Species affected by BTB

Bovine tuberculosis (BTB) is a reality in wildlife and domestic animal populations which needs to be addressed. Population genetics and conservation biology can combine to assist in managing the disease as well as assist in conserving species that are threatened by the disease.

M. bovis is widely distributed in both wild and domestic animal populations. The disease is more prevalent in older animals, under nutritional or productive stress. The disease is most likely spread when wild and domestic species share the same pasture or territory. *M. bovis* has an extremely broad host range and has been diagnosed and reported in several wild and domestic species in southern Africa, some of which are indicated in Table 1.2 and Table 1.3 (Michel *et al*., 2006).

Table 1.2 Wild animal species infected with *M. bovis*
African buffalo *(Syncerus caffer)*
Lion *(Panthera leo)*
Leopard *(Panthera pardus)*
Cheetah *(Acinonyx jybatus)*
Large spotted genet *(Genetta trigrina)*
Hyena *(Crocuta crocuta)*
Honey badger *(Mellivora capensis)*
Kudu *(Tragelaphus strepsiceros)*
Eland *(Taurotragus oryx)*
Impala (*Aepyceros melampus)*
Warthog *(Phacochoerus porcus)*
Chacma baboon *(Papio ursinus)*
Bushpig *(Potamochoerus porcus)*

Table 1.3 Domestic species infected with *M. bovis*
Cattle breeds *(Bos primigenius)*
Goats *(Capra hircus)*
Sheep *(Ovis aries)*
Domestic pigs

All affected species are not equally susceptible to the disease, some are maintenance hosts, while others are spill-over or end hosts. Maintenance hosts of BTB allow the persistence of the infection in wildlife populations without recurring introduction from a known reservoir species and enable transmission of the pathogen between different species. African buffaloes act as reservoirs and maintenance hosts of the disease, maintaining the disease within the KNP (De Vos *et al*., 2001). Maintenance of the disease in buffaloes within the Park can be as a result of their relatively high numbers and the successful disease transmission between individuals, within the KNP (De Vos *et al*., 2001). Spill-over hosts only have a limited capacity to maintain BTB within their population in the absence of a persistent alternate source of infection, as provided by the maintenance host. Leopard and cheetah appear to be spill-over hosts as they cannot maintain the infection in the absence of an infected maintenance host within their system (Renwick *et al*., 2006).

Within the KNP, approximately 100 African buffalo herds, with an average herd size of approximately 244 individuals are distributed across 22 000 km^2 (Greyling, 2007). Disease lesion pattern development suggests that BTB transmission takes place *via* aerosol. Their social behaviour and dispersal characteristics make this gregarious species an ideal maintenance host in southern Africa (Michel *et al*., 2006). There is no sexual bias in disease susceptibility. There is, however, an age related increase in disease prevalence. BTB development and spread throughout a population is affected by several variables, some of which include, animal genetic resistance, nutritional status, seasonal fluctuations, environmental variables and body condition (Cross and Getz, 2006).

2.4 Bovine microsatellite markers

The Bovini tribe forms part of the mammalian ungulate family Bovidae, which has played an important role throughout history for its contribution to several agricultural, economic, cultural and religious sections. Within this Bovini tribe, there are typically four extant genera, namely the *Bos, Bison, Bubalus* and *Syncerus*. Microsatellite data indicate that the *Syncerus* genera have emerged as the most divergent group (Ritz *et al*., 2000). To establish the phylogenetic relationships among the various Bovini genera, Ritz *et al*. applied microsatellite markers. These markers provide a medium to determine genetic linkage, population structure, mating system analysis, as well as the reconstruction of phylogenetic relationships among populations (Ritz *et al*., 2000).

Population bottlenecks and habitat fragmentation have influenced the dynamics and genetic diversity of the African buffalo population (Van Hooft *et al*., 2000). It has thus been important to establish the current status and genetic diversity of buffalo populations in Africa. Specific microsatellite markers have not yet been developed for the African buffalo. A large number of microsatellite markers have, however, been characterized in domestic cattle which amplify successfully and show a high level of allelic diversity in

the African buffalo and can be applied to population genetic studies related to the African buffalo directly (Van Hooft *et al.*, 1999).

2.5 Inter- and intra-population genetic structure and relatedness evaluation

In population genetics, relatedness (similarity by descent) can be expressed as the coefficient of relatedness (of which several types of coefficients are available, depending on the algorithm used). Concepts associated with genetic relatedness and the measurement thereof among individuals form the basis of population genetics. Several population genetic fields such as genetic analysis, conservation genetics, evolutionary biology and artificial selection, consider the concept of relatedness as an important part of the study (Oliehoek et al., 2006).

The social organization and behaviour of a population play a crucial role in the spread and distribution of disease amongst individuals. A recent study conducted on white-tailed deer *(Odocoileus virginianus)* in Michigan in the United States, incorporated molecular genetic markers to determine relatedness among individuals and test whether BTB infected deer are more closely related than non-infected deer (Blanchong *et al.*, 2007). Samples were collected from known locations from BTB infected deer, which were genotyped to reveal their genealogical relationships. Several non-infected deer were also sampled and genotyped. Their work suggests that contact among related deer, within a particular population or social group, contributed to BTB transmission. Contact between relatives within social groups, therefore, form an important factor of BTB transmission among deer, as a high level of relatedness among BTB infected deer, within a close proximity to one another was observed. It can therefore be concluded that individuals that were more closely related had a higher disease prevalence (Blanchong *et al.*, 2007).

2.6 Tools applied in Population Genetic studies

2.6.1 Deoxyribonucleic acid (DNA) marker technology (Microsatellites)

Microsatellite markers, also known as short tandem repeats (STR) are short, simple repeat sequences, randomly dispersed throughout the entire genome. Sequences vary in length and configuration. The di-nucleotide repeats most commonly found are (CA)n or (TG)n repeats, where n represents the number of repeats. These base pair sequences are generally between two and six nucleotides in length. Details of microsatellite markers can be viewed at the University of Vermont website at <http://www.uvm.edu/~cgep/Education/microsatellite.html>.

These short sequence repeats are used as location markers during genome mapping due to their abundance, high polymorphic nature and the fact that they are found in non-coding regions of the genome (Fairbanks and Anderson, 1999). Microsatellite lengths vary, based on slippage and mismatch repair during replication. The exact length of each allele is determined by sequencing and fragment analyses. Electrophoresis of fragments amplified by polymerase chain reaction (PCR) allows DNA products to be visualized. Details of microsatellite markers can be viewed at the University of Wyoming website at <http://www.uwyo.edu/dbmcd/popecol/Maylects/PopGenGloss.html>.

Microsatellites have several beneficial characteristics which make them ideal for population genetic studies. Microsatellites can easily be amplified *via* the polymerase chain reaction (PCR), therefore, only small amounts of starting material is required and samples that have been collected in a non-invasive manner or partially degraded samples can be analyzed. Analysis of these samples is also open to automation (Greyling, 2007). Microsatellites are highly polymorphic due to their high mutation rates. These mutation rates result in numerous alleles which are present in most populations, which suggest that significant variation and genetic relatedness can be observed and identified *via* microsatellite analysis (Greyling, 2007). A few drawbacks of

microsatellite markers in population genetic studies include the high initial development costs, stutter bands which may develop and complicate scoring, heterozygotes which may be misclassified as homozygotes when null alleles appear and the lack of information available regarding their mutational processes (Greyling, 2007).

2.6.2 Polymerase chain reaction (PCR)

PCR is a technique used to amplify a specific DNA segment in order to selectively increase the amount of target DNA to detectable levels using a mixture of specific primers, dNTP building blocks and *taq* polymerase enzyme. PCR has revolutionised the field of molecular biology and genetics, and is central to numerous applications, some of which include pathogen diagnosis, typing genetic markers, detecting point mutations, genomic DNA cloning, DNA sequencing and *in vitro* mutagenesis (Hartl and Clark, 2007). PCR is of great importance in population genetic studies, as it directly determines the amount of nucleotide sequence variation within a natural population. Furthermore, PCR is a useful technique as it generates large quantities of a specific DNA sequence (Hartl and Clark, 2007).

Multiplex PCR is a form of PCR where more than one locus is simultaneously amplified within the same reaction. This method provides a rapid, cost effective, convenient manner for screening of samples in both clinical and research laboratories. Multiplex PCR enables one to perform large scale genotyping with specific applications, including gene expression, whole genome sequencing, paternity evaluation and forensic analysis. A specific protocol should be followed during the PCR multiplexing procedure. The PCR multiplex procedure is, however technically demanding. Several undesirable artefacts may be generated during multiplexing, of which allelic dropout (dropped alleles/false alleles) is one of the most frequently encountered problems (Greyling, 2007).

To obtain the best PCR performance within a system, optimization and control of the temperature, cycle times and reaction component concentrations is

required. Optimal PCR conditions vary between different assays, as it is influenced by several factors. A simple block titration can be designed to optimise the PCR, with respect to the template, magnesium concentrations, primer concentrations, annealing temperatures, enzyme concentration, optimal reaction pH and any other variable that may influence the reaction.

2.6.3 Software for Population Genetic studies

High-throughput techniques and new statistical methods have made the estimation of relatedness associated with genotyping of microsatellite markers more common and are therefore more frequently used. Several population genetics computer programs have been designed to calculate values reflecting relatedness, some of which include, FSTAT, GENAIEX and Spagedi.

FSTAT version 2.9.3.2 is a computer based program used to estimate and test gene diversities and differentiation statistics from co-dominant genetic markers. FSTAT also computes Nei's (1973) estimators of gene diversities as well as Weir and Cockerham's (1984) estimators of gene diversities. F-statistics and Hamilton's (1971) relatedness estimates can also determined using FSTAT.

GENAIEX version 6.2, a population genetic analysis program within Microsoft Excel allows for the analysis of co-dominant markers, haploid data and binary genetic data (Peakall, R. and Smouse P.E 2006). For co-dominant data, GENAIEX can calculate standard population genetic statistics, such as the number of alleles, number of private alleles, allele frequencies as well as the expected and observed heterozygosities. Frequency based analyses can also be conducted. This program is particularly useful for Random Amplification of Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) data. GENAIEX provides direct export data to other genetic software formats, such as CERVUS, ARLEQUIN, GENEPOP and POPGENE. It also provides indirect export to FSTAT and GENEPOP (Peakall and Smouse, 2006).

GENEPOP version 4.0 is a computer software program which implements several traditional techniques as well as more recent developments. Important functions of this programme include exact tests for Hardy-Weinberg equilibrium, population differentiation and genotypic disequilibrium among pairs of loci. F-statistics, null allele frequencies and the allele sizes based on statistics for microsatellites are also estimated with GENEPOP 4.0. From pairwise comparisons of individuals and populations, isolation by distance analyses can also be performed.

2.7 Population genetic concepts

2.7.1 Population size (N) and Effective population size (Ne)

A population's size in the context of population genetics, depends on how genetic variation changes over time. *N* represents the actual number of individuals in a population. The effective population size *(Ne)* is the genetic size of a population and represents the number of individuals that actually contribute gametes to the next generation (Hamilton, 2009) or alternatively the effective population size represents the number of individuals in a theoretical population with the same amount of genetic drift as the actual population. Three approaches are used to calculate the effective population size (*Ne)*, these include inbreeding, variance and eigenvalue (Hendrick, 2000).

2.7.2 Allele frequency, allelic richness

Allele frequency is the proportion of alleles of a particular gene, while the genotype frequency is the proportion of genotypes in a population and therefore the distribution of alleles among genotypes in a population (Hamilton, 2009). Allelic richness (A), also known as allelic diversity, represents the mean number of alleles per locus. A disadvantage of using allelic richness (A) is that it is highly dependant on population size *(N)*, which makes comparisons between samples of different sizes difficult. It should therefore be standardized to adapt to different sample sizes via the rarefaction technique developed by Hurlbert (1971). Rarefaction provides a means to

compare allelic richness in species and samples of uneven sizes (Petit *et al.*, 1998). Private alleles are those unique alleles, found only in one sub-population (Hartl and Clark, 2007).

2.7.3 Genetic distance

Standard genetic distance *(D)* is a population genetic parameter often applied during evolutionary studies and classification of species. Standard genetic distance can either be defined as an average value across all loci examined, or it can be defined at each locus. Genetic distance is used to determine the amount of variation shared among groups. It assists in constructing relationships among animal groups and therefore, measures the level of differentiation among closely related groups (Hendrick, 2000). Several variations of genetic distance are known, which depend on the type of molecular data applied. The three most commonly used measures of distance include Nei's (1972) genetic distance, Weir and Cockerham's (1984) genetic distance and Cavalli-Sforza chord measure.

2.7.4 Polymorphism and the polymorphic information content (PIC)

Polymorphism represents the genetic variation within populations. It is the occurrence of two or more alleles with high frequency levels, within the same population, at the same locus (Hendrick, 2000). A marker is said to be polymorphic when it has at least two alleles (Shele *et al.*, 2000). The degree of polymorphism can be measured by two distinct values which include heterozygosity and the PIC value. PIC is used to assess marker genotype data (Shele *et al.*, 2000). The PIC value is often used as a measure to establish the genetic informativeness of genetic markers (Guo and Elston, 1998).

2.7.5 Fixation index

Wright's F-statistics determine the degree to which populations are sub-divided (Hartl and Clark, 2007). The fixation index *(F)* is a measure of genetic

structure, developed by Sewall Wright (1969). It represents a set of tools to divide heterozygote deficiency into 'within population' and 'among population' components. The fixation index *(F)* therefore assists in measuring the level of structure in samples and natural populations (Hartl and Clark, 2007). Specific fixation index parameters include *Fst, Fit, Fis.* *Fst* measures the difference between the average heterozygosity *(He)* of subpopulations and the heterozygosity *(He)* of the total population. *Fst* therefore measures the heterozygote deficit among populations. *Fit* measures variation in individuals, relative to variation in the total set of sub-populations. *Fit* is a measure seldom used in population genetics. The inbreeding coefficient *(Fis)* compares the average observed heterozygosities *(Ho)* in each subpopulation and expected *He* of individuals for all sub-populations. *Fis* measures the heterozygote deficit within populations (Hamilton, 2009).

2.7.6 Hardy-Weinberg Equilibrium (HWE)

The Hardy-Weinberg principle is one of the most important concepts in population genetics. It provides certain genotypic pattern expectations within a population, deviations from the predicted genotypic pattern can provide important insights into genetic and evolutionary changes of a population. The Hardy Weinberg model relates allele frequencies to genotype frequencies and represents a null model, it is, therefore, a test hypothesis. It is not meant to be an exact description of an actual population (Fairbanks and Anderson, 1999). Hardy-Weinberg Equilibrium (HWE) is a situation where allele and genotype frequencies remain constant from one generation to the next if certain assumptions are met. These eight unrealistic assumptions include random mating, no genetic migration, no genetic selection, diploid organisms, sexual reproduction, generations are non-overlapping, mutation is negligible and may be ignored and genetic drift is also negligible.

2.7.7 Gene flow and migration

Genetic migration (often referred to as gene flow), is the permanent movement of genes, individuals or groups of individuals from one population

to another (Slatkin, 1987). Genetic migration sets a limit as to how much genetic divergence can take place and can either restore genetic variation among isolated populations or reduce genetic variation if it persists (Hartl and Clark, 2007). The extent of gene flow among populations determines their genetic drift potential. The number of migrants *(Nm)* represents the total number of migrants that enter a sub-population in each generation and is an important factor in population genetics as it influences the dynamics of a population over time (Hartl and Clark, 2007).

2.8 Research problem (hypothesis)

- *M. bovis* infected buffaloes are more closely related to each other than to uninfected buffaloes, in the same population or prevalence group.
- Buffaloes infected with the same strain of BTB are more closely related to each other than to uninfected buffaloes or those infected with a different strain within a single population, i.e. not separated by time and space.

2.9 Research objectives

Specific objectives were formulated to address the above mentioned research problem (hypothesis), these include:

- Extraction of DNA and genotyping of animals from incurred samples which were previously collected from buffaloes in the Kruger National Park (KNP) and to compile a comprehensive database of the genotypes.
- Evaluation of the genotypic data for its genetic informativeness.
- Determine the intra-and inter population relationships of the animals to evaluate whether animals affected with the same strain of BTB are more closely related than those that are affected with a different strain or not affected.

- To produce recommendations for the monitoring and management of animals from buffalo populations based on the results of the relatedness evaluation and subsequent better quantification of within herd disease clustering.

2.10 Significance of the study

The outcomes of this study have both practical and theoretical significance. These include the establishment of a comprehensive reference database of genotypes of African buffalo within the KNP and the determination of genetic relationships between and within sampled buffalo populations. Data generated will assist with the future sampling and management strategy of buffaloes and other wild bovine species (for instance establishing the season of sampling and the amount of samples to collect). Results will improve the understanding of the transmission and maintenance of BTB within the KNP buffaloes. Support for the hypothesis could indicate a genetic basis for BTB resistance/susceptibility in buffaloes and provide a link to further investigation of this. The results will provide direct clues regarding the significance of behaviour (close contact) on the risk of infection.

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Chapter 2

Population and group evaluation of African buffalo *(Syncerus caffer)* sampled throughout the Kruger National Park

Abstract

Historical records indicate that during the 19th century approximately 3 million African buffaloes inhabited almost the whole of sub-Saharan Africa. Several factors such as disease, habitat fragmentation, over-hunting and drought reduced the buffalo population size to approximately 400 000 by 1990. Disease has played an important role in the status and current dynamics of African buffalo populations across the African continent. The African buffalo are host to several sub-acute diseases, some of which include bovine tuberculosis (BTB), foot-and-mouth disease (FMD) and corridor disease (CD) (Greyling, 2007). BTB is an airborne, multi-host pathogen, which is currently re-emerging worldwide, in both wildlife and livestock (Renwick *et al*., 2006).

Approximately 190, mainly lymph tissue samples were selected from the collection at the OVI, based on the BTB culture status of the sample. BTB positive as well as BTB negative samples were randomly selected from 43 different locations, primarily in the KNP.

Genomic amplification and characterization was based on DNA extraction from the selected samples and multiplex PCR conditions. Data analysis was based on fragment and statistical analysis of the samples using computer based programmes. Molecular genetic markers (microsatellites) are ideal tools to generate genetic data for further analysis with specific population genetic computer programmes. Twelve microsatellite markers were used to evaluate 181 samples from the KNP and nine samples from four different (outlier) areas in South Africa. Specific population genetic parameters (which

include allele frequencies, private alleles, observed and expected heterozygosities and polymorphism) revealed information based on the intra-population relationships as well as on the inter-population relationships of African buffalo populations dispersed throughout the KNP. These genetic parameters were calculated at a population level as well as at a prevalence group level.

Several factors influenced the outcome of our results, some of which include sample size, social organization and environmental factors.

Results presented in this chapter provide baseline information of the genetic status of the African buffalo in the KNP, based on a relatively small sample set.

1. Introduction

A range of 12 molecular markers and different statistical programmes (MS Toolkit, GENAlEX version 6.2 (Peakall and Smouse, 2006), FSTAT version 2.9.3 (Goudet, 2001), GENEPOP version 4.0 and POPGENE (POPGEN 32)) were employed to evaluate specific genetic indices associated with the African buffalo samples. Results are presented in two different sections. Section one provides genetic parameter information on a population level, while section two provides genetic parameter information on a prevalence group level. These prevalence groups were identified based on their sample location and the BTB culture status associated with that area (Michel, A. July 2010, personal communication). The genetic variation associated with each of these groups was evaluated.

2. Materials and Methods

2.1 Animals and Samples

African buffalo samples used in this study were provided by the Onderstepoort Veterinary Institute of the Agricultural Research Council (ARC-

OVI) - South Africa. Buffalo groups and populations in the different regions of the KNP had been mostly randomly selected for sampling over a time interval of 11 years within the KNP, for BTB disease monitoring, research and management purposes from 1996 to 2007 by the South African National Parks. Samples collected from Black Heron, Cork, Dzombo, Letaba, Kumana Dam, Macetse, Malahlapanga, Maloponyane, Mpongolo, Nyunyani, Shawu, Stamp-en-Stoot and Woodlands were sampled using live sampling.

Approximately 190, mainly lymph nodes (negative lymph nodes represent a negative animal), were selected from the collection at the OVI, based on the BTB status, which was determined by culture at the time. BTB positive as well as BTB negative samples were selected. No duplicate samples were used. Samples had been collected from 43 different locations, primarily in the KNP. Buffalo samples collected from Hoedspruit, Klaserie and Kimberley regions, as well as samples from the Hluhluwe-iMfolozi Park (HiP) were included as outliers for reference purposes. Table 2.1 represents population data referred to in this chapter and throughout the thesis. Each population was assigned a specific number. These numbers are referred to throughout the thesis. Throughout the thesis, the term population has been used to describe a small collection of buffaloes, sampled from the same location (other literature sources describe this small collection of buffaloes as a herd). The year in which samples were collected as well as the number of samples collected at each location site are also provided. The prevalence group regions represent the region within the KNP that samples were collected. These regions relate to the BTB prevalence within a particular area as discussed in section two of this chapter. Figure 2.1 is a geographical representation of the sampled collection sites within the KNP. Each red dot represents a sampled African buffalo population within the KNP. The size of the dot may vary, based on the number of samples collected at a particular location.

Table 2.1 Population numbers, names, year of sample collection, number of samples collected in each year and prevalence group to which each population is associated to

Population number	Population name	Year of sample Collection	Number of samples Collected	Prevalence group Region
1	Mahlanganzwane Dam	1996	4	South
2	Duke	1996	4	South
3	Mpanamana Dam	1996	1	South
4	Langtoon Dam	1996	1	North
5	Kostini	1996	1	North
6	Manzimhlope	1998	6	Central
7	Rietpan	1998	7	Central
8	Silolweni Dam	1998	3	Central
9	Mafortini	1998	7	Central
10	Sonop windmill	1998	6	Central
11	Gudzane Dam	1998	6	Central
12	Letaba	1998	3	North
		2000	3	
13	Mhlangazwane	1998	1	South
14	Macetse	2000	3	North
15	Stamp en Stoot	2000	1	North
16	Shawu	2000	1	North
17	Woodlands	2000	1	North

18	Black Heron	2003	1	North
19	Maloponyane	2003	1	North
20	Nyunyani	2007	2	North
21	Dzombo	2007	1	North
22	Mpongolo	2007	2	North
23	Malahlapanga	2007	1	North
24	Byamiti spruit	2005	7	South
25	Nwatimhiri	2005	15	South
26	Nyamundwa	2005	4	South
27	Lukimbi Lodge	2005	6	South
28	Mpanamane	2005	12	South
29	Zambhala	2005	8	South
30	Blinkwater windmill	2005	14	South
31	Satara	2001	2	Central
32	Lower Sabie	2003	2	South
		2004	6	
33	Cork	1999	1	South
34	Lupisi Gate	1999	1	South
35	MtemushaMhlandanyati	1996	3	South
36	Kumana Dam	1997	1	Central
37	Hoedspruit	2004	1	Outlier
		2007	2	
38	Klaserie	2001	1	Outlier

39	Kimberley	2007	1	Outlier
40	HiP	1996	2	Outlier
		2003	2	
41	Nzikazi spruit	2005	8	South
42	Napi Boulder	2005	9	South
43	Stolznek Dam	2005	15	South

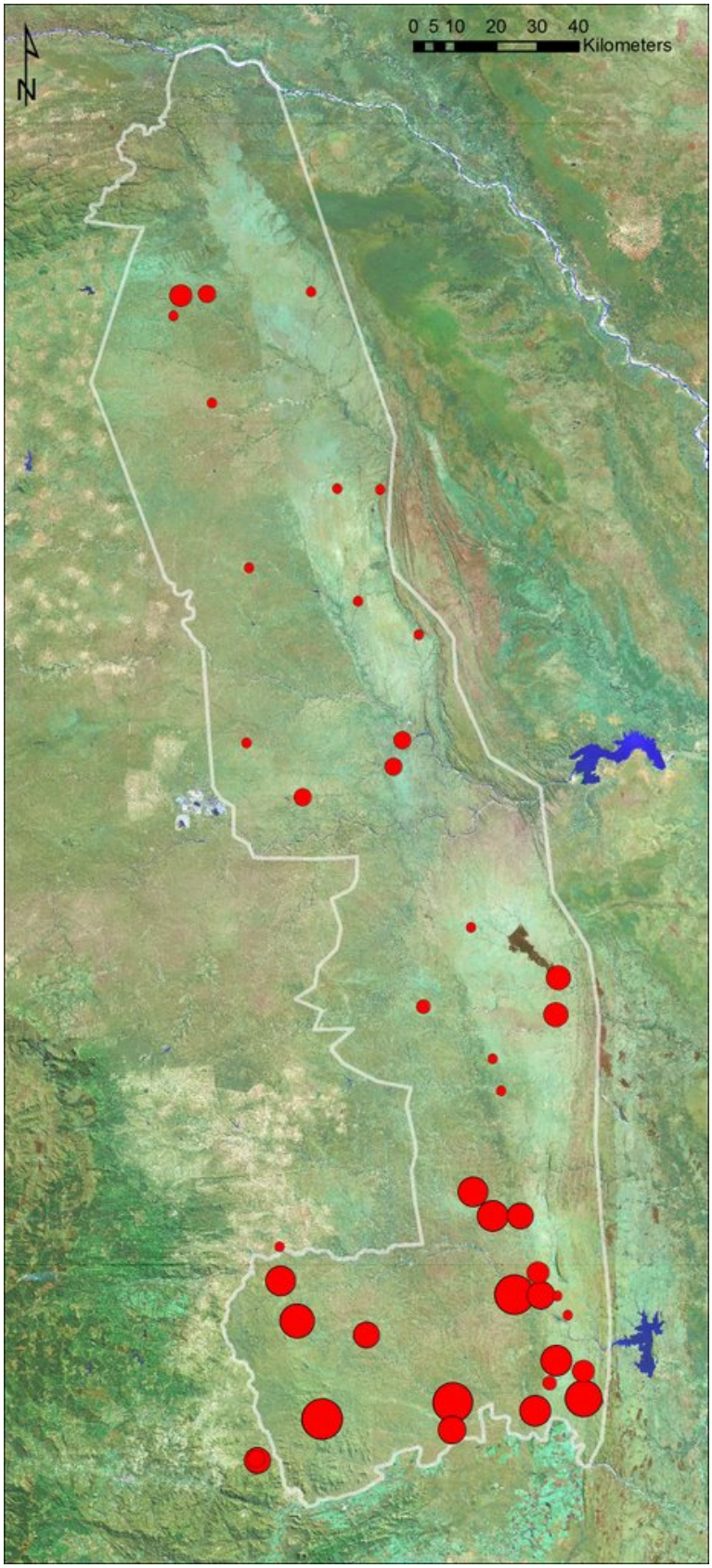

Fig 2.1 Geographical map representing the sample collection sites in the KNP
(The size of the dot may vary, based on the number of samples collected at a particular location. The more samples collected at a site, the bigger the dot.)

3. Genomic amplification and characterization

3.1 DNA extraction

Lymph tissue samples used during the study had been stored at – 20 ℃ at the ARC-OVI before DNA extraction. DNA extraction from BTB infected and uninfected samples were done at the ARC-OVI. Samples were handled in a biosecurity laboratory (in order to prevent transmission of the disease), using protective gear and appropriate procedures due to the zoonotic potential of the material. A Puregene Kit (Gentra Systems) was used to accurately extract and isolate DNA from the samples, which was done according to the manufacturer's instructions. DNA extracts were diluted to between 20 - 50 ng/µl genomic DNA and stored at the Veterinary Genetics Laboratory (VGL), Onderstepoort on behalf of SANparks.

3.2 Multiplex PCR conditions

A panel of 12 microsatellite markers was selected according to Greyling *et al.,* 2008, based on the level of polymorphism and consistency in amplification in the African buffalo and the ability to co-amplify in multiplex PCR reactions. The primers were co-amplified in three multiplexes (Table 2.2).

Table 2.2 Multiplex reactions with associated primers

Multiplex	Primers
Multiplex 1 (M 1):	TGLA227; BM1824; ETH10; SPS115
Multiplex 2 (M 2):	INRA006; INRA128; TGLA159; BM3205
Multiplex 3 (M 3):	CSSM19; BM719; ILSTS026; TGLA263

Reaction conditions were optimized according to (Greyling *et al*., 2008). Table 2.3 represents a summary of all primers used, including fluorescent labels, primer sequences and primer concentrations (nM).

A total reaction volume of 20 µl was used. The PCR multiplex contained 25 units *Taq* DNA polymerase (Applied Biosystems), 1.2 µl (25mM) $MgCl_2$

(Applied Biosystems), 2 µl (1 X) Supertherm Gold buffer (Applied Biosystems), 0.5 µl (0.5 mM) dNTP's (Thermo Fisher Scientific), 0.5 µl (50 - 100 ng) genomic DNA, primer mix and water.

PCR was performed on a Veriti 96 Well Thermal Cycler (Applied Biosystems) using the following parameters: enzyme activation at 94℃ for 10 minutes, followed by 35 cycles of denaturation at 94℃ for 45 seconds, annealing at 58℃ for 2 minutes and extension at 72℃ for 2.5 minutes and the final extension at 72 ℃ for 1 hour.

Following PCR, 1 µl PCR product was added to 10 µl formamide (Applied Biosystems) and 0.25 µl Genescan Liz 500 size standard (Applied Biosystems) and denatured at 94 ℃ for 2 minutes. Samples were then loaded as a single injection on an ABI 3130 xl Genetic Analyzer (Applied Biosystems).

Table 2.3 Summary of the 12 microsatellite markers used to profile African buffalo *S. caffer caffer* detailing the label, primer sequence and concentrations used for each multiplex

Msat ID	Label	Multi-plex	Forward primer sequence	Reverse primer sequence	Primer concentrations (nM)
BM1824	Fam	M1	gAg CAA ggT gTT TTT CCA ATC	CAT TCT CCA ACT gCT TCC TTg	6.4
ETH10	Ned	M1	gTT CAg gAC Tgg CCC TgC TAA CA	CCT CCA gCC CAC TTT CTC TTC TC	6
SPS115	Pet	M1	AAA gTg ACA CAA CAg CTT CTC Cag	AAC gAg TgT CCT AgT TTg gCT gTg	18
TGLA227	Fam	M1	CgA ATT CCA AAT CTg TTA ATT TgC T	ACA gAC AgA AAC TCA ATg AAA gCA	16
INRA006	Fam	M2	Agg AAT ATC TgT ATC AAC CTC AgT C	CTg AgC Tgg ggT ggg AgC TAT AAA TA	12.8
INRA128	Ned	M2	TAA gCA CCg CAC AgC AgA TgC	AgA CTA gTC Agg CTT CCT AC	12.8
TGLA159	Vic	M2	gCA TCC Agg gAA CAA ATT ACA AAC	TTT ATT TCg AAT CTC TTg AgT ACA g	18
BM3205	Pet	M2	TCT TgC TTC CTT CCA AAT CTC	TgC CCT TAT TTT AAC AgT CTg C	18
CSSM19	Fam	M3	TTg TCA gCA ACT TCT TgT ATC TTT	TgT TTT Aag CCA CCC AAT TAT TTg	14
BM719	Ned	M3	TTC TgC AAA Tgg gCT AgA gg	CAC ACC CTA gTT TgT AAG Cag C	11
ILSTS026	Pet	M3	CTg AAT Tgg CTC CAA Agg CC	AAA CAg AAg TCC Agg gCT Gc	17.6
TGLA263	Pet	M3	CAA gTg CTg gAT ACT ATC TgA gCA	TTA AAg CAT CCT CAC CTA TAT ATg C	13

4. Data analysis

4.1 Fragment analysis through capillary gel electrophoresis

Data generated by the 3130xl Genetic Analyzer was analyzed using STRand software to produce the genotypes used for further population analysis. (Toonen and Hughes, 2001) <http://www.vgl.ucdavis.edu/STRand>

4.2 Statistical data analysis

Several population genetic programmes were used to evaluate the data, these included MS Toolkit, GENAlEX version 6.2 (Peakall and Smouse, 2006), FSTAT version 2.9.3 (Goudet, 2001), GENEPOP version 4.0 and POPGENE (POPGEN 32).

Statistical analysis of the data was conducted on a population level as well as on a prevalence group level. Population size estimates were calculated with GENAlEX ver 6.2. Allele frequencies were determined with GENAlEX ver 6.2 as well as MS Toolkit. The number of private alleles associated with each population was calculated with GENAlEX ver 6.2. The level of gene diversity was established with FSTAT ver 2.9.3. Unbiased heterozygosity, observed heterozygosity and number of alleles were calculated with MS Toolkit. The level of polymorphism was determined with GENAlEX ver 6.2. F-statistics were calculated with FSTAT ver 2.9.3 as well as POPGENE (POPGEN 32). Hardy-Weinberg equilibrium (HWE) tests were calculated with GENAlEX ver 6.2 as well as with GENEPOP ver 4.0. The level of migration (gene flow) was established with GENEPOP ver 4.0.

5. Results and Discussion

Section one

5.1 Population level investigation

A total of 181 samples (individuals) distributed in 39 populations within the KNP and 9 samples (individuals) distributed in four outlier populations (HiP, Hoedspruit, Kimberley and the Klaserie) were genotyped. Several indices of genetic variation were calculated between individuals, including allele patterns, allele frequencies, number of private alleles, observed heterozygosities *(Ho)*, expected heterozygosities *(He)*, level of polymorphism, F-statistics and Hardy-Weinberg tests. Genetic variation and differentiation between all 43 populations were performed with population genetic software which included MS Toolkit, GENAlEX version 6.2, FSTAT version 2.9.3, GENEPOP version 4.0 and POPGENE (POPGEN 32).

Greyling *et al* (2008) designed an automated profiling system using a panel of seventeen autosomal bovine microsatellite markers. This development provides a high-throughput, high-resolution and cost-effective typing system, which serves as an ideal method for forensic and population genetic analyses. Their work confirmed their method as a suitable approach for forensic and population genetic studies. It also provides a valuable alternative typing method for Bonsmara cattle and confirms the probability of a broader applicability to other members of the Bovini tribe in Africa (Greyling *et al*., 2008).

5.1.1 Intra – population analysis

Intra-population analysis provides population genetic parameter estimates for each of the 43 sampled populations individually.

5.1.1.1 Allele frequencies

From the 43 populations, 190 samples and 12 loci investigated during the course of this study, 143 alleles were identified.

Allele frequencies represent the frequency of the different alleles amplified of a particular locus within or among a population(s). Allele frequencies were calculated by population, for each allele and for each locus, using GENAIEX ver 6.2 (Appendix 2.1). Appendix 2.1 represents the alleles that occur with the highest frequencies within each population, at each locus. Six populations were compared at a time and presented within the Appendix. These frequencies are represented as a percentage value in brackets.

5.1.1.2 Private alleles

Twenty-two exclusive (private) alleles were identified from a total of 143 alleles throughout the entire dataset and are associated with 10 populations and 13 individuals. These alleles occur in different populations with varying frequencies, as shown in Table 2.4. These populations include population 6 (Manzimhlope), population 12 (Letaba), population 13 (Malangazwane), population 24 (Byamiti Spruit), population 25 (Nwatimhiri), population 28 (Mpanamane), population 29 (Zanbhala), population 41 (Nzikazi Spruit), population 42 (Napi Boulder) and an outlier population, population 39 (Kimberley). Population 42 has the highest number of private alleles at 7 and locus SPS 115 has the highest number of private alleles at 4.

Table 2.4 Summary of private alleles at specific loci measured within different populations

Population number	Locus	Allele	*Freq
6	TGLA263	188	0.167
12	CSSM19	158	0.083
13	INRA128	115	0.500
24	SPS115	245	0.100
25	BM1824	173	0.033
	BM3205	220	0.033
	CSSM19	128	0.033
	INRA128	164	0.036
	SPS115	243	0.056
28	INRA128	175	0.042
29	INRA006	107	0.063
39	INRA128	113	0.500
	INRA006	168	0.500
	SPS115	247	0.500
41	ILSTS026	165	0.071
	TGLA263	108	0.063
42	BM1824	182	0.056
	BM1824	188	0.056
	ETH10	209	0.056
	ETH10	213	0.056
	TGLA227	77	0.056
	TGLA227	79	0.056

***Freq = Frequency of private alleles within population**

5.1.1.3 Population genetic variation

Population statistics for each of the 43 populations were determined across all 12 loci with MS Toolkit (Table 2.5). These estimates include the expected or unbiased heterozygosity, observed heterozygosity and number of alleles within each group with standard deviations. The observed heterozygosity represents the sum of frequencies of all the heterozygotes that have been observed within the population across all loci. The expected heterozygosity (also known as genetic diversity) represents the average expected

heterozygosity across all loci associated with a particular population. Most populations have a moderate level of heterozygosity. Each of these populations, therefore, has moderate genetic diversity. Four populations (population 18, 21, 34 and 36) do, however, have low heterozygosity levels (values below 0.500) and are genetically less diverse. These populations with low levels of heterozygosity have a small sample size (only one individual in each population and therefore a single sample) which may influence the level of heterozygosity.

Table 2.5 Population parameters including sample size, number of loci typed, unbiased heterozygosity, observed heterozygosity and the number of alleles

Population number	Sample size	Loci typed	[1]Exp Hz	Exp Hz SD	[2]Obs Hz	Obs Hz SD	[3]Mean no of alleles	Mean no of alleles SD
1	4	12	0.6310	0.0831	0.5625	0.0716	3.75	1.86
2	4	12	0.6690	0.0632	0.6042	0.0773	3.33	1.15
3	1	12	0.5000	0.1508	0.5000	0.1443	1.50	0.52
4	1	11	0.7273	0.1408	0.7273	0.1343	1.73	0.47
5	1	11	0.6364	0.1521	0.6364	0.1450	1.64	0.50
6	6	12	0.6552	0.0858	0.5778	0.0599	4.50	2.11
7	7	12	0.6944	0.0712	0.6242	0.0571	4.67	2.15
8	3	12	0.7861	0.0793	0.6528	0.0829	3.67	1.44
9	7	12	0.7654	0.0518	0.6540	0.0546	5.42	1.93
10	6	12	0.7340	0.0790	0.6250	0.0587	5.42	2.35
11	6	12	0.7448	0.0585	0.7194	0.0549	4.75	1.54
12	6	12	0.6662	0.0707	0.5889	0.0592	4.92	1.93
13	1	12	0.6667	0.1421	0.6667	0.1361	1.67	0.49
14	3	12	0.7167	0.0770	0.6111	0.0824	3.50	1.17
15	1	12	0.6667	0.1421	0.6667	0.1361	1.67	0.49
16	1	12	0.7500	0.1306	0.7500	0.1250	1.75	0.45
17	1	11	0.7273	0.1408	0.7273	0.1343	1.73	0.47

18	1	12	0.4167	0.1486	0.4167	0.1423	1.42	0.51
19	1	12	0.5833	0.1486	0.5833	0.1423	1.58	0.51
20	2	12	0.7083	0.0798	0.6667	0.0983	2.50	0.80
21	1	12	0.3333	0.1421	0.3333	0.1361	1.33	0.49
22	2	12	0.7083	0.1028	0.6250	0.0988	2.83	1.03
23	1	12	0.8333	0.1124	0.8333	0.1076	1.83	0.39
24	7	12	0.6988	0.0585	0.5766	0.0559	4.75	1.91
25	15	12	0.7511	0.0452	0.6401	0.0370	7.67	3.03
26	4	12	0.6873	0.0720	0.5694	0.0738	3.83	1.59
27	6	12	0.6926	0.0580	0.6944	0.0551	4.83	1.34
28	12	12	0.6923	0.0693	0.6313	0.0418	5.83	2.37
29	8	12	0.7386	0.0480	0.6200	0.0514	5.58	1.93
30	14	12	0.7366	0.0603	0.6303	0.0383	7.00	2.80
31	2	12	0.7222	0.0829	0.5833	0.1051	2.42	0.79
32	8	12	0.6681	0.0594	0.6473	0.0501	4.83	1.59
33	1	12	0.5000	0.1508	0.5000	0.1443	1.50	0.52
34	1	11	0.4545	0.1575	0.4545	0.1501	1.45	0.52
35	3	12	0.7250	0.0768	0.5556	0.0852	3.25	1.14
36	1	12	0.4167	0.1486	0.4167	0.1423	1.42	0.51
37	3	12	0.7000	0.0882	0.6667	0.0797	3.42	1.16
38	1	10	0.9000	0.1000	0.9000	0.0949	1.90	0.32
39	1	12	0.5833	0.1486	0.5833	0.1423	1.58	0.51
40	4	12	0.7565	0.0376	0.6042	0.0721	4.00	1.28

41	8	12	0.6852	0.0661	0.5818	0.0517	5.50	1.88
42	9	12	0.6702	0.0747	0.5911	0.0489	5.25	1.86
43	15	12	0.7362	0.0297	0.6261	0.0370	6.33	2.06

[1]**Exp Hz = Expected heterozygostity and their standard deviation**

[2]**Obs Hz = Observed heterozygosity and their Standard deviation**

[3]**Mean number of alleles represents the mean number of alleles within each population**

The level of polymorphism among the loci ranged between 0 and 100 %. Table 2.6 represents the percentage polymorphic loci within each population as well as the mean percentage polymorphic loci and its standard error.

Within the 43 populations, 16 populations are highly polymorphic with percentages of 100 %. These highly polymorphic populations include populations 2, 7, 9, 11, 24, 25, 26, 27, 28, 29, 30, 32, 40, 41, 42 and 43. Within these populations, high genetic diversity is expected. Opposed to these highly polymorphic populations, is the least polymorphic population, population 21, with a polymorphic loci content of 33.3 %. This low level of polymorphism within population 21 may be as a result of its small population size (consist of only one individual). The percentage polymorphism expected within a population may be influenced by the population size (number of individuals in population). Populations consisting of one individual (3, 4, 5, 13, 15, 16, 17, 18, 19, 21, 23, 33, 34, 36, 38 and 39) therefore exhibit the lowest levels of polymorphism.

Table 2.6 Percentage polymorphic loci among all the populations

Population number	* % P
1	91.67%
2	100.00%
3	50.00%
4	66.67%
5	58.33%
6	91.67%
7	100.00%
8	91.67%
9	100.00%
10	91.67%
11	100.00%
12	100.00%
13	66.67%
14	91.67%
15	66.67%
16	75.00%
17	66.67%
18	41.67%

19	58.33%
20	91.67%
21	33.33%
22	83.33%
23	83.33%
24	100.00%
25	100.00%
26	100.00%
27	100.00%
28	100.00%
29	100.00%
30	100.00%
31	91.67%
32	100.00%
33	50.00%
34	41.67%
35	91.67%
36	41.67%
37	91.67%
38	75.00%
39	58.33%
40	100.00%
41	100.00%
42	100.00%
43	100.00%
Mean	**82.36%**
SE	**3.19%**

*** % P = Percentage Polymorphism**

A measure of *Fis* within each population measured across all 12 loci was calculated with the population genetic programme POPGENE (Appendix 2.2). Population 31 has the highest inbreeding coefficient (*Fis*) of 0.333 and therefore may experience relatively high levels of inbreeding. Population 27 on the other hand has the lowest inbreeding coefficient (*Fis*) of –0.004. Several populations have *Fis* values which are not applicable (NA) due to their

small sample size (only one individual present in each population) these include populations 3, 4, 5, 13, 15, 16, 17, 18, 19, 21, 23, 33, 34, 38 and 39.
The Hardy-Weinberg model is a test hypothesis as it represents a null model. The probability (P) associated with the chi squared test, relates to the probability that chance alone is responsible for deviation between observed and expected values, which in turn influences the significance of the Hardy – Weinberg model. No significant deviation from HWE was observed when an analysis was carried out for each population and across all loci. A per locus analysis across each population, revealed that 8 loci (ETH10, INRA128, INRA006, TGLA227, SPS115, BM3205, BM1824, CSSM19) may deviate from HWE due to a heterozygote deficit.

5.1.2 Inter-population analysis

Inter-population analysis calculates the genetic indices between all 43 populations and therefore yields an overall, global estimate.

5.1.2.1 Allele frequencies

Allelic patterns were established by population, across the entire data set. The grand mean values with their standard errors, were calculated over all 12 loci and 43 populations. A mean, overall estimate of sample size *(N)*, number of different alleles *(Na)* and the effective number of alleles *(Ne)* were calculated with GENAlEX ver 6.2 (Table 2.7). Results indicate the mean number of alleles *(Na),* as 3.45 different alleles (SD = 0.1), this measure of genetic variation implies a relatively low genetic diversity per locus. The effective number of alleles *(Ne)* is an estimate of genetic diversity. The mean number of effective alleles *(Ne)* was calculated across all 12 loci, this value of 2.7 (SD = 0.071) suggests a medium level of genetic diversity across all populations.

Table 2.7 Mean and Standard error (SE) values calculated across all 43 populations and 12 loci

	N	Na	Ne
Mean	4.151	3.455	2.714
SE	0.167	0.1	0.071

Gene diversity was calculated per locus across all populations by FSTAT ver 2.9.3 (Table 2.8), to reveal the most genetically diverse locus. Locus ILSTS026 has the highest gene diversity (0.885) and locus ETH10 the lowest diversity at 0.307.

Table 2.8 Gene diversity per locus calculated across all populations

Locus	Gene diversity
BM1824	0.876
BM3205	0.865
BM719	0.83
CSSM19	0.831
ETH10	0.307
ILSTS026	0.885
INRA128	0.57
INRA006	0.62
SPS115	0.859
TGLA159	0.815
TGLA227	0.498
TGLA263	0.757

Allele frequencies represent the fraction of loci that a particular allele occupies within the population. Allele frequency distributions for buffaloes within the global population were calculated at each locus with MS Toolkit and are shown in Appendix 2.3. The most common allele per locus is shown in bold. These include allele 204 at locus ETH10, allele 176 at locus INRA128, allele 113 at locus INRA006, alleles 237 and 239 at locus SPS115, allele 72 at locus TGLA227 and alleles 118 and 122 at locus TGLA263. These alleles are regarded as genetically most diverse within the populations they are associated with.

5.1.2.2 Population genetic variation

An overall estimate of genetic variation across all 43 populations revealed important facts with regards to the global situation across all 12 loci, calculated with MS Toolkit. These estimates include the expected heterozygosity, observed heterozygosity and number of alleles. For each of these estimates their associated standard deviation levels were also calculated. The primary indicator of genetic variation is heterozygosity. A global heterozygosity value of 0.7257 (SD = 0.0535) was established. A moderate to high level of genetic diversity is, therefore, expected across all 43 populations. Approximately 72.57 % of individuals are heterozygous (Table 2.9). The mean number of alleles across the entire data set is very high at 11.92 (SD = 3.60)

Table 2.9 Population statistics including sample size, number of loci typed, expected heterozygosity, observed heterozygosity and the number of alleles, each presented here with their standard deviation values

Population	Sample size	Loci typed	Exp Hz	Exp Hz SD	Obs Hz	Obs Hz SD	No Alleles	No SD	Alleles
Global	190	12	0.7257	0.0535	0.6183	0.0105	11.92	3.60	

The mean percentage polymorphism across all populations and 12 polymorphic loci is 82.36 % (SE 3.19 %). This indicates that over all populations a high level of polymorphism can be expected, with high genetic diversity (Table 2.6).

The inbreeding coefficient *(Fis)* measures the heterozygote deficit per population, across all loci or on a per locus basis. The global inbreeding coefficient *(Fis)* across all loci was 0.148 (Table 2.10). This suggests a medium to low level of inbreeding and a heterozygote deficit across all samples in the study.

Table 2.10 Global inbreeding coefficient *(Fis)* across all loci

Locus	*Fis* value
BM1824	0.043
BM3205	0.094
BM719	0.083
CSSM19	0.087
ETH10	0.352
ILSTS026	0.085
INRA128	0.31
INRA006	0.091
SPS115	0.204
TGLA159	0.083
TGLA227	0.408
TGLA263	0.224
All	**0.148**

GENEPOP ver 4.0 and the Markov chain method (dememorization: 1000; batches: 20; iterations: 1000) was used to calculate the unbiased estimates of Hardy-Weinberg exact probability tests (exact P values), across all 12 loci and 190 samples.

Monomorphic loci were identified in 26 of the 43 populations. Each of the 12 loci was monophorphic at a specific population. Locus ETH10 had the highest monophorphic count and locus BM1824 the lowest monophorphic count. Across all populations, 10 out of the 12 loci used, deviate significantly ($P < 0.05$) from HWE, due to heterozygote deficit. Only loci CSSM19 and ILSTS026 did not deviate from HWE. The probability (P) value associated with the chi-square test of the global population is, therefore, highly significant (Table 2.11).

Table 2.11 Inbreeding coefficient *(Fis)* estimates of the global population

Global	*Fis* estimates

population			
Locus	**[1]P-value**	**[2]S.E.**	**[3]W&C**
BM1824	0.0022	0.0022	0.04
BM3205	0.0062	0.0061	0.09
BM719	0.0003	0.0003	0.08
CSSM19	0.0518	0.0159	0.09
ETH10	0.0000	0.0000	0.35
ILSTS026	0.2388	0.0328	0.08
INRA128	0.0000	0.0000	0.00
INRA006	0.0000	0.0000	0.09
SPS115	0.0000	0.0000	0.20
TGLA159	0.0234	0.0087	0.08
TGLA227	0.0000	0.0000	0.41
TGLA263	0.0000	0.0000	0.22

[1]P-value = Probability value

[2]S.E. = Standard error

[3]W&C = Weir & Cockram estimate

Genetic migration (often referred to as gene flow), is the movement of genes, individuals or groups of individuals from one population to another (Slatkin, 1987). The number of migrants *(Nm)* represents the total number of migrants that enter a sub-population in each generation. The number of migrants was calculated with GENEPOP ver 4.0 and incorporated private alleles. A mean sample size of 4.2 with a mean frequency of private alleles of 0.014 was calculated across all 43 populations. The number of migrants (1.45) was determined after several sample size corrections were made, 1.45 number of migrants can therefore be expected in every generation. A low level of migration between animals in different populations can, therefore, be expected.

Section two

5.2 Prevalence group level investigation based on the three prevalence groups associated with the KNP

5.2.1 BTB prevalence zones (groups)

Three BTB prevalence zones are identified within the KNP (Fig 2.2). The high prevalence zone is in the southern region of the Park, south of the Sabie River. From the 181 samples taken in the KNP, 123 samples were collected from the southern, high BTB prevalence region. The central region of the KNP is regarded as a moderate BTB prevalence region (areas between Sabie an Olifants Rivers). Thirty-eight samples were collected in this region. The northern region of the park is a relatively low BTB prevalence region (area north of the Olifants River) the disease is, however, on the increase in this area. Twenty samples were collected from this region. Figure 2.3 indicates the percentage of BTB positive animals within the southern region of the park, increasing from the initial 27.11% in 1992 to 38.16% in 1998 and decreasing to 30.7% in 2005. Figure 2.4 indicates the BTB prevalence associated with the central region of the park. An increase in BTB prevalence is noted, from 4.35% in 1992 to 16.02% in 1998. The BTB prevalence in the northern regions of the park increased dramatically from 1992 to 2008 (Fig 2.5) (Van Schalkwyk, L. July 2010, personal communication.

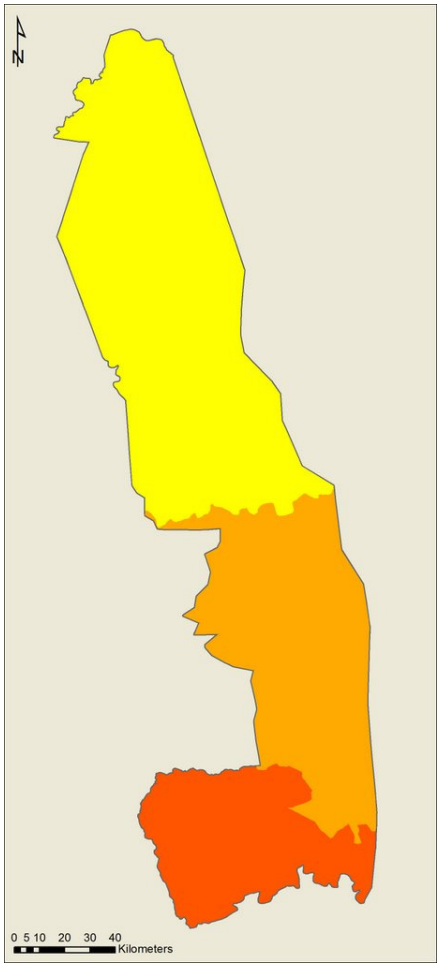

Fig 2.2 Map of the BTB prevalence zones in the KNP. Red represents the high BTB prevalence zone (southern KNP), orange the moderate BTB prevalence zone (central KNP) and yellow the low BTB prevalence zone (northern KNP).

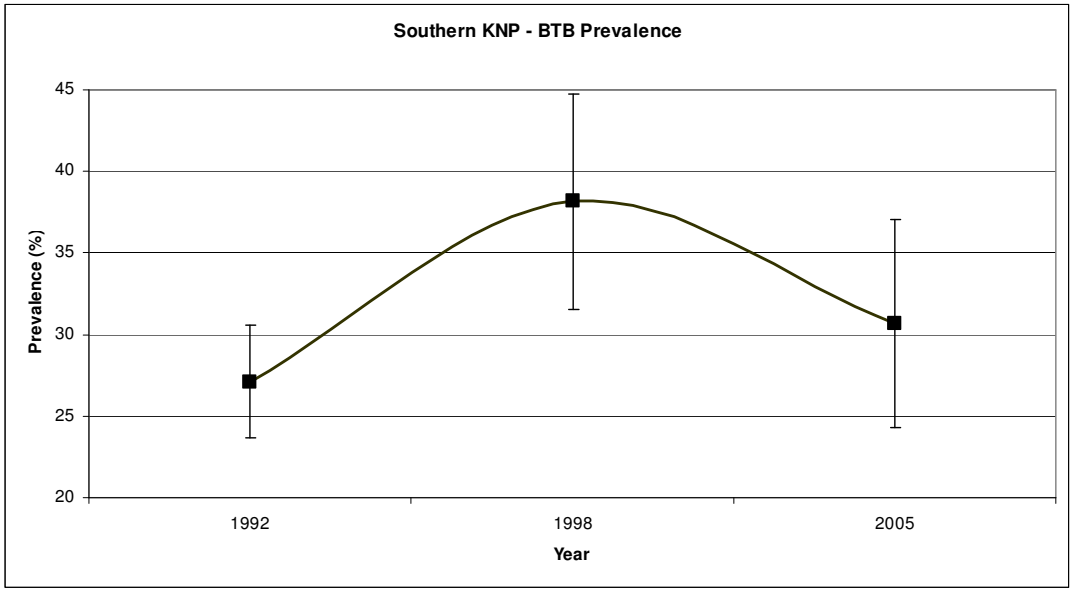

Figure 2.3 BTB prevalence (percentage value) measured against year in the southern regions of the KNP

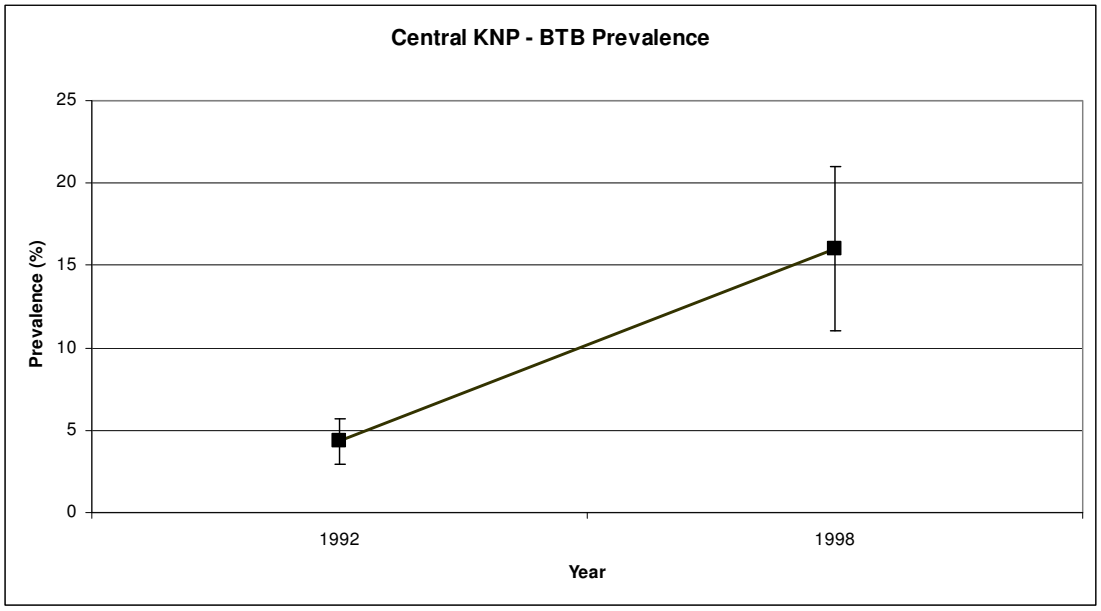

Figure 2.4 BTB prevalence (percentage value) measured against year in the central regions of the KNP

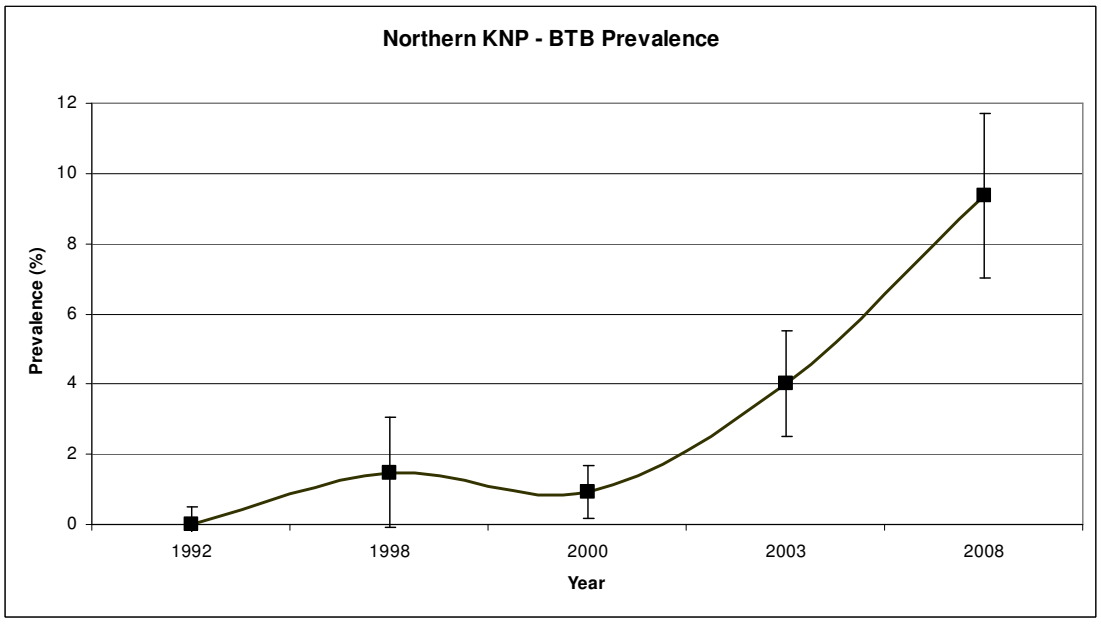

Figure 2.5 BTB prevalence (percentage value) measured against year in the northern regions of the KNP

5.2.2 Gender, BTB status and age distribution of animals sampled in KNP

The gender, age and BTB strain of several samples associated with this dataset, were not known, which made sex biased as well as age biased analysis difficult. From the 181 samples collected in the KNP, the gender of only 154 individuals was known (82 females and 72 males). Age related BTB distribution was hampered by the lack of available data. From the group of 181 sampled animals, the ages of only 143 were available. These ages ranged between newborns and animals twelve years of age. From the group of 82 females, 50 individuals were BTB positive, 31 BTB negative and one individual from Mpanamane with an unknown (suspect) BTB culture status. From the group of 72 males, 31 individuals were BTB positive and 41 were BTB negative. From the 181 buffaloes sampled throughout the KNP, the BTB strains of only 27 individuals were known. Eighteen individuals were infected with the dominant C8 strain and nine individuals were infected with variant C8v strains, were identified. One individual sampled at Woodlands (located in the northern region of the Park) had both dominant and variant strains. The BTB strains of the remaining 153 buffalo samples were not typed during the initial sampling procedure (refer to Table 2.1 for population names).

Table 2.12 provides a summary of the number of individuals, their gender and BTB culture status within each population. Several populations consist of individuals of unknown gender, these individuals along with their BTB culture statuses are represented in the last two columns of the table.

Table 2.12 Number of individuals of known and unknown gender and the BTB status of these individuals present in each population

Population number	Nr. of individuals	Nr. males	Male BTB status	Nr. Females	Female BTB status	Nr. of individuals with unknown gender	Individuals of unknown gender BTB status
1	4	2	2 positive	2	2 positive	-	-
2	4	1	1 positive	3	3 positive	-	-
3	1	-	-	-	-	1	Positive
4	1	1	1 negative	-	-	-	-
5	1	1	1 negative	-	-	-	-
6	6	2	2 positive	4	3 positive 1 negative	-	-
7	7	5	2 positive 3 negative	2	1 positive 1 negative	-	-
8	3	-	-	3	2 positive 1 negative	-	-
9	7	2	1 positive 1 negative	5	4 positive 1 negative	-	-
10	6	3	1 positive 2 negative	3	3 positive	-	-
11	6	5	2 positive 3 negative	1	1 negative	-	-
12	6	2	1 positive 1 negative	1	1 negative	3	2(Positive) 1(Negative)

13	1	-	-	1	1 positive	-	-
14	3	-	-	-	-	3	1(Positive) 2(Negative)
15	1	-	-	-	-	1	Negative
16	1	-	-	-	-	1	Positive
17	1	-	-	-	-	1	Positive
18	1	-	-	-	-	1	Positive
19	1	-	-	-	-	1	Positive
20	2	1	1 negative	2	1 negative	-	-
21	1	-	-		1 positive	-	-
22	2	1	1 negative	1	1 positive	-	-
23	1	-	-	1	1 positive	-	-
24	7	5	2 positive 3 negative	2	2 positive	-	-
25	15	8	2 positive 6 negative	7	3 positive 4 negative	- -	- -
26	4	-	-	4	2 positive 2 negative	-	-
27	6	4	3positive 1 negative	2	2 negative	-	-
28	12	8	3 positive 5 negative	4	3 positive 1 negative	-	-
29	8	3	2 positive	5	2 positive	-	-

			1 negative		3 negative		
30	14	4	1 positive	10	6 positive	-	-
			3 negative		4 negative		
31	2	2	1 positive	-	-	-	-
			1 unknown				
32	8	-	-	-	-	8	4(Positive)
							4(Negative)
33	1	-	-	-	-	1	Positive
34	1	-	-	-	-	1	Positive
35	3	-	-	-	-	3	Positive
36	1	-	-	-	-	1	Positive
41	8	4	2 positive	4	3 positive	-	-
			2 negative		1 negative		
42	9	5	1 positive	4	2 positive	-	-
			4 negative		2 negative		
43	15	4	2 positive	11	6 positive	-	-
			2 negative		5 negative		

5.2.3 Prevalence group size and structure

Population genetic programmes, GENAIEX version 6.2, POPGENE (POPGEN 32), GENEPOP version 4.0 and FSTAT version 2.9.3 were used to evaluate each group. Evaluation of group size was based on gene diversity and allele frequencies. Evaluation of group structure (genetic variation) was conducted within each prevalence group, at each of the 12 loci and was based on genetic variation, F-statistics, Hardy-Weinberg evaluation, chi-squared tests, degrees of freedom and probability values.

Group 1 (high BTB prevalence zone), is located in the southern region of the KNP and consists of 18 populations, which include populations 1, 2, 3, 13, 24, 25, 26, 27, 28, 29, 30, 32, 33, 34, 35, 41, 42, and 43.

Group 2 (moderate BTB prevalence zone), is located in the central region of the KNP and consists of 8 populations, which include populations 6, 7, 8, 9, 10, 11, 31 and 36.

Group 3 (low BTB prevalence zone) is located in the northern region of the KNP and consists of 13 populations, which include populations 4, 5, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22 and 23.

Group 4 (outlier populations), samples were collected in the HiP, Hoedspruit, Klaserie and Kimberley regions. Populations 37, 38, 39, and 40 form part of this group. This group was, however, not used for further analysis, as samples within this group are widely dispersed throughout the country and can not be viewed as a single entity.

5.2.3.1 Group size

Gene diversity was calculated across all 12 loci and for each group using FSTAT ver 2.9.3. Group 1 shows high gene diversity, especially at locus ILSTS026 (0.886) and relatively low diversity at locus ETH10 (0.341). Within Group 2 high gene diversity was detected at locus BM1824 (0.902), locus

ETH10 (0.205) had the lowest level of diversity. Group 3 has high gene diversity at locus TGLA159 (0.87) with low diversity at locus ETH10 (0.256). Locus ETH10 therefore has low gene diversity levels throughout all three groups evaluated (Table 2.13).

Table 2.13 Gene diversity per locus and prevalence group (1, 2 and 3)

Locus	1	2	3
BM1824	0.876	0.902	0.855
BM3205	0.865	0.858	0.854
BM719	0.809	0.877	0.854
CSSM19	0.822	0.847	0.817
ETH10	0.341	0.205	0.256
ILSTS026	0.886	0.893	0.861
INRA128	0.531	0.602	0.637
INRA006	0.607	0.636	0.604
SPS115	0.836	0.879	0.864
TGLA159	0.803	0.822	0.87
TGLA227	0.505	0.482	0.406
TGLA263	0.745	0.775	0.775
Average across all loci:	**0.718**	**0.731**	**0.721**

The allele frequency distributions for sampled buffaloes within the KNP (Group 1, 2 and 3) were calculated at each locus with MS Toolkit and are shown in Appendix 2.3.

5.2.3.2 Group structure (Genetic variation)

An overall estimate across all three prevalence groups were calculated over all 12 loci with MS Toolkit. These estimates include the expected heterozygosity, observed heterozygosity and number of alleles within each group. For each of these estimates their associated standard deviation levels were also calculated. The primary indicator of genetic variation is heterozygosity, which measures the genetic variability within a population

(group) and reveals the amount of variation distributed among alleles of loci examined. Observed and expected heterozygosities, therefore, reveal a lot about a population's structure and history. All three prevalence groups have moderately high unbiased heterozygosity values which indicate moderate to high levels of genetic diversity within each group. The expected heterozygosity for each prevalence group is greater than 0.7. Group 1 has the largest sample size (121 individuals) and therefore also has the highest mean allele count at 11.08 (SD = 3.40) in comparison to the other two groups (Table 2.14).

Table 2.14 Population statistics including sample size, number of loci typed, expected heterozygosity, observed heterozygosity and the mean number of alleles, each presented here with their standard deviation values

Group	Sample size	Loci typed	Exp Hz	Exp Hz SD	Obs Hz	Obs Hz SD	Mean no alleles	No alleles SD
Group 1	121	12	0.7185	0.0516	0.6135	0.0132	11.08	3.40
Group 2	38	12	0.7298	0.0618	0.6266	0.0237	8.92	3.40
Group 3	22	12	0.7184	0.0590	0.6158	0.0304	7.83	3.19

An important estimate of F-statistics is the measure of variation in 'sub-populations' relative to the total population (*Fst*). *Fst* is one of the most widely used estimators of genetic distance between populations and is used to quantify the level of genetic differentiation between populations. FSTAT ver 2.9.3 was used to calculate *Fst* among all groups and revealed a low degree of genetic differentiation. The *Fst* value among all groups ranged between 0.003 and 0.007. The genetic differentiation among all three prevalence groups and therefore, sampled African buffaloes in the KNP, is very low.

The inbreeding coefficient *(Fis)* measures the heterozygote deficit within each group. *Fis* was calculated for each group and over all loci with FSTAT ver 2.9.3. All three groups have a medium to low level of inbreeding and a therefore, a heterozygote deficit (*Fis)* for each group ranges between 0.143 and 0.147 (Table 2.15). Social and reproductive behaviour of buffaloes within the Park might be responsible for creating this low inbreeding status within each group.

Table 2.15 Inbreeding coefficient *(Fis)* calculated per group (Group 1, 2 and 3)

Locus	1	2	3
BM1824	0.07	-0.016	-0.01
BM3205	0.088	0.101	0.095
BM719	0.08	-0.014	0.202
CSSM19	0.113	0.006	0.11
ETH10	0.36	0.604	-0.116
ILSTS026	0.076	0.077	0.115
INRA128	0.34	0.326	0.073
INRA006	0.086	0.131	0.097
SPS115	0.142	0.305	0.319
TGLA159	0.057	0.078	0.164
TGLA227	0.411	0.563	0.328
TGLA263	0.235	0.151	0.263
All	**0.147**	**0.143**	**0.146**

GENEPOP ver 4.0 and the Markov chain method (dememorization: 1000; batches: 20; iterations: 1000) was used to calculate the unbiased estimates of Hardy-Weinberg exact probability tests (exact P-values), across all 12 loci and 181 samples.

As mentioned previously, the probability (P) value associated with the chi squared test, relates to the probability that chance alone is responsible for deviation between observed and expected values, which in turn influences the significance of the Hardy-Weinberg model. The probability (P) value associated with the chi square test of group 1 and group 2, reveals a highly significant value, which suggests that samples deviate significantly from HWE and is, therefore, not in HWE. Group 3 has a P-value of 0.0001, which deviates from HWE ($P < 0.05$) and is, therefore, not in HWE (Tables 2.16 to Table 2.18). From a combination of these three groups, an overall estimate was taken across all loci, which revealed a highly significant P-value, which suggest that the group combination is not in HWE and a heterozygote deficit is expected, this may be as a result of one of the Hardy-Weinberg assumptions which has not been met.

Table 2.16 Inbreeding coefficient *(Fis)* estimates for group 1

Group 1			Fis estimate
Locus	**[1]P-value**	**[2]S.E.**	**[3]W&C**
BM1824	0.0005	0.0002	0.0695
BM3205	0.0123	0.0009	0.0876
BM719	0.0154	0.0009	0.0804
CSSM19	0.0747	0.0036	0.1134
ETH10	0.0000	0.0000	0.3600
ILSTS026	0.3012	0.0060	0.0759
INRA128	0.0000	0.0000	0.3404
INRA006	0.0037	0.0004	0.0862
SPS115	0.0229	0.0020	0.1417
TGLA159	0.7257	0.0034	0.0570
TGLA227	0.0000	0.0000	0.4112
TGLA263	0.0000	0.0000	0.2351
Across all loci:	**Probability highly significant**		

[1]P-value = Probability value
[2]S.E. = Standard error
[3]W&C = Weir & Cockram estimate

Table 2.17 Inbreeding coefficient *(Fis)* estimates for group 2

Group 2			**Fis estimate**
Locus	**[1]P-value**	**[2]S.E.**	**[3]W&C**
BM1824	0.3435	0.0069	-0.0158
BM3205	0.4894	0.0052	0.1009
BM719	0.0878	0.0029	-0.0136
CSSM19	0.5137	0.0050	0.0063
ETH10	0.0001	0.0000	0.6044
ILSTS026	0.3859	0.0048	0.0774
INRA128	0.0000	0.0000	0.3263
INRA006	0.1197	0.0017	0.1309
SPS115	0.0834	0.0026	0.3048
TGLA159	0.2211	0.0029	0.0783
TGLA227	0.0000	0.0000	0.5634
TGLA263	0.0069	0.0007	0.1514
Across all loci:	**Probability highly significant**		

[1]P-value = Probability value

[2]S.E. = Standard error

[3]W&C = Weir & Cockram estimate

Table 2.18 Inbreeding coefficient *(Fis)* estimates for group 3

Group 3			**Fis estimate**
Locus	**[1]P-value**	**[2]S.E.**	**[3]W&C**
BM1824	0.9510	0.0025	-0.0101
BM3205	0.2387	0.0056	0.0951
BM719	0.0431	0.0015	0.2015
CSSM19	0.0872	0.0038	0.1099
ETH10	1.0000	0.0000	-0.1163
ILSTS026	0.1205	0.0033	0.1148
INRA128	0.0663	0.0663	0.0014
INRA006	0.7053	0.0019	0.0968
SPS115	0.0007	0.0002	0.3191
TGLA159	0.0194	0.0007	0.1642
TGLA227	0.1214	0.0007	0.3280
TGLA263	0.0192	0.0005	0.2627
Across all loci:	**Probability is 0.0001**		

[1]P-value = Probability value

[2]S.E. = Standard error

[3]W&C = Weir & Cockram estimate

The probability (P) value was also calculated among group pairs, across all loci based on Fisher's model. These group comparisons showed no significant deviation from Hardy-Weinberg, which implies that chance alone, could be responsible for deviations between the prevalence groups (Table 2.19).

Table 2.19 P-value for each population pair, taken across all loci (Fisher's method)

Group	Pairs	[1]Chi[2]	[2]df	[3]P-value
1	& 2	33.944	24	0.08567
1	& 3	24.976	24	0.40704
2	& 3	25.503	24	0.37885

[1]Chi[2] = Chi squared

[2]df = Degrees of freedom

[3]P-value = Probability value

A mean sample size of 56.63 with a mean frequency of private alleles of 0.012 was calculated across all three prevalence groups. The number of migrants (11.68) was determined after several sample size corrections were made. One can, therefore, expect 11.68 buffaloes to migrate between the different prevalence groups every generation. A high level of gene flow, maintains genetic similarity among populations and prevalence groups.

5.2.4 Discussion

Results and discussions are based on our dataset of 181 samples from the KNP.

The intra- and inter-population relationships between buffalo populations provide an overall description of the sampled buffalo data within the KNP. The genetic characteristics of each population as well as across the entire dataset were determined. Samples were also separated on a prevalence group level, which provided a better representation of the results, as samples were grouped based on their BTB culture status and the area sampled.

Work previously conducted by Greyling *et al* (2008) on African buffaloes within the KNP was used as platform for comparisons with results generated in this study. Their study was based on microsatellite data from 485 sampled individuals throughout the KNP as well as on a sub-population level (83 samples from northern KNP and 182 samples from southern KNP).

From our data set, a total of 181 samples, 43 populations and 143 alleles were identified. These samples and populations were subjected to several population genetic tests to reveal important population genetic estimates.

Several genetic indices are descriptors of the global population size. The effective number of alleles *(Ne)* plays an important role as it provides information on the genetic diversity of the global population across all loci. The mean number of effective alleles *(Ne)* was calculated across all 12 loci as 2.7 (SD = 0.071). Calculation of the effective number of alleles *(Ne)* provided

sufficient information to conclude that medium level of genetic diversity is expected for all individuals sampled across all loci.

In this study a total of 143 alleles were identified from the sampled African buffaloes, compared to the study of Greyling, 2007 with 158 alleles. This difference in the number of alleles identified could be as a result of the difference in sample size (181 samples vs. 485 samples in this study and Greyling's study respectively) or due to the different microsatellite marker panels used (12 loci vs. 17 loci, in this study and Greyling's study respectively).

Private alleles indicate how unique a certain population might be. In this study 22 private alleles were identified out of a total of 143 alleles (16 % of the total number of alleles). These alleles were associated with 10 different populations. Ten out of a total of 43 populations are, therefore, genetically unique. From the 9 individuals that were sampled at Napi Boulder (population 42), 7 alleles were exclusive to this population. A relatively large proportion of alleles are, therefore, not shared between this population and other populations identified in this study, which contributes to the unique genetic characteristics of individuals associated with this population. Greyling, (2007) identified 89 private alleles from his 485 KNP samples (18 % of the total number of alleles were regarded as private alleles when compares to HiP). Sample size is again suspected to be responsible for the difference in the number of private alleles identified between the two studies. Genetic drift may also have influenced the occurrence of private alleles.

Genetic indices associated with the global population genetic variation, include estimates of unbiased heterozygosity, and the observed heterozygosity, mean number of alleles and allelic richness. Expected heterozygosity *(He)* is the primary indicator of genetic variation. Heterozygosity differs between Greyling's (2007) dataset and the current dataset, with the current dataset (values) showing higher heterozygosity values and therefore, higher levels of genetic differentiation. Fewer microsatellite markers (12), with varying degrees of polymorphism were used

in the current study compared to the 17 markers used in Greyling's (2007) study. Heterozygosity may vary based on the level of polymorphism of loci. The global population also represents four outlier populations from Hluhluwe-iMfolozi Park (HiP), Kimberley, Klaserie and Hoedspruit areas which may have contributed to the higher heterozygosity levels observed in the global population.

A global inbreeding coefficient *(Fis)* was calculated across all samples and loci, indicating a medium to low level of inbreeding (0.14) as well as a heterozygote deficit. The social and reproductive behaviour of the African buffalo may affect the level of inbreeding. This medium to low inbreeding coefficient *(Fis)* value may be attributed to the social organization of the African buffalo, where dispersing bulls or heifers may enter their native population at high frequencies in a non-random manner or as a result of female philopatry (Greyling, 2007). The sampling procedure may also have contributed to the low level of inbreeding observed among populations.

Tests to evaluate conformance to HW proportions revealed that 10 out of the 12 loci used in this study, deviated significantly ($P < 0.05$) from HWE, due to heterozygote deficit, (only locus CSSM19 and ILSTS026 did not deviate from HWE). The probability (P) value associated with the chi square test of the global population is, therefore, highly significant. These deviations from HWE may be as a result of one of the assumptions which have not been met. A low level of migration was observed between individuals from various populations. From the 17 microsatellite markers used by Greyling, only five (BM1824, TGLA227, TGLA159, BM4028 and INRA128) deviated significantly from HWE, due to heterozygote deficit (Greyling, 2007).

Genetic migration or gene flow is influenced by the social behaviour of the African buffalo, habitat fragmentation, human habitation, environmental factors and geographical barriers. The number of migrants (1.45) was determined after several sample size corrections were made between samples associated with the global population. Approximately 1.45 numbers of migrants can, therefore, be expected to be exchanged in every generation.

A low level of migration between animals in different populations is, therefore, expected. Random genetic drift may be the result of a low level of migration between individuals from different populations (Hartl and Clark, 2007). The time period when samples were collected (non-breeding season) is an important factor which may also have influenced the African buffalo's migratory patterns between the various populations. The time period of sample collection (late dry season) coincides with environmental factors such as low water and food availability as well as food quality, which may also have contributed to the low level of migration. The group organization of the African buffalo (male and heifer dispersal and female philopatry) varies throughout the year, which may be responsible for the low level of migration observed from a global population perspective. Geographic barriers between the populations may have hindered migration and thus contributed to the low level of migration between the populations.

Three BTB prevalence zones have been identified within the KNP, each varying in size and structure. The amount of variation produced by alleles of all loci was established by determining the amount of heterozygosity *(He)* in each group. All three groups showed moderately high unbiased (expected) heterozygosity (above 0.7). Samples from the central region of the KNP are, however, slightly more heterozygous than samples from the southern or northern regions. Greyling also conducted research on a sub-population level, by dividing his samples in southern (182 samples) and northern (83 samples) groups. Genetic diversity based on heterozygosity levels were significantly higher in the northern region (0.66, SD = 0.037) compared to the southern region (0.62, SD = 0.035). He attributed this difference to buffaloes migration in the northern region of the Park, prior to completion of the Park's fence (Greyling, 2007).

A very small degree of genetic differentiation between the prevalence groups was observed from pairwise *Fst* values between groups. These three prevalence groups can thus be regarded as being genetically similar. The inbreeding coefficient *(Fis)* which measures the heterozygote deficit within each group was calculated over all 12 loci. A medium to low level of

inbreeding is expected for each prevalence group, which might be as a result of sampling of related individuals or null alleles.

An overall estimate of HWE revealed that samples associated with prevalence group's one, two and three deviate significantly from HWE. This may be as a result of HWE assumptions which have not been met, for example migration (buffalo migration was relatively high between the various prevalence groups as indicated by the relatively high level of migration between the groups).

As mentioned previously, the number of migrants *(Nm)* is an important factor in population genetics as it influences the dynamics of the group over time, as well as the rate of spread of a disease such as BTB. The number of migrants expected between prevalence groups was 11.68. This high level of gene flow, maintains genetic similarity among individuals of the different prevalence groups as a result of a high migration rate. The social organization, male biased dispersal and movement of heifers between herds via splinter groups (Greyling, 2007), may be responsible for this relatively high level of gene flow between the various prevalence groups.

5.3 Conclusion

Genetics, ecology and evolution interact and influence the outcome of population genetic estimates. To better understand these interactions a variety of approaches including descriptive observations, theoretical models, as well as statistical models are required and have been applied throughout this study (Hartl and Clark, 2007). Data generated from population genetic estimates can assist in future conservation and management strategies of wildlife in southern Africa.

Animal dispersal behaviour, group organization and sample size are important factors that may have influenced the outcome of our study. Animal dispersal behaviour (natal or breeding dispersal) influences the demography, social structure and genetic composition of populations (Banks *et al*., 2002). The group organization of the African buffalo which includes male dispersal, young

female (heifer) migration and adult female philopatry varies throughout the year. The relatively limited sample size of 181 African buffaloes used during the study may have influenced the results to some degree.

Individuals were divided into specific prevalence groups based on whether BTB occurs at high, medium or low levels within the sampled region. Evidence suggests a medium to high level of genetic diversity within each group, with samples from the central region (Group 2) being slightly more heterozygous. Low to medium levels of inbreeding was determined within each group, which confirms the relatively high levels of genetic diversity within each group. The relatively high level of migration observed between these three groups indicates gene flow between these groups. The social organization of the African buffalo (male dispersal and heifer migration) within the KNP may have influenced this value.

Appendix 2.1 Highest allele frequencies observed at ea narker (Allele frequencies were calculated by population, for locus. This appendix represents the alleles that occur with the highest frequencies within each population, at each locus. Six populations were compared at a time. These frequencies are represented as a percentage value in brackets).

Locus	Population number					
	1	2	3	4	5	6
BM1824	183 (37.5 %)	181 (50%)	191,195 (50%)	185,199 (50%)	181,195 (50%)	181 (50%)
BM3205	208 (25%)	214 (33.3%)	206,214 (50%)	206,214 (50%)	204 (100%)	204,206,208,214 (16.4%)
BM719	148 (62.5%)	148 (50%)	146,148 (50%)	148,154 (50%)	154 (100%)	146,148 (25%)
CSSM19	146 (50%)	144(50%)	146,148 (50%)	144,150 (50%)	144,156 (50%)	146 (33.3%)
ETH10	204 (75%)	204 (87.5%)	204 (100%)	204 (100%)	204,206 (50%)	204 (100%)
ILSTS026	141 (25%)	149, 157 (25%)	159,161 (50%)	zero alleles	151,157 (50%)	149 (33.3%)
INRA128	176 (100%)	176 (75%)	174 (100%)	176,178 (50%)	176 (100%)	176 (33.3%)
INRA006	113 (62.5%)	113 (83.3%)	111 (100%)	115,119 (50%)	113,115 (50%)	113 (58.3%)
SPS115	239 (50%)	239 (50%)	231,239 (50%)	233 (100%)	zero alleles	237,239 (33.3%)
TGLA159	223 (37.5%)	225 (50%)	231(100%)	227 (100%)	221,229 (50%)	227 (40%)
TGLA227	72 (87.5%)	72 (62.5%)	72 (100%)	72,74 (50%)	74 (100%)	72 (83.3%)
TGLA263	122 (62.5%)	122 (50%)	122 (100%)	118,120 (50%)	114,122 (50%)	122 (58.3%)

Appendix 2.1 Continued

Locus	Population number					
	7	8	9	10	11	12
BM1824	175,177,181,187 (16.7%)	189 (33.3%)	185,197 (21.4%)	185,197 (20%)	181 (33.3%)	181 (41.7%)
BM3205	202 (50%)	202,204 (33.3%)	206,214 (33.3%)	202 (41.7%)	208,214 (25%)	202,204,206 (25%)
BM719	154 (41.7%)	138,140,142,148,150,154 (16.7%)	148,152 (21.4%)	142,154 (20%)	154 (33.3%)	148 (41.7%)
CSSM19	148 (28.6%)	148 (33.3%)	146,150 (35.7%)	138,142,146,150 (16.7%)	150 (33.3%)	146 (50%)
ETH10	204 (85.7%)	204 (66.7%)	204 (83.3%)	204 (100%)	204 (83.3%)	204 (80%)
ILSTS026	151 (30%)	151 (33.3%)	151,159,163 (20%)	159 (25%0	151 (33.3%)	149 (33.3%)
INRA128	176 (71.4%)	178(33.3%)	178 (35.7%)	176 (66.7%)	176 (50%)	176 (75%)
INRA006	113 (85.7%)	113 (50%)	115 (42.9%0	113 (41.7%)	113 (66.7%)	113 (75%)
SPS115	223,225 (33.3%)	235,239 (50%)	237 (33.3%)	237 (37.5%0	227,229.231,239 (25%)	233 (40%)
TGLA159	223,225,227 (25%)	223 (50%)	223,227 (35.7%)	227 (33.3%)	231 (25%)	227 (33.3%)
TGLA227	72 (51.7%)	72 (100%)	72 (64.3%)	72 (66.7%)	72 (66.7%)	72 (91.7%)
TGLA263	118 (42.95%)	120,122 (33.3%)	118 (35.7)	122 (50%)	122 (58.3%)	122 (50%)

Appendix 2.1 Continued

Locus	Population number					
	13	14	15	16	17	18
BM1824	187 (100%)	183 (33.3%)	181,185 (50%)	181,191 (50%)	181,197 (50%)	181,187 (50%)
BM3205	202,218 (50%)	202 (50%)	198,218 (50%)	204 (100%)	202,206 (50%)	202,204 (50%)
BM719	148,152 (50%)	134 (33.3%)	146,148 (50%)	140,152 (50%)	148,156 (50%)	144,154 (50%)
CSSM19	146,148 (50%)	146 (33.3%)	146,159 (50%)	134,144 (50%)	136,148 (50%)	138,146 (50%)
ETH10	204,206 (50%)	204 (66.7%)	204 (100%)	204 (100%)	204 (100%)	204 (100%)
ILSTS026	151,157 (50%)	149 (33.3%)	149,159 (50%)	147,157 (50%)	149,163 (50%)	149 (100%)
INRA128	109,115 (50%)	178 (50%)	176 (100%)	166,178 (50%)	168,176 (50%)	168 (100%)
INRA006	176 (100%)	113 (100%)	109,115 (50%)	113,115 (50%)	109,115 (50%)	115 (100%)
SPS115	239 (100%)	225,235,237,239 (25%)	239 (100%)	227,231 (50%)	zero alleles	239 (100%)
TGLA159	225,231 (50%)	225 (50%)	227,235 (50%)	221,225 (50%)	225 (100%)	223,231 (50%)
TGLA227	72 (100%)	72 (66.7%)	72 (100%)	72 (100%)	72 (100%)	72 (100%)
TGLA263	122,126 (50%)	118 (66.7%)	118,120 (50%)	122,124 (50%)	122,126 (50%)	126 (100%)

Appendix 2.1 Continued

Locus	Population number					
	19	20	21	22	23	24
BM1824	169,183 (50%)	181 (75%)	185,191 (50%)	181 (25%)	191,197 (50%)	181,197 (25%)
BM3205	204,208 (50%)	204 (50%)	206 (100%)	204,208,210,212 (25%)	202,206 (50%)	202 (35.7%)
BM719	142,156 (50%)	134,148 (50%)	146 (100%)	146 (50%)	144,152 (50%)	148 (35.7%)
CSSM19	136,150 (50%)	146 (50%)	146 (100%)	146 (50%)	146,150 (50%)	148 (35.7%)
ETH10	204 (100%)	204 (100%)	204 (100%)	204 (100%)	204,206 (50%)	204 (91.7%)
ILSTS026	151,153 (50%)	159 (50%)	153 (100%)	149 (50%)	149,157 (50%)	145,149 153 (21.4%)
INRA128	176 (100%)	174 (100%)	168,176 (50%)	176 (75%)	168,176 (50%)	176 (57.1%)
INRA006	113 (100%)	113,115 (50%)	111,113 (50%)	115 (50%)	113 (100%)	113 (50%)
SPS115	229,233 (50%)	229,239 (50%)	237,239 (50%)	227,229,235,237 (25%)	239 (100%)	239 (50%)
TGLA159	223 (100%)	223,229,231,233 (25%)	223 (100%)	223,227,235,239 (25%)	223,235 (50%)	227 (40%)
TGLA227	72 (100%)	72 (75%)	72 (100%)	74 (50%)	72,74 (50%)	72 (57.1%)
iLA263	124,126 (50%)	124 (50%)	122 (100%)	122 (100%)	122,124 (50%)	

Appendix 2.1 Continued

Locus	Population number					
	25	26	27	28	29	30
BM1824	181 (23.3%)	187,193 (25%)	181 (58.3%)	181 (33.3%)	181 (56.3%)	195 (14.3%)
BM3205	202 (16.7%)	200,206,214 (25%)	214 (33.3%)	214 (22.7%)	204 (43.8%)	206 (72.3%)
BM719	148 (32.1%)	148 (62.5%)	152,154 (33.3%)	134 (25%)	154 (35.7%)	134,152 (21.4%)
CSSM19	146 (40%)	144 (37.5%)	146 (33.3%)	148 (29.2%)	146 (37.5%)	148, 150 (21.4%)
ETH10	204 (56.7%)	204 (87.5%)	204 (83.3%)	204 (95.5%)	204 (75%)	204 (85.5%)
ILSTS026	153,157 (20.8%)	157,163 (25%)	151 (33.3%)	163 (25%)	147 (25%)	149 (26.9%)
INRA128	174 (71%)	176 (87.5%)	176 (83.3%)	176 (54.2%)	176 (62.5%)	176 (75%)
INRA006	113 (56.7%)	111,113 (37.5%)	113 (66.7%)	113 (58.3%)	113 (43.8%)	113 (53.6%)
SPS115	237 (27.8%)	221,239 (33.3%)	235 (37.5%)	239 (41.7%)	239 (25%)	239 (27.3%)
TGLA159	227 (42.9%)	227,231 (50%)	223 (33.3%)	225,117 (27.8%)	223 (33.3%)	223 (33.3%)
TGLA227	72 (73.3%)	72 (75%)	72 (58.3%)	72 (75%)	72 (68.8%)	72 (57.1%)
TGLA263	122 (43.3%)	118 (50%)	118 (33.3%)	122 (58.3%)	122,126 (31.3%)	118 (28.6%)

Appendix 2.1 Continued

Locus	Population number					
	31	32	33	34	35	36
BM1824	181 (50%)	181 (37.5%)	179,187 (50%)	183,185 (50%)	183,187 (33.3%)	181,195 (50%)
BM3205	206,218 (50%)	202 (31.3%)	202,214 (50%)	206 (100%)	214 (33.3%)	212 (100%)
BM719	142,148,152,154 (25%)	148 (50%)	148,158 (50%)	134,148 (50%)	142 (50%)	144,148 (50%)
CSSM19	146,148 (50%)	146 (31.3%)	144 (100%)	146 (100%)	150 (50%)	148 (100%)
ETH10	204 (75%)	204 (87.5%)	204 (100%)	204 (100%)	204 (100%)	204 (100%)
ILSTS026	161 (50%)	153 (31.3%)	149,163 (50%)	151,153 (50%)	151 (50%)	151,157 (50%)
INRA128	176 (75%)	176 (75%)	176 (100%)	166,176 (50%)	178 (66.7%)	176 (100%)
INRA006	109 (50%)	113 (68.8%)	113 (100%)	113 (100%)	111,113,115 (33.3%)	113 (100%)
SPS115	237,239 (50%)	239 (40%)	239 (100%)	zero alleles	223,231,237,249 (25%)	241 (100%)
TGLA159	229 (50%)	227 (42.9%)	227,231 (50%)	223,227 (50%)	231 (33.3%)	223,225 (50%)
TGLA227	72 (75%)	72 (75%)	72 (100%)	72 (100%)	74 (50%)	74 (100%)
TGLA263	118,120 (50%)	120,122 (31.3%)	114,126 (50%)	118 (100%)	118 (66.7%)	122,126 (50%)

Appendix 2.1 Continued

Locus	Population number						
	37	38	39	40	41	42	43
BM1824	181 (33.3%)	185,193 (50%)	181,187 (50%)	181 (37.5%)	197 (25%)	181 (27.8%)	181,187,197 (20%)
BM3205	206,212 (33.3%)	210,218 (50%)	204 (100%)	204 (37.5%)	202 (37.5%)	214 (31.3%)	202 (32.1%)
BM719	146,148,154 (33.3%)	148,152 (50%)	148 (100%)	158 (37.5%)	148 (43.8%)	148 (33.3%)	152 (26.7%)
CSSM19	148 (50%)	zero alleles	140,150 (50%)	146 (37.5%)	144 (50%)	146 (33.3%)	146 (46.7%)
ETH10	204 (100%)	204,206 (50%)	204 (100%)	204 (62.5%)	204 (93.8%)	204 (88.9%)	204 (60%)
ILSTS026	159 (50%)	149,151 (50%)	145,159 (50%)	163 (66.7%)	149 (28.6%)	151,153,163 (16.7%)	151 (40%)
INRA128	166,176 (33.3%)	176 (100%)	109,113 (50%)	176 (62.5%)	176 (75%)	176 (83.3%)	176 (56.7%)
INRA006	113 (83.3%)	109,113 (50%)	168,176 (50%)	115 (37.5%)	113 (68.8%)	113 (81.3%)	113 (56.7%)
SPS115	225,237,239,249 (25%)	zero alleles	233,237 (50%)	221,227 (25%)	239 (37.5%)	229 (25%)	235 (38.9%)
TGLA159	227 (50%)	223,227 (50%)	225 (100%)	227 (50%)	227 (31.3%)	223 (28.6%)	227 (34.6%)
TGLA227	72 (83.3%)	72,74 (50%)	74,76 (50%)	76 (50%)	72 (62.5%)	72 (72.2%)	72 (56.7%)
TGLA263	118,124 (33.3%)	120,122 (50%)	118 (100%)	118 (37.5%)	122 (43.8%)	122 (44.4%)	122 (40%)

Appendix 2.2 Inbreeding coefficient *(Fis)* for each population for the 12 loci

	BM1824	BM3205	BM719	CSSm19	ETH10	ILSTS026	Inra 128	Inra006	SPS115	TGLA159	TGLA227	TGLA263	All
Pop1	-0.2	-0.043	-0.2	0.294	1	-0.043	NA	0.2	0.368	0.429	0	-0.286	0.124
Pop2	-0.5	0.333	-0.5	-0.333	0	0.217	0.5	0	0.2	0.6	0.625	-0.125	0.108
Pop3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Pop4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Pop5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop6	-0.136	-0.071	0.057	0.02	NA	0.02	-0.111	0.211	0.273	0.314	1	0.268	0.132
pop7	-0.071	-0.111	0.216	-0.151	-0.091	0.111	0.429	-0.091	0.667	-0.143	0.478	0.032	0.117
pop8	-0.091	0.667	0	-0.091	0.5	0.333	0.6	0.111	NA	0.5	NA	-0.2	0.234
pop9	0.065	0.2	-0.105	-0.108	1	0.158	0.294	0.262	0.273	0.048	0.489	-0.043	0.16
pop10	-0.053	-0.064	-0.053	-0.071	NA	0.107	0.706	-0.111	0.429	0.231	0.706	0.362	0.163
pop11	0.057	0.057	0.02	0	1	0.02	-0.067	-0.29	0	-0.132	0.394	-0.053	0.037
pop12	0	0.038	-0.064	0.583	-0.143	0.074	-0.111	-0.154	0.556	0.038	0	0.2	0.128
pop13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop14	0.333	0.2	0.6	-0.091	-0.143	0.333	-0.5	NA	0	0.111	0.5	0.5	0.178
pop15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop18	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop20	0	-0.333	1	-0.333	NA	-0.333	0.5	-1	NA	0	0	0.5	0.097
pop21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop22	-0.333	0	-0.333	0.5	NA	0.5	0	0.5	0	0	0.5	NA	0.167
pop23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop24	0.273	0	0.077	0.438	0	0.04	0.564	-0.034	-0.379	0.059	0.556	0.75	0.186
pop25	0.04	0.132	0.062	-0.077	0.375	0.072	0.548	-0.194	0.262	0.152	0.012	0.468	0.153
pop26	-0.091	0.182	-0.2	0.143	0	-0.091	0	0.7	0.667	-1	1	0.4	0.188
pop27	0.024	0.231	-0.087	0.02	-0.053	-0.154	-0.053	0.118	-0.2	-0.136	0.231	0.02	-0.004
pop28	-0.071		0.316	-0.08	0	-0.057	0.089	-0.135	-0.064	0.213	0.614	0.341	0.092

pop29	0.282		-0.029	0.023	0.391	0.176	0.382	0.176	-0.043	0.038	0.525	0.097	0.17
pop30	0.246		0.172	0.112	0.469	0.226	0.35	-0.217	0.282	-0.175	0.313	0.161	0.149
pop31	-0.333		0	1	NA	-0.333	0	0.5	NA	0.5	0	1	0.333
pop32	-0.217		-0.017	0.106	-0.077	-0.043	-0.167	0.051	-0.333	0.463	0.451	0.239	0.032
pop33	NA		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop34	NA		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop35	0.273		0.6	-0.333	NA	-0.5	1	1	0	0.333	0.111	0.5	0.273
pop36	NA		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop37	-0.091		0.2	0.2	NA	-0.333	0.273	0	0	0.2	0	0.273	0.059
pop38	NA		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop39	NA		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
pop40	0.429		0.143	-0.263	0.571	0.5	0.625	-0.6	0.5	0.2	-0.125	0.455	0.23
pop41	0.03		-0.126	0.157	0	0	0.176	0.276	0.455	-0.032	0.594	0.341	0.163
pop42	0.089		0.172	0.026	0.5	0.038	-0.091	-0.105	0.273	0.014	0.324	0.216	0.125
pop43	0.022		0.094	0.289	0.453	0.311	0.38	0.06	0.15	-0.152	0.533	-0.034	0.154

Appendix 2.3 Allele frequency distributions of the panel of 12 molecular markers obtained from 181 African buffalo sampled at random throughout the Kruger National Park.

Group 1																			
Locus																			
BM 1824	Allele	169	173	175	177	179	181	182	183	185	187	188	189	191	193	195	197	199	201
	Frequency	4.20	0.42	1.26		2.10	26.05	0.42	11.76	9.24	11.34	0.42	2.10	3.36	5.88	7.56	10.50	1.68	1.68
BM3205	Allele	198	200	202	204	206	208	210	212	214	216	218	220	222					
	Frequency	5.26	3.51	21.49	12.72	17.54	7.89	2.19	6.58	17.11	3.51	0.88	0.44	0.88					
BM719	Allele	134	138	140	142	144	146	148	150	152	154	156	158						
	Frequency	10.26	0.43	0.43	2.99	2.56	10.68	32.48		20.94	14.53	1.71	2.99						
CSSm19	Allele	128	134	136	138	140	142	144	146	148	150	152	154	156	158				
	Frequency	0.42	0.42	4.24	2.97	2.12	2.54	13.56	31.36	16.53	17.37	5.93	1.69	0.85					
ETH10	Allele	202	204	206	208	209	213												
	Frequency	4.20	80.25	11.76	2.94	0.42	0.42												
ILSTS026	Allele	137	141	145	147	149	151	153	155	157	159	161	163	165	167				
	Frequency	0.86	3.88	4.74	6.90	16.38	15.95	11.21	1.72	12.50	6.03	2.59	15.52	0.43	1.29				
Inra 128	Allele	109	113	115	164	166	168	170	172	174	175	176	178	180					
	Frequency	0.42		0.42	0.42	4.17	2.92	11.25	0.42	5.00	0.42	67.08	6.67	0.83					
Inra 006	Allele	105	107	109	111	113	115	117	119	168	176								
	Frequency	0.84	0.42	11.76	7.98	58.40	18.49		1.26		0.84								
SPS 115	Allele	221	223	225	227	229	231	233	235	237	239	241	243	245	247	248	249		
	Frequency	1.92	8.33	3.85	4.49	8.97	3.85	3.85	16.03	12.18	32.69		0.64	0.64		1.28	1.28		
TGLA 159	Allele	221	223	225	227	229	231	233	235	237	239								
	Frequency	2.43	20.87	16.02	31.55	2.91	14.56		9.71	1.46	0.49								

TGLA 227	Allele	70	72	74	76	77													
	Frequency	4.96	66.94	20.66	6.61	0.41	0.41												
TGLA 263	Allele	108	110	112	114	116	118	120	122	124	126	188							
	Frequency	0.41	1.24	0.41	2.89	1.24	26.86	8.26	40.08	8.26	10.33								
Group 2																			
Locus																			
BM 1824	Allele	169	173	175	177	179	181	182	183	185	187	188	189	191	193	195	197	199	201
	Frequency	5.56		4.17	4.17		23.61		4.17	8.33	11.11		5.56	6.94	5.56	4.17	11.11	2.78	2.78
BM3205	Allele	198	200	202	204	206	208	210	212	214	216	218	220	222					
	Frequency	2.86	1.43	24.29	12.86	17.14	10.00	2.86	5.71	18.57	1.43	2.86							
BM719	Allele	134	138	140	142	144	146	148	150	152	154	156	158						
	Frequency	8.33	1.39	1.39	8.33	5.56	8.33	19.44	8.33	12.50	22.22	1.39	2.78						
CSSm19	Allele	128	134	136	138	140	142	144	146	148	150	152	154	156	158				
	Frequency			2.63	7.89	3.95	7.89	3.95	23.68	22.37	19.74	2.63	3.95	1.32					
ETH10	Allele	202	204	206	208	209	213												
	Frequency	4.05	89.19	2.70	4.05														
ILSTS026	Allele	137	141	145	147	149	151	153	155	157	159	161	163	165	167				
	Frequency		4.41	2.94	8.82	13.24	20.59	11.76	4.41	4.41	10.29	2.94	14.71		1.47				
Inra 128	Allele	109	113	115	164	166	168	170	172	174	175	176	178	180					
	Frequency					1.35	12.16	6.76	2.70	2.70		60.81	13.51						
Inra 006	Allele	105	107	109	111	113	115	117	119	168	176								
	Frequency			18.42	6.58	55.26	17.11	2.63											
SPS 115	Allele	221	223	225	227	229	231	233	235	237	239	241	243	245	247	248	249		
	Frequency		5.56	5.56	2.78	16.67	5.56	2.78	11.11	22.22	22.22	5.56							

TGLA 159	Allele	221	223	225	227	229	2				239								
	Frequency		24.24	16.67	27.27	12.12	10.61		6.06	3.03									
TGLA 227	Allele	70	72	74	76	77	79												
	Frequency	2.63	68.42	23.68	5.26														
TGLA 263	Allele	108	110	112	114	116	118	120	122	124	126	188							
	Frequency		1.32	1.32	3.95	1.32	22.37	13.16	39.47	6.58	7.89	2.63							
Group 3																			
Locus																			
BM 1824	Allele	169	173	175	177	179	181	182	183	185	187	188	189	191	193	195	197	199	201
	Frequency	2.27			2.27	2.27	34.09		9.09	9.09	6.82		2.27	6.82	6.82	4.55	11.36	2.27	
BM3205	Allele	198	200	202	204	206	208	210	212	214	216	218	220	222					
	Frequency	4.55	2.27	20.45	27.27	18.18	9.09	4.55	4.55	4.55		2.27		2.27					
BM719	Allele	134	138	140	142	144	146	148	150	152	154	156	158						
	Frequency	9.09		2.27	2.27	9.09	13.64	29.55		15.91	13.64	4.55							
CSSm19	Allele	128	134	136	138	140	142	144	146	148	150	152	154	156	158				
	Frequency		2.27	11.36	4.55			11.36	38.64	9.09	11.36	4.55		4.55	2.27				
ETH10	Allele	202	204	206	208	209	213												
	Frequency	2.38	85.71	11.90															
ILSTS026	Allele	137	141	145	147	149	151	153	155	157	159	161	163	165	167				
	Frequency		2.38	2.38	4.76	30.95	9.52	11.90	4.76	9.52	11.90		11.90						
Inra 128	Allele	109	113	115	164	166	168	170	172	174	175	176	178	180					
	Frequency					4.55	15.91			4.55		56.82	15.91	2.27					
Inra 006	Allele	105	107	109	111	113	115	117	119	168	176								
	Frequency			11.36	2.27	56.82	27.27		2.27										

SPS 115	Allele	221	223	225	227	229	2				239	241	243	245	247	248	249		
	Frequency	5.88		5.88	8.82	11.76	2				29.41								
TGLA 159	Allele	221	223	225	227	229	231	233	235	237	239								
	Frequency	6.82	22.73	18.18	18.18	9.09	9.09	2.27	11.36		2.27								
TGLA227	Allele	70	72	74	76	77	79												
	Frequency	4.55	75.00	20.45															
TGLA 263	Allele	108	110	112	114	116	118	120	122	124	126	188							
	Frequency				4.76		19.05	7.14	40.48	14.29	14.29								

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Chapter 3

Relatedness evaluation of BTB infected and BTB uninfected African buffalo *(Syncerus caffer)* sampled in the Kruger National Park

Abstract

The African buffalo is host to a variety of diseases such as bovine tuberculosis (BTB), foot-and-mouth disease (FMD) and corridor disease (CD) (Greyling, 2007). Disease has influenced African buffalo populations throughout the continent and more specifically the Kruger National Park (KNP) and is largely responsible for the fact that buffaloes are restricted to enclosed areas with strict regulations imposed on their movement. The social organization of animals influences the distribution and spread of a disease (Blanchong *et al*., 2007), especially in the case of the African buffaloes in the KNP (Greyling, 2007). The emergence of BTB in one of the largest conservation areas in South Africa (the KNP), pose a threat to the survival of African buffaloes as well as to the survival of several endangered species (Michel *et al*., 2006). Concerns have also been raised at the wildlife-livestock-human interface, where spill-over of the infection from wildlife to neighbouring communal cattle, may affect human health conditions (Michel *et al*., 2006). The potential economic losses to the livestock and wildlife industries, associated with this disease are excessive.

In this study, data was divided into various groups based on the BTB culture status of the individuals as well as the location of their sampling collection site within the KNP. The computer based programme GENAlEX ver 6.2 was used to evaluate the familial relationships among the African buffalo populations within the KNP based on pairwise genetic relatedness calculations. These familial relationships include half siblings, full siblings and parent-offspring

which were classified according to Queller and Goodnight's estimates (Queller and Goodnight, 1989). The mean level of relatedness was calculated at different levels, which include an intra- and inter-population level, among all pairs of BTB infected and BTB uninfected African buffalo populations within the KNP and among BTB infected and BTB uninfected African buffaloes based on the relevant BTB prevalence groups from which they were sampled within the KNP.

Results presented in this chapter are based on a relatively small data set and provide baseline information into the genetic relationships, BTB culture status and transmission as well as BTB strains associated with the sampled buffaloes from the KNP.

1. Introduction

The African buffalo (*Syncerus caffer)* is, according to the IUCN, a lower risk conservation dependant species, affected by habitat fragmentation and loss, hunting pressures and disease (Van Hooft *et al*., 2002).

Bovine Tuberculosis (BTB) is a chronic, progressive bacterial disease caused by *Mycobacterium bovis*, currently re-emerging worldwide, in both wildlife and livestock. Most mammalian species are susceptible to the disease (Cross and Getz, 2006). The number of wild African species affected by BTB is currently on the rise (Renwick *et al*., 2006). BTB was first diagnosed in cattle in South Africa in 1880 and in wildlife in 1928 (Renwick *et al*., 2006). The greater kudu (*Tragelaphus strepsiceros*) in the Eastern Cape region, was the first reported wildlife species to be infected with the disease (Renwick *et al*., 2006). It is suspected that BTB was transmitted by domestic cattle to African buffaloes during the 1950's in the south eastern corner of the KNP before fencing of the southern boundary of the reserve had been completed (Michel *et al*., 2009). The disease was however only discovered in the KNP in 1990 (Renwick *et al*., 2006). By 1992 prevalence of the disease was estimated at 27.1 % in the southern region, 4.4 % in the central region and 0 % in the northern regions of the Park. By 1998, prevalence of the disease drastically increased to 38.2 %

in the southern and 16 % in the central regions of the Park (Michel *et al*., 2006). The disease has, therefore, spread in a northerly direction, with an high prevalence in the southern region of the Park (Michel *et al*., 2006). BTB development and spread throughout a population is affected by several factors, including individual resistance, nutritional status, seasonal fluctuations and changes in environmental and body condition (Cross and Getz, 2006). Potential for disease transmission between domestic animals and susceptible wildlife, and *vice versa*, is the highest when pasture or territory is communally shared (Renwick *et al*., 2006). The two main transmission routes of BTB include the alimentary and respiratory pathways (Renwick *et al*., 2006). The spread of *M. bovis* within ruminants and large carnivores is a relatively slow process and clinical signs may take years to appear. Most infected animals are sub-clinically affected and only once advanced stages of the disease have been reached, disseminated lesions will appear. The severity of the disease is associated with the infectious dose, route of infection and immune status of the individual (Renwick *et al*., 2006).

Microsatellite markers are ideal for establishing familial relationships as well as the genetic structure of a population (Taylor *et al*., 1997). Although specific microsatellite markers have not yet been developed for the African buffalo, numerous microsatellite markers have been characterized in domestic cattle which can be applied to population genetic studies related to the African buffalo (Greyling, 2007).

Relatedness has been defined by Hamilton as the expected proportion of alleles between two individuals that are identical by descent. Different levels of relatedness exist (Hamilton, 2009). A recent study conducted on white-tailed deer *(Odocoileus virginianus)* in Michigan in the United States, incorporated molecular genetic markers to determine relatedness among individuals and test whether BTB infected deer were more closely related than non-infected deer (Blanchong *et al*., 2007). Their work suggests that contact among related deer, within a particular population or social group, contributed to BTB transmission, since individuals that were more closely related, exhibited higher levels of disease prevalence than non-related individuals

(Blanchong *et al*., 2007). During the course of our study, relatedness was determined at various levels to determine whether BTB infected African buffaloes were more closely related to one another than to BTB uninfected African buffaloes within the KNP.

2. Materials and Methods

2.1 Animals and Samples

Sampled buffalo groups and populations from the different regions of the KNP (southern, central and northern regions) were selected for sampling (based on the sample collector's preference) over a time interval of 11 years, for BTB disease monitoring and management purposes by the South African National Parks (SANparks). Samples collected from Black Heron, Cork, Dzombo, Letaba, Kumana Dam, Macetse, Malahlapanga, Maloponyane, Mpongolo, Nyunyani, Shawu, Stamp-en-Stoot and Woodlands were sampled using live sampling. The number of infected and uninfected African buffalo samples, the location where they were sampled, as well as year of collection has previously been described in chapter two (Table 2.1). The age, gender and BTB culture status of several of our samples was not known. As mentioned in chapter 2, the term population has been used to describe a small collection of buffaloes sampled from the same location.

3. Genomic amplification and characterization

3.1 DNA extraction and Multiplex PCR conditions

DNA extraction was conducted with the Puregene Kit (Gentra Systems), at the Agricultural Research Council – Onderstepoort Veterinary Institute (ARC-OVI), as described in chapter two. The DNA extracts were stored at the Veterinary Genetics Laboratory (VGL), Onderstepoort on behalf of SANparks. A panel of 12 microsatellite markers was used and placed into three core multiplexes (Table 2.2). Reaction conditions were optimized and executed according to Greyling *et al* (2008). Table 2.3 is a summary of each multiplex,

fluorescent labels applied and primers used with their forward primer sequences as well as their reverse primer sequences.

4. Data analysis

4.1 Fragment and genetic data analysis

Data generated by the 3130xl Genetic Analyzer was analyzed using STRand software to produce the genotypes used for further population analysis. (Toonen and Hughes, 2001) <http://www.vgl.ucdavis.edu/STRand>

The computer based programme GENAlEX version 6.2 (Peakall and Smouse, 2006) was used for the statistical analysis of data. Pairwise genetic relatedness was calculated at three different levels, to determine the familial relationships, based on the mean level of relatedness shared between individuals. The mean level of relatedness was calculated based on 99 permutations and 1000 bootstraps, with an upper and lower confidence level of 95 %. These levels included an intra- and inter-population level, among all pairs of BTB infected and BTB uninfected African buffalo populations within the Park and lastly among BTB infected and BTB uninfected African buffaloes, based on the relevant prevalence groups within the Park from which they were sampled. Genetic relatedness was calculated from genetic markers using the method developed by Queller and Goodnight (1989). Relatedness parameters based on Queller and Goodnight's (1989) work included unrelated individuals ($r = 0$) in terms of half-sib, full-sib and parent-offspring relationships, half siblings ($r \geq 0.25$) and full siblings or parent-offspring ($r \geq 0.5$). Coefficient of relatedness values ranged from -1 to +1, with a value of zero equal to a pair that is no more or less related than average relatedness within the population (Queller and Goodnight, 1989). The different BTB strains (dominant C8 and variant C8v) were also assessed to determine whether buffaloes infected with the same strain of BTB were more closely related than uninfected buffaloes, or those infected with a different strain within a single population.

5. Results and Discussion

Pairwise relatedness evaluation was conducted on three different levels, firstly on an intra- and inter -population level across the entire sample set, secondly among all pairs of BTB infected and BTB uninfected African buffalo populations within the Park and thirdly among BTB infected and BTB uninfected African buffaloes based on their relevant prevalence groups within the Park. The third pairwise relatedness evaluation was conducted to determine the importance of geographical proximity in relation to distribution and spread of the disease. The BTB culture status of each individual (with known BTB culture status) was evaluated to determine whether any correlation between the genetic relatedness of African buffaloes within the KNP and their disease status could be established.

5.1 Intra- and inter – population evaluation of pairwise relatedness across the entire sample set

5.1.1 Intra - population relatedness evaluation

The mean level of relatedness within populations (sampled in the KNP with a sample size greater than two individuals) was calculated. Sampled populations and the number of samples collected at each sample site are represented in Table 3.1.

Table 3.1 Sampled populations (consisting of more than two individuals), with the number of samples collected within each population

Population number	Population name	Number of samples collected (N)
1	Mahlanganzwane Dam	4
2	Duke	4
6	Manzimhlope	6
7	Rietpan	7
8	Silolweni Dam	3
9	Mafortini	7
10	Sonop windmill	6
11	Gudzane Dam	6
12	Letaba	6
14	Macetse	3
24	Byamiti spruit	7
25	Nwatimhiri	15
26	Nyamundwa	4
27	Lukimbi Lodge	6
28	Mpanamane	12
29	Zambhala	8
30	Blinkwater windmill	14
32	Lower Sabie	8
35	MtemushaMhlandanyati	3
41	Nzikazi spruit	8
42	Napi Boulder	9
43	Stolznek Dam	15

The mean level of relatedness among all populations varied between -0.113 and 0.097. The mean level of relatedness and associated upper and lower confidence intervals (95 %), within each sampled population, indicates that no individual population had a notably higher relatedness value than another population. Populations with marginally higher familial relationships include those sampled at Manzimhlope (central KNP), Lower Sabie (southern KNP), Mahlanganzwane Dam (southern KNP) and Rietpan (central KNP), which are presented in bolded text in Table 3.2. Populations 1, 8, 14 and 35, have a relatively large variance between their upper and lower confidence levels which may be as a result of a sample size artefact (Table 3.2).

Table 3.2 Mean level of relatedness within each sampled population that included more than two sampled animals

Population number	Prevalence group region	Number of samples (N)	[1]Mean	[2]L	[3]U	[4]Mean value between L and U confidence interval
1	South	4	**0.077**	-0.164	0.218	0.027
2	South	4	-0.01	-0.225	0.157	-0.034
6	Central	6	**0.09**	-0.172	0.134	-0.019
7	Central	7	**0.076**	-0.134	0.132	-0.001
8	Central	3	-0.047	-0.284	0.301	0.0085
9	Central	7	-0.113	-0.15	0.11	-0.02
10	Central	6	-0.055	-0.147	0.121	-0.013
11	Central	6	-0.035	-0.198	0.147	-0.0255
12	North	6	0.014	-0.171	0.127	-0.022
14	North	3	-0.096	-0.248	0.206	-0.021
24	South	7	0.007	-0.135	0.139	0.002
25	South	15	-0.053	-0.073	0.079	0.003
26	South	4	0.039	-0.248	0.147	-0.0505
27	South	6	0.03	-0.173	0.13	-0.0215
28	South	12	0.031	-0.116	0.097	-0.0095
29	South	8	0.051	-0.108	0.088	-0.01
30	South	14	-0.039	-0.104	0.064	-0.02
32	South	8	**0.097**	-0.143	0.104	-0.0195
35	South	3	-0.036	-0.312	0.272	-0.02
41	South	8	0.067	-0.115	0.079	-0.018
42	South	9	0.043	-0.111	0.104	-0.0035
43	South	15	-0.049	-0.092	0.073	-0.0095

[1]Mean represents the mean level of relatedness as calculated within each sampled population consisting of more than two animals

[2]L represents the lower confidence level (95 %)

[3]U represents the upper confidence level (95 %)

[4]Mean value calculated between lower and upper confidence intervals

5.1.2 Inter – population relatedness evaluation

A pairwise relatedness evaluation between all 181 animals and 39 populations in the KNP was performed, based on Queller and Goodnight's (1989) coefficient of relatedness.

Results show that from the 16 289 pairwise comparisons made between African buffaloes sampled in the KNP, 1293 half sib pairs and 64 full sib pairs or parent-offspring pairs, were identified. The greater majority of our sampled African buffaloes were, therefore, unrelated in terms of sibling and parent-

offspring relationships (approximately 14 932 unrelated pairwise comparisons were observed). A total of 8 % were, therefore, half sibs and 0.4 % full sibs or parent-offspring related. As can be expected, the greater majority of individuals were unrelated at 91.6 %. The relatedness value of $r \geq 0.5$, may suggest either full sib or parent-offspring relationships. It is, however, more likely that this value of 0.4 % represents the level of parent-offspring relationships throughout the sampled populations. These outcomes may have been influenced by the fact that samples were collected during different time periods (different years). As a result of our lack of sufficient age and gender data, we can only speculate reasons for parent-offspring relatedness. Adult bulls may migrate between populations and thus have offspring in more than one population. Migrating heifers may also contribute to parent-offspring relatedness between herself and her parents in her native herd. The level of relatedness ($r \geq 0.5$) may also be as a result of young bulls (in bachelor herds) that migrate to new populations (during the breeding season), with half or full siblings in their population of origin or due to a chance event.

5.2 Pairwise relatedness evaluation of BTB infected and BTB uninfected African buffalo populations

The sample set of 181 African buffaloes was further divided into two groups, the BTB infected group and the BTB uninfected group. Individuals within populations were grouped according their BTB culture status. Pairwise relatedness evaluation was conducted within each population associated with a particular group (BTB infected or BTB uninfected) as well as across all populations associated with a particular group. Pairwise relatedness was, therefore, calculated between all pairs of BTB infected buffalo as well as between all pairs of BTB uninfected buffalo within populations, as well as across all populations.

5.2.1 BTB infected group

The BTB infected group consists of 101 individuals. Table 3.3 represents populations within the infected group, along with the number of infected

individuals associated with each particular population and the total number of individuals sampled per population.

Table 3.3 The number of BTB infected individuals per population

Population number	Population name	No. of BTB infected buffalo	Total number of buffalo sampled
1	Mahlanganzwane Dam	4	4
2	Duke (Mahlangazwane)	4	4
3	Mpanamana Dam	1	1
6	Manzimhlope	5	6
7	Rietpan	3	7
8	Silolweni Dam	2	3
9	Mafortini	5	7
10	Sonop windmill	4	6
11	Gudzane Dam	2	6
12	Letaba	3	6
13	Mhlangazwane	1	1
14	Macetse	1	3
16	Shawu	1	1
17	Woodlands	1	1
18	Black Heron	1	1
19	Maloponyane	1	1
21	Dzombo	1	1
22	Mpongolo	1	2
23	Malahlapanga	1	1
24	Byamiti Spruit	4	7
25	Nwatimhiri	5	15
26	Nyamundwa	2	4
27	Lukimbi Lodge	3	6
28	Mpanamane	6	12
29	Zambhala	4	8
30	Blinkwater windmill	7	14
31	Satara	2	2
32	Lower Sabie	4	8
33	Cork	1	1
34	Lupisi Gate	1	1
35	MtemushaMhlandanyati	3	3

36	Kumana Dam	1	1
41	Nzikazi Spruit	5	8
42	Napi Boulder	3	9
43	Stolznek Dam	8	15

Results indicated that certain populations exhibited slightly higher levels of relatedness than others and *vice versa*. The mean level of relatedness among all BTB infected animals, across all populations varied between - 0.184 and 0.154 (Table 3.4). The three populations with the highest levels of relatedness among infected individuals include Lower Sabie (southern KNP), Napi Boulder (southern KNP) and Rietpan (central KNP) as indicated in bolded text in Table 3.4. The age and gender information of several individuals from these populations was not available, which made it difficult/impossible to elaborate on possible age/sex affects that influence relatedness.

Pairwise relatedness was also calculated between individuals across the whole BTB infected group, across all populations to reveal familial and genetic relationships. From the 5050 pairwise comparisons made between BTB infected African buffalo samples, 383 half sib pairs (7.5 %) and 14 full sib pairs or parent-offspring pairs (0.27 %) were identified. A total of 397 pairwise relationships (sibling/parent-offspring) were, therefore, observed among the sampled BTB infected individuals across all populations associated with the BTB infected group. Only 7.77 % of sampled BTB infected individuals were genetically related, the greater majority (92.23 %) of sampled BTB infected buffaloes can, therefore, be regarded as unrelated in terms of sibling and parent-offspring relatedness. Appendix 3.1 represents the full sibling or parent-offspring relatedness associated with the BTB infected individuals across all populations, with the particular prevalence group region from which they were sampled. Sample ID 1 and sample ID 2 refer to individual buffaloes sampled and compared to one another with their mean level of relatedness calculated based on Queller and Goodnight's (1989) classification. Appendix 3.2 represents the level of sibling relatedness as estimated across the BTB infected African buffalo group. This Appendix consists of 4 columns (A, B, C, D), each representing two samples (Sample ID 1 and Sample ID 2) that have

been compared and the mean level of relatedness calculated between them. For example sample 656 and sample 667 have a mean relatedness value of 0.262.

GENAIEX ver 6.2 was used to determine the mean level of relatedness between all individuals associated with the BTB infected group. Results indicate an overall mean level of relatedness, calculated across sampled populations associated with the BTB infected group, to be 0.013. A relatively low level of relatedness can therefore be expected between individuals associated within BTB infected populations (Table 3.4).

Table 3.4 Mean levels of relatedness among BTB infected individuals within populations

Population number and name	[1]No. BTB infected animals	[2]No. animals sampled per population	[3]Mean	[4]U	[5]L
1 Mahlanganzwane Dam	4	4	0.075	0.163	-0.207
2 Duke (Mahlangnzwane)	4	4	-0.015	0.155	-0.231
6 Manzimhlope	5	6	0.121	0.173	-0.167
7 Rietpan	3	7	**0.134**	0.237	-0.247
9 Mafortini	5	7	-0.097	0.187	-0.175
10 Sonop windmill	4	6	-0.093	0.198	-0.192
12 Letaba	3	6	-0.184	0.221	-0.341
24 Byamiti Spruit	4	7	0.033	0.185	-0.226
25 Nwatimhiri	5	15	-0.068	0.157	-0.184
27 Lukimbi Lodge	3	6	0.034	0.226	-0.339
28 Mpanamane	6	12	0.075	0.154	-0.173
29 Zambhala	4	8	-0.043	0.2	-0.218
30 Blinkwater Windmill	7	14	-0.038	0.092	-0.1
32 Lower Sabie	4	8	**0.154**	0.173	-0.193
35 Mtemusha-Mhlandanyati	3	3	-0.043	0.267	-0.276
41Nzikazi Spruit	5	8	0.102	0.173	-0.124
42 Napi Boulder	3	9	**0.146**	0.257	-0.25
43 Stolznek Dam	8	15	-0.062	0.131	-0.098
[6]N			**18**		
[7]Mean			**0.013**		
[8]SE			**0.023**		
[9]Min.			**-0.184**		
[10]Max.			**0.154**		

[1]No. of BTB infected animals represents the number of BTB infected individuals within each sampled population associated with the BTB infected group

[2]No. of animals per population represents the total number of animals sampled within each population

[3]Mean represents the mean level of relatedness as calculated among infected buffalo within each sampled, population (associated with the BTB infected group) consisting of more than two animals

[4]U represents the upper confidence level (95%)

[5]L represents the lower confidence level (95%)

[6]N represents the total number of populations associated with the BTB infected group

[7]Mean represents the mean level of relatedness among all sampled, infected individuals associated with the BTB infected group

[8]SE represents the standard error

[9]Min. represents the minimum, mean level of relatedness measured across all BTB infected individuals measured across all populations associated with the BTB infected group

[10]Max. represents the maximum, mean level of relatedness measured across all BTB infected individuals measured across all populations associated with the BTB infected group

5.2.2 BTB uninfected group

The BTB uninfected group consists of 80 BTB uninfected individuals. Table 3.5 represents populations associated with the uninfected group along with the number of uninfected individuals within each particular population and the total number of individuals associated with a particular population.

Table 3.5 The number of BTB uninfected individuals per population

Population number	Population name	*No. of BTB uninfected buffalo	*Total number of buffalo sampled
4	Langtoon Dam	1	1
5	Kostini	1	1
6	Manzimhlope	1	6
7	Rietpan	4	7
8	Silolweni Dam	1	3
9	Mafortini	2	7
10	Sonop windmill	2	6
11	Gudzane Dam	4	6
12	Letaba	3	6
14	Macetse	2	3
15	Stamp en stoot	1	1
20	Nyunyani	2	2
22	Mpongolo	1	2
24	Byamiti Spruit	3	7
25	Nwatimhiri	10	15
26	Nyamundwa	2	4
27	Lukimbi Lodge	3	6
28	Mpanamane	6	12
29	Zambhala	4	8
30	Blinkwater windmill	7	14
32	Lower Sabie	4	8
41	Nzikazi Spruit	3	8
42	Napi Boulder	6	9
43	Stolznek Dam	7	15

[1]No. of BTB un infected animals found within each population

[2]Total number of buffalo sampled within each population

The mean level of relatedness among uninfected individuals within populations, ranged between -0.153 and 0.374 (Table 3.6). Results indicate that individuals within certain populations may be slightly more related than others. The three populations with the highest levels of relatedness among uninfected individuals are represented in bolded text in Table 3.6 and include individuals associated with Letaba (northern KNP), Lukimbi Lodge (southern KNP) and Zambhala (southern KNP). Age and sex information with regards to these and other populations were not available, which made it difficult to elaborate on age and sex related effects on levels of relatedness.

As with the infected group, pairwise relatedness was also calculated between individuals across the whole BTB uninfected group, across all populations. From the 3240 pairwise comparisons made, 221 half sib pairs and 12 full sib pairs or parent-offspring pairs were identified. Only 7.19 % of the pairwise comparisons (233 pairwise comparisons) revealed relatedness among uninfected individuals. The greater majority of uninfected African buffaloes can thus be regarded as unrelated in terms of sibling or parent-offspring relationships. Appendix 3.3 represents the level of relatedness (full sibling/parent-offspring) among uninfected individuals across all sampled populations associated with the uninfected group, with the particular prevalence group region from where each sample was collected. Sample ID 1 and sample ID 2 refer to individual buffalo sampled and compared to one another with their mean level of relatedness calculated based on Queller and Goodnight's classification. Appendix 3.4 represents the half sibling relatedness estimated between all uninfected individuals, across all populations. This Appendix consists of 4 columns (A, B, C, D), each column representing two samples (Sample ID 1 and Sample ID 2) that have been compared and the mean level of relatedness between them determined.

GENAIEX ver 6.2 was used to calculate an overall mean level of relatedness across populations associated with the BTB uninfected group. The mean level of relatedness across the entire BTB uninfected group was 0.012 (Table 3.6). A relatively low level of relatedness can therefore be expected among individuals associated with the BTB uninfected group.

Table 3.6 Mean level of relatedness within populations consisting of more than two individuals, associated with the BTB uninfected group

Population number and name	[1]No. of BTB uninfected animals	[2]No. animals sampled per population	[3]Mean	[4]U	[5]L
7 Rietpan	4	7	0.065	0.134	-0.257
11 Gudzane Dam	4	6	0.002	0.202	-0.254
12 Letaba	3	6	**0.374**	0.218	-0.466
24 Byamiti Spruit	3	7	-0.153	0.215	-0.317
25 Nwatimhiri	10	15	-0.064	0.084	-0.131
27 Lukimbi Lodge	3	6	**0.095**	0.262	-0.381
28 Mpanamane	7	12	0.048	0.093	-0.191
29 Zambhala	4	8	**0.075**	0.176	-0.203
30 Blinkwater Windmill	7	14	-0.074	0.116	-0.166
32 Lower Sabie	4	8	0.042	0.177	-0.236
41 Nzikazi Spruit	3	8	-0.077	0.247	-0.362
42 Napi Boulder	6	9	-0.032	0.111	-0.187
43 Stolznek Dam	7	15	-0.142	0.105	-0.167
[6]N			**13**		
[7]Mean			**0.012**		
[8]SE			**0.038**		
[9]Min.			**-0.153**		
[10]Max.			**0.374**		

[1]No. of BTB uninfected animals represents the number of BTB uninfected individuals within each sampled population associated with the BTB uninfected group

[2]No. of animals per population represents the total number of animals within each population

[3]Mean represents the mean level of relatedness as calculated among uninfected buffalo within each sampled population (associated with the BTB uninfected group) consisting of more than two individuals

[4]U represents the upper confidence level (95%)

[5]L represents the lower confidence level (95%)

[6]N represents the total number of populations associated with the uninfected group

[7]Mean represents the mean level of relatedness among all the sampled uninfected individuals associated with the BTB uninfected group

[8]SE represents the standard error

[9]Min. represents the minimum, mean level of relatedness measured across all BTB uninfected individuals measured across all populations associated with the BTB uninfected group

[10]Max. represents the maximum, mean level of relatedness measured across all BTB uninfected individuals measured across all populations associated with the BTB uninfected group

5.2.3 BTB infected and BTB uninfected group comparisons

To determine whether the level of relatedness between BTB infected and BTB uninfected African buffaloes were statistically different, populations associated with both groups were compared (Table 3.7). Comparisons were based on the mean level of relatedness calculated between infected and uninfected individuals within populations. The levels of relatedness between infected and uninfected individuals within the same population, varied amongst populations (Table 3.7). For instance, populations 7, 24, 32, 41 and 42, infected individuals exhibited a higher level of relatedness than uninfected individuals associated with the same populations. Within populations 12, 27 and 29, uninfected individuals were more highly (closely) related compared to infected individuals associated with the same populations. The levels of genetic relatedness between infected and uninfected individuals within populations 25, 30 and 43, were similar.

No statistically significant difference could be demonstrated between the mean level of relatedness calculated between BTB infected and BTB uninfected animals, sampled from the same populations (Table 3.7).

Table 3.7 Mean level of relatedness associated with BTB infected and BTB uninfected populations within the same populations

	BTB infected group		BTB uninfected group		
Population number	[1]No. BTB infected animals	[2]Mean	[3]No. of BTB uninfected animals	[4]Mean	[5]P-value
7	3	0.134	4	0.065	0.319
12	3	-0.184	3	0.374	0.696
24	4	0.033	3	-0.153	0.419
25	5	-0.068	10	-0.064	0.281
27	3	0.034	3	0.095	0.175
28	6	0.075	7	0.048	0.335
29	4	-0.043	4	0.075	0.191
30	7	-0.038	7	-0.074	0.385
32	4	0.154	4	0.042	0.512
41	5	0.102	3	-0.077	0.254
42	3	0.146	6	-0.032	0.451
43	8	-0.062	7	-0.142	0.728

[1]No. of BTB infected animals represents the number of sampled BTB infected individuals within populations (associated with the BTB infected group) consisting of more than two animals

[2]Mean represents the mean level of relatedness among sampled infected individuals within populations associated with the infected group

[3]No. of BTB uninfected animals represents the number of sampled BTB uninfected individuals (associated with the BTB uninfected group) consisting of more than two animals

[4]Mean represents the mean level of relatedness among sampled uninfected individuals within populations associated with the uninfected group

[5]P-value relating to the statistical significance in the difference between the mean values of BTB infected and BTB uninfected animals, associated with similar populations

A comparison between the mean level of relatedness among all individuals (BTB infected and BTB uninfected groups), across all populations is summarized in Table 3.8. Group statistics information based on the number of populations associated with each group, the mean level of relatedness among all populations associated with each group, the standard error and the minimum and maximum mean values associated with each particular group is presented. No statistically significant difference could be demonstrated

between the overall mean level of relatedness within each group (0.013 and 0.012, p = 0.108, student's t-test).

Table 3.8 Summary of the group statistics information relating to the mean level of relatedness among individuals associated with the BTB infected group and BTB uninfected group respectively

	BTB infected group statistics	BTB uninfected group statistics
***N**	101	80
***Mean**	0.013	0.012
***SE**	0.023	0.038
***Min.**	-0.184	-0.153
***Max.**	0.154	0.374

[1]**N represents the total number of BTB infected and BTB uninfected individuals within populations associated with each group**

[2]**Mean represents the mean level of relatedness among all BTB infected and BTB uninfected individuals within a population consisting of more than two individuals, respectively**

[3]**SE represents the standard error for both groups**

[4]**Min represents the minimum, mean level of relatedness measured across all BTB infected and BTB uninfected individuals at a 95 % CI**

[5]**Max represents the maximum, mean level of relatedness measured across all BTB infected BTB uninfected individuals at a 95 % CI**

5.3 Pairwise relatedness evaluation of BTB infected and BTB uninfected African buffalo groups based on their relevant prevalence group

As our hypothesis stated, we propose that *M. bovis* infected buffaloes are more closely related to each other than to uninfected buffaloes in the same population or prevalence group. This evaluation was conducted to determine whether geographical proximity influenced BTB relatedness among African buffaloes, sampled in the same location or BTB prevalence area. The infected and uninfected African buffalo groups were, therefore, further divided into three prevalence groups (Fig 2.5).

5.3.1 Infected African buffalo group

Prevalence group one (southern KNP region), consists of 66 infected individuals. From the 2144 pairwise relatedness combinations, 10 full sibs or parent-offspring pairs and 145 half sib pairs were identified based on Queller and Goodnight's classification system (Queller and Goodnight, 1989). In the southern region of the KNP, a total of 0.46 % full sib pairs or parent-offspring pairs were BTB infected and 6.7 % half sibs were BTB infected. Prevalence group two (central KNP region), consists of 24 infected individuals. Pairwise relatedness estimates of buffaloes associated with prevalence group two revealed no full sib or parent-offspring relationships and 18 half sib pairs from the 275 pairwise comparisons. The central KNP therefore, consists of approximately 6.5 % BTB infected half sibs. Prevalence group three (northern KNP region), consists of 11 infected buffaloes. From the 54 pairwise comparisons made between infected buffaloes in the northern region of the KNP, no full sib or parent-offspring pairs were observed. Two half sib pairs (3.7 %) were, however, identified. A higher level of genetic relatedness and familial relationships among BTB infected buffaloes was shared among individuals associated with the southern region of the KNP (7.16 %), compared to those sampled from the central (6.5 %) and northern regions (3.7 %). This may be as a result of the lower number of individuals sampled from each region (more samples were collected from the southern regions of the KNP, 66 individuals), compared to samples collected from the central and northern regions, 24 and 11 individuals respectively. Previous studies have shown that the prevalence of BTB among buffalo populations from the southern regions of the KNP is higher, compared to populations from the central and northern regions (Michel *et al.*, 2009), hence a higher probability exists for sampling infected individuals in the south of the Park.

Appendix 3.1 represents the sibling or parent-offspring relationships between BTB infected animals. Most pairwise comparisons revealed, that a genetic relationship between individuals, originated from the southern region of the KNP. Two pairwise comparisons, however, revealed relationships between samples originating from the southern regions and samples originating from

the central regions of the KNP. The African buffalo group organization or male migration may be responsible for the above mentioned familial relationships associated between the different prevalence regions.

5.3.2 Uninfected African buffalo group

Prevalence group one (southern KNP) associated with the uninfected African buffalo populations consists of 57 uninfected individuals. Pairwise relatedness estimates revealed one full sib pair or parent-offspring pair and 108 half sib pairs from the pairwise relatedness comparisons made. In the southern region of the KNP, a total of 0.06 % full sibs or parent-offspring were BTB uninfected and 7 % half sibs were BTB uninfected. Prevalence group two (central KNP) consists of 14 individuals, one full sib pair or parent-offspring pair and one half sib pair was identified from the pairwise relatedness comparisons. One percent of the BTB uninfected animals in the central region of the KNP were, therefore, full sibs or parent-offspring and 1 % were half sibs. Prevalence group three (northern KNP) consists of 9 uninfected buffaloes, no full sib pairs, parent-offspring relationships or half sib pairs were identified from the pairwise relatedness comparisons. BTB uninfected African buffaloes sampled in the southern region of the KNP, therefore, appears to share the highest amount of relatedness with a total of 7.06 % being related, compared to individuals sampled from the central and northern regions, with 2 % and 0 % genetic relatedness shared among individuals sampled from these regions, respectively. This low level of genetic relatedness among individuals sampled from the central and northern regions of the KNP may be ascribed to the small number of samples collected from both these regions.

Appendix 3.3 represents full-sibling or parent-offspring relationships between BTB uninfected individuals. Data suggests that related samples are not all associated with the same BTB prevalence region. For example sample 224 was sampled from the northern regions of the KNP and is related to sample 4978, which was sampled from the southern region of the KNP. Genetic relatedness between individuals may, therefore, span over vast distances and disease prevalence zones. The African buffalo's social group organization and

migratory behaviour may be responsible for the level of relatedness between sampled individuals from different prevalence regions. Sampling bias within and among populations may also have affected the outcome of this result.

5.3.3 BTB infected and BTB uninfected prevalence region/zone comparisons

Evaluation of *M. bovis* infected buffaloes based on their BTB culture status and genetic relationships to one another within a particular prevalence region/zone, was hampered by the variation in the number of samples collected from the different prevalence zones, especially samples collected from the central and northern regions of the KNP. Equal numbers of BTB infected and uninfected samples were collected from the southern regions of the Park. More substantial relatedness values could, therefore, be compared between BTB infected and uninfected animals, sampled from the southern region of the KNP. Sixty-six BTB infected animals were sampled from the southern regions of the Park, with a 7.16 % genetic relatedness (half sibs, full sibs and parent offspring) shared among them compared to the 57 BTB uninfected animals sampled from the same region, with a 7.06 % genetic relatedness (half sibs, full sibs and parent offspring) shared among them. Results therefore, indicate that BTB infected and uninfected African buffaloes sampled from the southern region of the KNP (prevalence group one) share similar levels of genetic relatedness. The majority of individuals (BTB infected and uninfected) sampled from the southern region of the Park can, therefore, be expected to be unrelated (with regards to sibling and parent-offspring relatedness) (Table 3.9).

Table 3.9 The level of genetic relatedness shared between sampled BTB infected and BTB uninfected buffaloes within the KNP, sampled from different prevalent groups

	BTB infected group	BTB uninfected group
Southern KNP (Prevalence group one)	66 (7.16 %)	57 (7.06 %)
Central KNP (Prevalence group two)	24 (6.5 %)	14 (2 %)
Northern KNP (Prevalence group three)	11 (3.7 %)	9 (0 %)

*** Values within Table 3.9 represents the number of animals within each prevalence region associated with either BTB infected or BTB uninfected groups (e.g. 66 BTB infected and 57 uninfected animals in southern KNP) as well as the percentage relatedness shared between individuals associated with a particular prevalence region in brackets**

5.4 BTB strain related data evaluation

From the 181 buffaloes sampled throughout the KNP, the BTB strains of only 27 individuals were known. Eighteen individuals were infected with the dominant C8 strain and nine individuals were infected with the variant C8v strain were identified. One individual sampled at Woodlands (located in the northern region of the Park) had both dominant and variant strains. The BTB strains of the remaining 153 buffalo samples were not typed during the initial sampling procedure. The lack of BTB strain data made it difficult to asses whether buffaloes infected with the same BTB strain were more closely related than uninfected buffaloes, or those infected with a different strain (due to limited sample numbers). From the limited data available, one could note that both the dominant C8 and variant C8v strains infected male and female buffaloes and was identified throughout the southern, central and northern region of the Park.

6. Discussion

Pairwise relatedness evaluation between African buffaloes sampled throughout the entire KNP revealed several familial relationships. Population organization plays a crucial role in establishing genetic relationships and correlations between various individuals. Male migration and dispersal

characteristics, random sampling procedure, time (year) of sample collection as well as gender and age distributions may all have influenced the outcome of the relatedness evaluation based on our sampled African buffalo populations (Cross *et al*., 2005).

The intra-population relatedness evaluation, revealed a mean level of relatedness within each sampled population (that consists of two or more individuals). Results indicated (as can be expected) that no individual population had notably higher relatedness values, compared to other populations. This may have been influenced by the original sampling strategy, the number of samples used and sampling methods, which were employed for disease monitoring and management strategies. Sampled populations with marginally higher familial relationships include those sampled at Manzimhlope, Lower Sabie, Mahlanganzwane Dam and Riepan located in the southern and central regions of the KNP. These higher relatedness values may be attributed to the year in which samples were collected and male migration, where populations will congregate at higher frequencies in their familial or native populations (Ryan *et al*., 2006).

The inter-population relatedness evaluation was determined between all 181 animals and 39 populations in the KNP, based on pairwise relatedness estimates. As can be expected, results show that the greater majority (91.6 %) of sampled individuals, dispersed throughout the KNP, were unrelated in terms of sibling and parent-offspring relationships. This high percentage of unrelated African buffaloes can be expected as a result of the original random sampling strategy that did not target specific family groups or members, or animals of a specific gender. Samples were collected from a wide geographic area within the KNP, populations that may be geographically separated by long distances or as a result of geographical barriers that hinder migration and breeding between different populations, may also be responsible for the large percentage unrelated, sampled African buffaloes. The high level of unrelated African buffaloes within the KNP may also be as a result of our small sample set of 181 individuals (approximately 0.64 % of the entire buffalo population in the KNP), compared to the 28 500 African buffaloes found within the KNP,

that are distributed across 100 herds with an average herd size of approximately 244 (Greyling, 2007). The high level of unrelated buffaloes can also be due to the large area that the KNP comprises (approximately 2 million ha) (Mabunda *et al*., 2003), which made it difficult to sample and obtain genetically related (in terms of half sibs, full sibs and parent-offspring) individuals (as mentioned in our materials and methods, samples were collected for previous African buffalo studies within the KNP and not for this study exclusively). Male and female buffaloes have different migratory patterns, which may contribute to the differences in genetic structure and relatedness characteristics of sampled African buffalo populations throughout the KNP. Males are viewed as the dispersing sex, young females (heifers) may also migrate while adult females and young males (younger than three years of age) remain in their native populations (De Vos *et al*., 2001).

Pairwise relatedness evaluation was conducted within each sampled population associated with a particular group (BTB infected or BTB uninfected group) as well as across all sampled individuals associated with a particular group. For both the infected and uninfected groups, results indicate that the greater majority of individuals were unrelated (in terms of sibling or parent-offspring relationships). A direct comparison between the BTB infected and uninfected groups, based on the mean level of relatedness associated with each group, revealed that the mean level of relatedness between infected and uninfected populations, did not differ significantly ($p = 0.108$, student's t-test).

As our hypothesis stated, we proposed that *M. bovis* infected buffaloes are more closely related to each other than to uninfected buffaloes in the same population or prevalence group. Table 3.2 represents sampled populations that consisted of two or more individuals. Samples collected from the central and northern regions (prevalence groups two and three respectively) of the KNP were too few to be included and further evaluated in separate prevalence group analyses. Prevalence group evaluation of both BTB infected and uninfected buffaloes were hampered by the variation in sample numbers between the three prevalence groups. The number of BTB infected and uninfected samples collected from the southern region of the Park were more

substantial and could therefore be compared to one another. Results indicated a similar level of relatedness between BTB infected and uninfected samples collected from the southern regions of the KNP. A small percentage of BTB infected (7.16 %) and uninfected (7.06 %) samples collected from the southern region of the Park were genetically related, irrespective of their BTB culture status (Table 3.9). The greater majority of African buffaloes sampled from prevalence group one can, therefore, be regarded as unrelated (no sibling or parent-offspring relationships), irrespective of their BTB disease status. The small sample set of 181 individuals, variations in population sizes (which range between 1 and 15 individuals), the original, sampling procedure, variation in prevalence group size as well as the time period of sample collection (1996 to 2007) may have influenced the outcome of our results. Several individuals associated with different prevalence group regions, were also related (full sibs or parent-offspring). Male migration, heifer migration, social behaviour and structure of the African buffaloes within the KNP as well as chance events due to sampling artefacts may have contributed to this observation.

The bovine tuberculosis (BTB) status of each individual was evaluated to determine whether any correlation between the genetic relatedness of individual African buffaloes within the KNP and their disease status (BTB infected or uninfected) could be observed. Evidence suggests that the BTB culture status and transmission between individuals, is not associated with the genetic relatedness (sibling or parent-offspring relatedness) of sampled African buffaloes within the KNP. The dynamic nature of the African buffalo herd may have influenced the spread of the disease from infected to uninfected animals, irrespective to whether they were genetically related or not. BTB disease transmission takes place *via* the respiratory route and short periods of mingling between individuals (as seen in the mixed buffalo herd which typically consists of adult cows and bulls, sub-adult cows and bulls, juveniles and calves), may be sufficient for the successful transmission of the disease. Buffalo dispersal (male or female) and movement of animals in and out of their native herds, may also have contributed to BTB transmission between sampled buffaloes within the KNP. The group dynamics of the

African buffalo population may be disturbed by several factors such as annual rainfall, breeding season, vegetation (availability of food) and communal drinking water, which may have influenced the spread of *M. bovis* throughout buffalo populations across the entire KNP and ultimately determined which animals were infected with the disease. Several factors that affect the dynamics of disease spread and transmission, however, are still poorly understood and unknown and merits further study.

From the 181 buffaloes sampled throughout the KNP, the BTB strains of only 27 individuals were known (one individual sampled at Woodlands had both dominant and variant strains). The BTB strains of the remaining 153 buffalo samples were not typed during the initial sampling procedure. The lack of BTB strain data made it difficult to asses whether buffaloes infected with the same BTB strain were more closely related than uninfected buffaloes, or those infected with a different strain (due to small sample numbers). From the limited data available, one could note that both the dominant C8 and variant C8v strains infected male and female buffaloes and was identified throughout the southern, central and northern region of the Park. Previous studies have shown that the dominant C8 strain was more commonly found in the southern region of the Park, while variant C8v strains were more commonly found in the central and northern regions of the Park (Michel, 2008).

7. Conclusion

An overall picture of the disease condition and genetic relatedness, within and between sampled buffalo populations (BTB infected and BTB uninfected populations) within the KNP, was sketched.

Results revealed that within each sampled population, no notable difference in their mean relatedness values was observed between BTB infected and BTB uninfected groups. The mean level of relatedness did not vary significantly within or between sampled populations of either infected or uninfected groups. Comparisons between the mean level of relatedness associated among each group, revealed that relatedness between them did not vary notably. The

greater majority of sampled individuals (BTB infected or uninfected), were genetically unrelated (in terms of sibling and parent-offspring relationships), irrespective of their BTB disease status. Prevalence group evaluation was hampered by the lack of samples collected from the central and northern regions of the Park. The number of samples collected from the southern region of the Park was substantial and similar for both infected and uninfected groups and were consequently used for further analysis. The greater majority of animals sampled from the southern region of the KNP, (whether BTB infected or not), were genetically unrelated in terms of sibling and parent-offspring relatedness.

M. bovis infected buffaloes sampled and used in our study are, therefore not more closely related to each other than to uninfected buffaloes in the same population or prevalence group.

Appendix 3.1 Full-sibling or parent-offspring relationships calculated across the whole BTB infected African buffalo group

Sample ID 1	Prevalence group region	Sample ID 2	Prevalence group region	*Mean
656	South	659	South	0.557
668	South	KNP32A	Central	0.788
KNP79	Central	4851	South	0.547
4832	South	5134	South	0.53
4852	South	5122	South	0.676
3887	South	1518	South	0.544
670	South	895	South	0.622
4851	South	895	South	0.546
668	South	5208	South	0.526
1516	South	5208	South	0.51
4832	South	5189	South	0.588
668	South	5154	South	0.513
5208	South	5154	South	0.594
5054	South	5166	South	0.543

***Mean level of relatedness calculated between Sample ID 1 and Sample ID 2**

Appendix 3.2 Half-sibling relationships calculated between all infected individuals, across all populations

Column A			Column B			Column C			Column D		
Sample ID 1	Sample ID 2	Mean	Sample ID 1	Sample ID 2	Mean	Sample ID 1	Sample ID 2	Mean	Sample ID 1	Sample ID 2	Mean
656	667	0.262	659	5001	0.263	660	5149	0.407	KNP32A	5208	0.443
663	667	0.497	660	5001	0.363	770	5149	0.262	KNP38	5208	0.309
667	KNP14	0.265	KNP18	5001	0.305	KNP8	5149	0.266	KNP58	5208	0.348
672	KNP14	0.344	660	5018	0.373	6423	5149	0.391	KNP221	5208	0.347
660	KNP18	0.337	668	5018	0.291	5018	5149	0.281	3896	5208	0.278
667	KNP18	0.355	KNP18	5018	0.395	5054	5149	0.368	6431	5208	0.295
668	KNP18	0.335	KNP32A	5018	0.284	656	3887	0.426	4850	5208	0.304
KNP14	KNP18	0.268	KNP58	5018	0.260	659	3887	0.344	4832	5208	0.271
656	KNP8	0.337	KNP149	5018	0.274	668	3887	0.426	5018	5208	0.428
656	KNP12	0.288	KNP221	5018	0.312	KNP18	3887	0.460	5010	5208	0.447
668	KNP12	0.384	656	5010	0.321	KNP12	3887	0.276	5153	5208	0.297
KNP8	KNP12	0.429	659	5010	0.308	KNP32A	3887	0.399	5151	5208	0.366
660	KNP10	0.266	668	5010	0.451	KNP58	3887	0.397	3887	5208	0.303
670	KNP35	0.330	KNP18	5010	0.442	3896	3887	0.345	5196	5208	0.436
KNP35	KNP32A	0.291	KNP32A	5010	0.371	6423	3887	0.432	5201	5208	0.423
672	KNP38	0.427	KNP58	5010	0.420	5010	3887	0.436	659	5189	0.288
656	KNP58	0.407	5006	5010	0.290	656	1516	0.258	668	5189	0.376
668	KNP58	0.282	5018	5010	0.345	668	1516	0.485	672	5189	0.263
KNP8	KNP58	0.257	KNP147	5045	0.314	KNP32A	1516	0.314	KNP18	5189	0.303
KNP32A	KNP58	0.370	660	5054	0.304	KNP58	1516	0.338	KNP12	5189	0.389
KNP47	KNP71	0.339	KNP18	5054	0.380	KNP171	1516	0.279	KNP58	5189	0.251
KNP47	KNP79	0.284	6423	5054	0.293	4832	1516	0.278	KNP147	5189	0.254
667	KNP70	0.281	5001	5054	0.369	5010	1516	0.253	1856	5189	0.366

672	KNP70	0.315	5018	5054	0.418	5153	1516	0.355	3896	5189	0.284
656	KNP150	0.270	5010	5054	0.264	656	1518	0.264	4850	5189	0.254
KNP8	KNP150	0.315	660	5053	0.304	659	1518	0.260	5001	5189	0.253
KNP58	KNP150	0.304	1854	5053	0.384	668	1518	0.391	5010	5189	0.383
660	KNP149	0.341	1865A	5053	0.371	KNP18	1518	0.288	5045	5189	0.348
667	KNP149	0.394	6431	5044	0.272	1856	1518	0.376	5134	5189	0.426
KNP10	KNP160	0.404	4852	5044	0.344	6423	1518	0.436	5153	5189	0.384
KNP32A	KNP160	0.270	656	5036	0.336	5006	1518	0.360	3887	5189	0.391
KNP32A	KNP221	0.274	KNP8	5036	0.356	5010	1518	0.452	1516	5189	0.321
KNP35	1853	0.264	KNP150	5036	0.274	5054	1518	0.313	5196	5189	0.291
KNP160	1853	0.258	670	5042	0.260	5149	1518	0.373	5208	5189	0.282
663	1854	0.260	770	5042	0.290	KNP10	896	0.336	668	5184	0.365
672	1854	0.316	KNP8	5042	0.288	KNP149	896	0.267	KNP18	5184	0.499
670	KNP466	0.344	KNP76	5042	0.258	5001	896	0.286	KNP32A	5184	0.292
670	1856	0.259	KNP150	5042	0.328	KNP35	895	0.330	4851	5184	0.312
KNP35	1856	0.391	5045	5042	0.280	1856	895	0.364	4852	5184	0.347
KNP58	1856	0.356	5036	5042	0.304	KNP47	883	0.294	4832	5184	0.342
660	1865A	0.276	663	5110	0.309	KNP65	883	0.418	5006	5184	0.293
672	1865A	0.312	KNP18	5109	0.298	4851	883	0.332	5018	5184	0.488
KNP18	1865A	0.350	1865A	5109	0.254	5130	883	0.467	5010	5184	0.444
1854	1865A	0.267	KNP14	5111	0.256	KNP14	1082	0.412	5133	5184	0.429
KNP71	3894	0.450	656	5112	0.312	6430	1082	0.280	3887	5184	0.285
KNP150	3894	0.258	KNP8	5112	0.315	4852	1082	0.258	895	5184	0.380
1865A	3894	0.306	KNP38	5112	0.283	668	5196	0.457	5196	5184	0.271
656	3896	0.464	KNP58	5112	0.400	672	5196	0.307	656	5183	0.406
659	3896	0.337	KNP171	5112	0.325	KNP14	5196	0.280	659	5183	0.420
668	3896	0.391	3896	5112	0.294	KNP35	5196	0.266	3896	5183	0.290
KNP12	3896	0.381	4980	5112	0.423	KNP32A	5196	0.447	4832	5183	0.323
KNP32A	3896	0.353	5018	5112	0.381	KNP160	5196	0.299	3887	5183	0.293

770	6423	0.338	656	5118	0.257	1856	5196	0.414	1518	5183	0.254
KNP8	6423	0.257	KNP8	5118	0.330	4850	5196	0.320	5201	5183	0.285
KNP12	6423	0.268	KNP12	5118	0.336	4832	5196	0.401	5208	5183	0.415
KNP10	6423	0.351	KNP58	5118	0.347	5006	5196	0.283	5189	5183	0.270
KNP58	6423	0.271	KNP149	5118	0.302	5010	5196	0.384	659	5154	0.291
KNP150	6423	0.403	KNP171	5118	0.265	5134	5196	0.390	663	5154	0.273
667	6430	0.286	1856	5118	0.327	5133	5196	0.296	672	5154	0.269
KNP65	6430	0.275	4850	5118	0.317	2649	5196	0.283	KNP32A	5154	0.476
KNP149	6430	0.251	5044	5118	0.325	3887	5196	0.444	4832	5154	0.328
KNP58	6431	0.311	672	5134	0.398	1516	5196	0.473	4953	5154	0.309
KNP150	6431	0.391	KNP14	5134	0.380	1518	5196	0.374	4977	5154	0.253
6423	6431	0.264	5006	5134	0.287	667	5197	0.272	5010	5154	0.251
KNP47	4851	0.254	KNP171	5123	0.325	KNP14	5197	0.396	5036	5154	0.268
1853	4851	0.342	5112	5123	0.299	1865A	5197	0.422	5132	5154	0.425
KNP14	4852	0.273	672	5133	0.257	5045	5197	0.297	5153	5154	0.298
KNP38	4852	0.252	KNP160	5133	0.288	5053	5197	0.368	1516	5154	0.499
KNP65	4852	0.385	1853	5133	0.482	656	5201	0.259	5196	5154	0.440
6430	4852	0.449	4851	5133	0.376	660	5201	0.454	5189	5154	0.264
667	4850	0.454	5006	5133	0.316	670	5201	0.258	5197	5160	0.251
KNP149	4850	0.351	KNP65	5122	0.419	KNP18	5201	0.483	659	5156	0.313
6431	4850	0.285	6430	5122	0.257	KNP8	5201	0.273	KNP14	5156	0.266
668	4832	0.414	4984	5122	0.406	KNP10	5201	0.317	KNP58	5156	0.258
KNP18	4832	0.382	5044	5122	0.335	KNP32A	5201	0.295	6430	5156	0.466
KNP47	4953	0.297	659	5132	0.279	KNP58	5201	0.428	4852	5156	0.442
KNP71	4953	0.432	672	5132	0.304	KNP149	5201	0.336	4984	5156	0.270
4851	4953	0.327	KNP8	5132	0.318	KNP160	5201	0.327	5006	5156	0.270
668	4977	0.268	KNP12	5132	0.259	6430	5201	0.343	5010	5156	0.276
KNP32A	4977	0.296	KNP32A	5132	0.359	4852	5201	0.331	5111	5156	0.299
KNP221	4977	0.286	668	2649	0.326	4850	5201	0.298	5122	5156	0.287

KNP182	4955	0.328	KNP14	2649	0.279	5018	5201	0.437	1082	5156	0.300
KNP14	4984	0.268	KNP18	2649	0.257	5010	5201	0.377	5201	5156	0.360
KNP70	4984	0.368	5006	2649	0.279	5036	5201	0.333	6430	5163	0.301
6430	4984	0.254	660	2837	0.326	5112	5201	0.305	5201	5163	0.347
656	4980	0.263	KNP18	2837	0.374	2837	5201	0.343	KNP8	5167	0.284
663	4980	0.333	KNP32A	2837	0.253	5149	5201	0.254	KNP12	5167	0.406
KNP32A	4980	0.312	5018	2837	0.404	KNP71	5207	0.325	1856	5167	0.442
KNP58	4980	0.352	5010	2837	0.285	4953	5207	0.275	5118	5167	0.259
6431	4980	0.384	5054	2837	0.294	656	5208	0.373	5132	5167	0.351
672	5006	0.252	5112	2837	0.323	659	5208	0.361	5197	5167	0.303
1853	5006	0.279	659	5153	0.295	667	5208	0.325	5018	5166	0.257
4984	5006	0.313	668	5153	0.373	KNP18	5208	0.346	2837	5166	0.293
KNP147	5153	0.252	KNP12	5153	0.330	KNP8	5208	0.254	6430	5155	0.343
5018	5151	0.294	KNP32A	5153	0.270	KNP12	5208	0.272	4852	5155	0.254
									4955	5155	0.283
									659	5140	0.448
									KNP150	5140	0.297

***Mean level of relatedness calculated between Sample ID 1 and Sample ID 2**

Appendix 3.3 Full-sibling or parent-offspring relationships calculated across the whole BTB uninfected African buffalo group

Sample ID 1	Prevalence group region	Sample ID 2	Prevalence group region	*Mean
KNP66	Central	KNP164	Central	0.665
KNP224	North	KNP219	North	0.525
KNP224	North	4978	South	0.605
KNP219	North	4978	South	0.501
KNP69	Central	5005	South	0.577
5037	South	5038	South	0.643
KNP219	North	5106	South	0.542
KNP66	Central	5127	South	0.568
1855	North	5127	South	0.525
KNP66	Central	5179	South	0.546
KNP186	Central	5144	South	0.572
KNP224	North	5161	South	0.564

***Mean level of relatedness calculated between Sample 1 ID and Sample ID 2**

Appendix 3.4 Half-sibling relationships calculated between all uninfected individuals, across all populations

Column A			Column B			Column C			Column D		
Sample ID 1	Sample ID 2	*Mean	Sample ID 1	Sample ID 2	*Mean	Sample ID 1	Sample ID 2	*Mean	Sample ID 1	Sample ID 2	*Mean
703	KNP175	0.401	KNP39	5019	0.402	4848	5137	0.297	4983	5176	0.32
KNP33	KNP175	0.363	KNP180	5019	0.256	KNP21	5136	0.266	5152	5176	0.382
KNP66	KNP174	0.264	4983	5045	0.261	5017	5136	0.389	KNP39	5169	0.288
KNP186	KNP174	0.301	KNP50	5049	0.464	5046	5136	0.254	4987	5169	0.392
KNP66	KNP180	0.28	KNP174	5049	0.31	5037	5136	0.309	5017	5169	0.263
KNP34	1855	0.345	KNP224	5049	0.275	5106	5136	0.31	5046	5169	0.296
KNP66	1855	0.492	KNP219	5049	0.321	KNP174	5124	0.28	5106	5169	0.418
KNP174	1855	0.398	6406	5049	0.404	6406	5124	0.265	5148	5169	0.252
KNP180	1855	0.369	KNP39	5041	0.354	KNP69	5117	0.398	KNP39	5180	0.283
KNP164	KNP224	0.255	KNP34	5041	0.385	1855	5117	0.307	KNP164	5180	0.265
1855	KNP224	0.305	KNP66	5041	0.286	KNP224	5117	0.387	KNP219	5180	0.258
KNP174	KNP219	0.363	KNP69	5041	0.278	4978	5117	0.414	1863	5180	0.269
KNP180	KNP219	0.291	4847	5041	0.397	5105	5117	0.37	4982	5180	0.374
1855	KNP219	0.36	4960	5041	0.32	4848	5150	0.305	4960	5180	0.269
KNP180	1858	0.3	4959	5041	0.277	5115	5150	0.316	5017	5180	0.252
KNP219	6406	0.261	KNP39	5046	0.346	KNP34	3895	0.305	5019	5180	0.33
KNP66	4848	0.257	1857	5046	0.437	KNP66	3895	0.268	3895	5180	0.378
KNP224	4848	0.391	4847	5046	0.363	1855	3895	0.322	5148	5180	0.339
692	4847	0.25	4982	5046	0.448	KNP219	3895	0.419	KNP34	5181	0.252
1863	4847	0.446	4960	5046	0.264	4978	3895	0.253	KNP66	5181	0.26
KNP21	4957	0.283	5017	5046	0.254	4987	3895	0.386	1855	5181	0.327
KNP180	4957	0.277	5011	5046	0.276	5019	3895	0.314	1857	5181	0.352
1855	4957	0.251	5019	5046	0.262	5127	3895	0.258	4848	5181	0.288

6407	4957	0.298	5041	5046	0.346	5117	3895	0.289	4982	5181	0.355
4847	4957	0.289	KNP39	5037	0.277	KNP39	5148	0.362	5046	5181	0.265
1857	4981	0.274	5017	5037	0.304	KNP34	5148	0.344	5180	5181	0.322
KNP66	4982	0.31	4982	5038	0.295	KNP69	5148	0.306	KNP33	5179	0.269
KNP180	4982	0.303	4978	5038	0.25	1855	5148	0.389	KNP164	5179	0.34
1855	4982	0.382	4954	5038	0.319	KNP224	5148	0.266	KNP180	5179	0.377
KNP174	4978	0.254	703	5040	0.341	1863	5148	0.352	1855	5179	0.457
1855	4978	0.398	KNP33	5040	0.265	6406	5148	0.27	KNP224	5179	0.271
1863	4978	0.287	KNP175	5040	0.44	4982	5148	0.421	4982	5179	0.384
6429	4978	0.304	KNP219	5040	0.276	4978	5148	0.332	5038	5179	0.462
4982	4978	0.36	4849	5040	0.261	4960	5148	0.3	5127	5179	0.479
KNP33	4983	0.297	4978	5040	0.482	4954	5148	0.253	5148	5179	0.431
KNP175	4983	0.308	KNP69	5105	0.317	5041	5148	0.476	5181	5179	0.351
KNP174	4983	0.493	KNP224	5105	0.274	5046	5148	0.3	KNP39	5141	0.366
1855	4983	0.346	1863	5105	0.383	5037	5148	0.257	KNP34	5141	0.396
KNP219	4983	0.274	4978	5105	0.281	5038	5148	0.324	5019	5141	0.257
4978	4983	0.33	KNP174	5106	0.267	5105	5148	0.411	5041	5141	0.303
KNP69	4960	0.301	6406	5106	0.334	5106	5148	0.352	KNP50	5144	0.295
6406	4960	0.26	5011	5106	0.271	3895	5148	0.255	5045	5144	0.271
4982	4960	0.341	5049	5106	0.344	KNP33	5198	0.257	5127	5144	0.295
703	4958	0.28	5046	5106	0.415	KNP180	5198	0.285	4849	5143	0.323
KNP174	4959	0.33	5037	5106	0.391	4987	5198	0.261	4983	5143	0.308
692	5005	0.298	KNP33	5115	0.34	5040	5198	0.288	5040	5143	0.258
1857	5005	0.271	KNP175	5115	0.279	5152	5198	0.294	5152	5143	0.269
4847	5005	0.284	5106	5115	0.283	KNP174	5195	0.366	703	5158	0.319
4960	5005	0.26	KNP164	5127	0.389	4987	5195	0.273	KNP33	5158	0.292
KNP39	4987	0.319	KNP174	5127	0.393	5017	5209	0.468	4979	5158	0.269
1855	4987	0.317	4982	5127	0.295	5037	5209	0.495	5176	5158	0.336
1863	4987	0.255	4959	5127	0.281	5136	5209	0.476	5141	5158	0.288

KNP39	5017	0.307	5041	5127	0.356	3895	5209	0.281	KNP219	5161	0.304
4982	5017	0.265	KNP50	5137	0.274	KNP33	5176	0.277	1857	5161	0.264
5017	5011	0.377	KNP148	5137	0.337	KNP175	5176	0.256	4978	5161	0.266
									4979	5161	0.317
									5106	5161	0.274

***Mean level of relatedness calculated between Sample ID 1 and Sample ID 2**

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Chapter 4

Concluding Remarks and Recommendations

4.1 Concluding Remarks

Our environment is constantly placed under stress, which is responsible for the rapid decline of our natural resources. The primary cause for this rapid decline is an increased human population growth, development and habitation. Population genetics and conservation biology encompass several important fields (conservation, wildlife management, animal breeding, ecology, systematics, computational and evolutionary biology), which assist in preserving species, their habitats and ecosystems. Population genetic principles and computer programmes applied during the course of this study are valuable tools that assist in the evaluation and management of populations and species, including the African buffalo *(Syncerus caffer).*

The African buffalo is one of Africa's most majestic, large mammalian species. Three distinct sub-species are recognized, the two savannah subspecies that include the Cape buffalo, *Syncerus caffer caffer* and *Syncerus caffer brachyceros* (also referred to as the Sudan or West African savannah buffalo) and the third subspecies, the forest buffalo, *Syncerus caffer nanus.*

The Cape buffalo is widely distributed throughout sub-Saharan Africa, and together with elephant (*Loxodonta africana*) and wildebeest (*Connochaetes taurinus*) form the bulk of the large herbivore biomass in Africa (Van Hooft *et al.*, 2002). The African buffalo are predominantly dispersed throughout the savannah biomes of eastern and southern Africa. This large gregarious herbivore is also abundant in the entire KNP (one of Africa's largest conservation areas) with a current population size estimated at 28 500 individuals. The herd structure of the African buffalo in the KNP is quite dynamic, and herd sizes vary from between 50 to 1000 individuals, the average herd consisting of approximately 244 individuals (De Vos *et al.*, 2001). The structural dynamics within herds is a function of social behaviour,

food supply and its abundance (due to persisting drought conditions), as well as habitat fragmentation and loss, hunting pressures and disease.

Historical events such as disease, drought and population control measures played vital roles in the population dynamics of the African buffalo. The rinderpest epidemic of the 1890's virtually destroyed the African buffalo population of southern Africa (Van Hooft *et al*., 2002). It is believed that the African buffalo population was so severely affected and depleted by the rinderpest epidemic, that approximately 95 % of South Africa's African buffalo population was destroyed. Population control measures implemented in the KNP between 1967 and 1981 also influenced the current status of the African buffalo, as approximately 25 800 animals were removed from the Park through culling. Environmental factors such as droughts (especially between 1992 and 1995 in the KNP) also impacted upon the population size of the buffalo, as approximately 14 000 animals within the Park died due to starvation. The African buffalo also suffer from sub-acute diseases such as BTB and corridor disease (CD), which ultimately influenced the current status and size of the species within the KNP (Greyling, 2007).

Bovine Tuberculosis (BTB) is a chronic, progressive bacterial disease caused by *Mycobacterium bovis*. The greater kudu (*Tragelaphus strepsiceros*) in the Eastern Cape region of South Africa, was the first reported wildlife species to be infected with the disease (Renwick *et al*., 2006). It is believed that the bacterium was transmitted from infected domestic cattle herds to the African buffalo during the 1960's in the south eastern corner of the KNP before fencing of the southern boundary of the reserve had been completed. The disease was however only officially diagnosed in the KNP in July 1990 in an emaciated buffalo bull (De Vos *et al*., 2001). The infection has since spread in a northerly direction throughout the susceptible African buffalo population and several other wildlife species within the Park's boundaries. It has been speculated that the spread of the disease takes place at a rate of 6 km / year (De Vos *et al*., 2001), supported by the fact that by 2007, *M bovis* infection was detected in all regions of the KNP (Van Schalkwyk, L. July 2010, personal communication). This high rate of spread can be attributed to high African buffalo population densities and their dispersal characteristics, within

the Park (De Vos *et al*., 2001). The disease is maintained as a sub-clinical infection in the buffalo, and clinical signs (emaciation) may take years to appear. It is however difficult to determine the time-span of the disease from its point of infection to the terminal stage (De Vos *et al*., 2001).

Species conservation strategies require multidisciplinary baseline information in order to devise actions to determine and minimize threats to animal survival (e.g. a decrease in genetic variation). Molecular genetic technologies such as microsatellite marker profiling, have contributed to a large degree to the management and conservation of several species and are ideal tools for assessing a range of important population genetic parameters. A panel of 12 microsatellite markers was used to evaluate the 181 samples from the KNP. Specific population genetic parameters and indices revealed information based on the intra- and inter -relationships at the population as well as prevalence group levels. Samples were placed into specific prevalence groups based on the sampling area (where samples were collected) and whether BTB occurs at high, medium or low levels within the sampled region. Evidence generated in chapter two, suggests a medium to high level of genetic diversity and variation distributed throughout populations sampled at the southern, central and northern regions of the Park. Each prevalence group had a greater than 70 % chance of being heterozygous, with samples from the central region showing slightly higher heterozygosity levels. Low to medium levels of inbreeding can be expected within each group, with the inbreeding coefficient (*Fis)* for each group ranging between 0.143 and 0.147. Relatively high level of migration was observed between the three groups which indicate relatively high levels of gene flow between individuals associated with the three prevalence groups. The number of migrants (11.68) expected between prevalence groups, was determined after several sample size corrections were made. The amount of gene flow between individuals from different prevalence groups may vary based on the period of sample collection, for example, buffaloes sampled during the breeding season (higher levels of gene flow would be expected as bulls migrate to mixed herds and compete for mates). Results related to the genetic status of the African buffalo within the KNP can be confirmed by work previously conducted by B.J

Greyling (as discussed in chapter two) (Greyling, 2007). The high level of genetic variation detected with our panel of 12 microsatellite markers, suggests and conforms to results from a previous study (Greyling, 2007), that the reported bottlenecks presumably caused by the outbreak of rinderpest during the last century, did not reduce the genetic variability of the African buffalo.

The division of samples on a 'per population' basis as well as on a 'per prevalence group' basis sketched an overall picture of the genetic relatedness (within populations and between individuals) as far as sibling and parent-offspring relationships are concerned. The social organization of the African buffalo (which varies during the course of the year) in the KNP may influence the genetic indices evaluation and genetic status calculation of the species within the Park.

Results revealed that the greater majority of sampled African buffaloes were unrelated in terms of Queller and Goodnight's relatedness parameters, irrespective of their disease status. The outcome of these results may have been influenced by several factors some of which include the limited sample size of 181 individuals as well as variations in population and prevalence group sizes. The limited sample size and population numbers may provide a negative bias of genetic relatedness between our sampled African buffaloes. Despite these factors however, a broad insight into the levels of relatedness between different groupings of buffaloes were obtained, especially for populations in the south of KNP for which more substantial sample numbers existed.

Two strain types of BTB have previously been identified within the KNP these include the C8 strain and the C8v strain. The dominant C8 strain is more commonly found in the southern region of the Park, while the variant C8v strain is more commonly found in the central and northern regions of the Park (Michel, 2008). The different BTB strains were assessed to determine whether buffaloes infected with the same strain of BTB were more closely related than uninfected buffaloes, or those infected with a different strain within a single

population. The association between the type of BTB strain and genetic relatedness of African buffaloes could not be calculated, due to limited strain data. This is true for our sample set of 181 African buffaloes within the KNP, the situation may, however be different for the entire African buffalo population within the Park.

One would expect that related animals share more time and space and therefore, have a higher risk of infecting one another due to shared temporal and spatial relationships. As our results indicate, the possibility of infection with *M. bovis* is not related to the spatial and temporal association between relatives. Other environmental, behavioural and demographic factors are likely to play a role in disease transmission between individuals within and across populations.

A comprehensive reference database of genotypes of African buffalo within the KNP was established. Evaluation of genetic relationships (based on sibling and parent-offspring relationships) between and within sampled buffalo populations was also completed. Results have highlighted the transmission and maintenance characteristics of BTB, within our sampled African buffaloes. Results revealed that the greater majority of sampled African buffaloes were unrelated (in terms of sibling and parent-offspring relatedness), irrespective of their disease status. We were, therefore, unable to support our hypothesis and could not identify any genetic basis for BTB resistance or susceptibility in buffaloes.

4.2 Recommendations

Within the KNP, BTB control in buffaloes is regarded as a priority and involves long-term planning, management and evaluation of the situation. Risk assessment is employed as a preventative measure. Details of the various animal species within the KNP can be viewed at the South African National Parks website at <http://www.sanparks.org/parks/kruger/>. Current BTB management options within the Park are limited, as complete eradication of

the disease is hampered by the large African buffalo population size, spill-over hosts and the limited success of the BCG vaccine (Cross *et al*., 2009).

For future population genetic studies on the African buffalo, the original sampling procedure needs to be well documented and data properly managed, especially with regards to the age, gender, BTB culture status and season when sampling was conducted. This will ensure a more accurate interpretation of data and results generated. To maximise sampling of relatives, smaller populations may be targeted. For management and conservation purposes it is recommend that more samples be collected throughout the year, during the breeding and non-breeding season, in order to obtain a random and diverse sample set, sampling as many individuals as possible will ultimately increase the reliability and accuracy of the calculated relatedness parameters (the smaller the data set, the less genetic material and gene diversity is available to analyse and compare and eventually draw accurate conclusions from). The current BTB sampling survey applied in the KNP, involves stratified random sampling of herds and animals (based on gender and age) (Van Schalkwyk, L. July 2010, personal communication). As our results indicate, the BTB culture status and transmission between individuals, is not associated with the genetic relatedness (sibling or parent-offspring relatedness) of sampled African buffaloes within the KNP. When designing a BTB prevalence survey, one therefore, does not need to consider clustering of the disease due to social or familial clustering. In the future continuous effort should also be applied to disease management and monitoring strategies with regards to BTB in African buffaloes within the KNP, in view of the role that the species play within the greater KNP ecosystem. Forthcoming conservation efforts should subsequently be based on sound scientific principles, encompassing all relevant disciplines.

The data set of 181 individuals represents a relatively small fraction of the entire African buffalo population within the KNP (approximately 28 500 individuals). This small data set did, however, provide similar results regarding the genetic variation of population and group evaluation conducted previously using a larger data set of 485 samples (Greyling *et al*., 2008). Microsatellites

were essential in generating population genetic indices, the number of microsatellites used for population genetic studies regarding the African buffalo is, however, debateable. Greyling *et al*. (2008) applied 17 microsatellite markers whilst we applied 12 markers. A smaller number of microsatellite markers will result in significant cost savings, while still retaining acceptable levels of accuracy and power of resolution (as noted in our study, use of 12 microsatellite markers provided accurate power and resolution).

Any future population genetic investigations based on herd or prevalence zone levels should regard the moderate to high levels of both genetic diversity and polymorphism (found among our sampled African buffalo group within the KNP), as a pre-requisite to their study.

King David, who defeated the mighty Goliath, uses the buffalo's power as an analogy in Psalm 92. Not the elephant, not the lion – which indicates the respect that this ostensibly understated animal created in the minds of people living 4000 B.C. A minute bacterium called *Mycobaterium bovis* is causing problems amongst African buffaloes and several other wildlife species worldwide. It is our duty to find a solution and eventually a cure, to restore the buffalo population and animal kingdom to its Biblical status.

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