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LIST OF ABBREVIATIONS

A	adenine
°C	degrees Celsius
C	cytocine
AEP	Addo Elephant National Park
Berg	Bergville
BLAST	basic local alignment search tool
CNP	Camdeboo National Park
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DVTD	Department of Veterinary Tropical Diseases
EDTA	Ethylenediaminetetraacetic acid
G	guanine
H	hour
HiP	Hluhluwe iMfolozi Park
HSP	Heat Shock Protein
KNP	Kruger National Park
MEGA	molecular evolutionary genetics analysis
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MNP	Mokala National Park
MTNZNP	Mountain Zebra National Park
ng	nanogram
OVI	Onderstepoort Veterinary Institute
PCR	polymerase chain reaction
pmol	picomoles
qPCR	quantitative polymerase chain reaction
RLB	reverse line blot
T	thymine
TMNP	Table Mountain National Park
µl	microliter
µM	micromolar

THESIS SUMMARY

ANAPLASMA CENTRALE IN SOUTH AFRICA: OCCURRENCE, PHYLOGENY AND GENETIC DIVERSITY

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Bovine anaplasmosis is one of the most important diseases of ruminants worldwide. It causes significant economic losses in the livestock industry particularly in tropical and subtropical areas including South Africa. The primary causative agent is *Anaplasma marginale*. *Anaplasma centrale* causes a milder form of anaplasmosis and is used as a live blood vaccine against *A. marginale.* However, there has been less interest in the epidemiology of *A. centrale,* and as a result, there are only a few reports on detecting natural infections of this organism. When detected in cattle, it is assumed that it is due to vaccination, and in most cases it is reported as co-infection with *A. marginale* without characterization of the strain.

This study was designed to (i) determine the genetic diversity of *A. centrale* strains using the *msp1aS* (major surface protein) gene, (ii) to establish whether wildlife species are reservoir hosts of *Anaplasma* species, (iii) to clarify and infer the phylogenetic relationship between *A. marginale* and *A. centrale* using sequence analysis of individual genes (16S rRNA, *groEL* and *msp4*) and (iv) to shed light on the possible tick species responsible for transmitting *A. centrale* in the absence of *Rhipicephalus simus* in Bergville, KwaZulu-Natal.

In this study a total of 380 blood samples from wild ruminant species and cattle collected from Biobanks, National Parks, and other regions of South Africa were used in duplex real-time PCR assays to simultaneously detect *A. marginale* and *A. centrale.* PCR results indicated high prevalence of *A. centrale* infections ranging from 25-100% in National Parks. Samples positive for *A. centrale* were further characterized using the *msp1aS* gene, a homolog of *msp1α* of *A.*

marginale which contains repeats at the 5' end that are useful for genotyping strains. A total of 47 Msp1aS repeats were identified which corresponded to 32 *A. centrale msp1aS* genotypes which were detected in cattle, buffalo and wildebeest. Our results demonstrated the diversity of *A. centrale* strains from cattle and wildlife hosts from South Africa and indicate the utility of *msp1aS* as a genotypic marker for *A. centrale* strain diversity.

The near full length 16S rRNA gene sequences, and the amino acid sequences deduced from *groEL* and *msp4* gene sequences from several isolates of *A. marginale* and *A. centrale* from around South Africa were obtained. The sequences were phylogenetically compared with that of the *A. marginale* type strain, St Maries, and the *A. centrale* Israel strain sequence and other reference sequences. Phylogenetic analyses of these sequences demonstrated that *A. centrale* forms a separate clade from *A. marginale* revealing that there is divergence between these two organisms. This was supported by high bootstrap values (≥90%). The distinctive differences in Msp1a/Msp1aS gene structure, as well as genome architecture provided further evidence that *A. centrale* is, in fact, a separate species.

In addition, 458 ticks were collected from 109 cattle from three dip tanks in the uThukela district in KwaZulu-Natal, South Africa between June 2015 and February 2016. Based on morphological characteristics, two genera and four species of ticks were identified: *R. evertsi evertsi* (50%), *R. appendiculatus* (30.1%), *R. microplus* (19.0%), and *Hyalomma rufipes* (0.9%). The screening of tick salivary glands and midguts using a duplex quantitative PCR (qPCR) assay revealed the presence of *A. marginale* and *A. centrale* infections in 53-100% of the samples. All cattle tested positive for the presence of both *A. marginale* and *A. centrale* DNA. Samples from five cattle infested with *R. evertsi evertsi* and *R. appendiculatus*, were selected for *A. centrale* strain characterization using *msp1aS* genotyping. The *msp1aS* genotyping revealed that genotypes Ac8 and Ac20 were present in DNA samples from one of the cattle and *R. evertsi evertsi* ticks that had fed on that animal. The findings from this study suggest that *R. evertsi evertsi* may be responsible for transmission of *A. centrale* infections in uThukela district, and *R. appendiculatus* and *R. microplus* may also be implicated.

In summary, this study presents a novel genetic test based on *msp1aS* to discriminate strains of *A. centrale* and shows that the vaccine strain is found widely distributed across South Africa and in animals that do not have a history of vaccination. Results also indicate the significance of wildlife as a reservoir host for *A. centrale.* The phylogenetic analysis presented, together

with differences in genome architecture, *msp1α*/*msp1aS* gene sequence, and the biology of tick transmissibility, provided sufficient divergence between *A. centrale* and *A. marginale* to classify them as separate species. The findings from this study furthermore suggested that *R. evertsi evertsi*, *R. appendiculatus* and *R. microplus* may be responsible for transmission of *A. centrale*. This study contributes greatly to the description and the taxonomic status of *A. centrale.*

CHAPTER 1

1. General Introduction

1.1 Background

Bovine anaplasmosis is one of the most important diseases of ruminants worldwide. The disease causes significant economic losses in the livestock industries in the tropical and subtropical areas of the world (Kocan et al., 2003), mainly due to the high morbidity and mortality in susceptible cattle herds. The primary causative agent is *Anaplasma marginale*, which is a Gram-negative obligate intracellular bacterium, and the type species for the genus *Anaplasma*. It is intra-erythrocytic, parasitizing the red blood cells. The disease is mainly characterized by progressive haemolytic anaemia associated with fever, weight loss, abortion, decreased milk production and in some cases death may result (Theiler, 1910, Theiler, 1911; Kocan et al., 2003).

Anaplasmosis has been described in domestic and wild animals including cattle, water buffalo, African antelopes, white-tailed deer and mule deer; however, clinical signs are most noticeable in cattle. Bovine anaplasmosis is infectious but not contagious; the disease is transmitted through tick bites, contaminated fomites and also transplacental transmission has been documented (Kocan et al., 2003). In many countries, especially in Africa, livestock can be in contact with wildlife species, indicating complex circulation dynamics of tick-borne pathogens between wild and domestic animals. This implicates wildlife species as a source of *A. marginale* persistent infections. However, there is limited information on the role played by wildlife species in the epidemiology of tick-borne pathogens such as *Anaplasma* in endemic areas; such information is important for both domestic and wild animal health. Livestock are susceptible to many tick-borne pathogens, while wildlife susceptibility is poorly studied and understood. There has been a case of wildlife mortality due to *Anaplasma* infection, where a giraffe died due to *A. marginale* infection (Neitz, 1935). There are studies showing the prevalence of *A. marginale* and *A. centrale* in African buffalo populations in some African countries (Debeila, 2012; Eygelaar et al., 2015; Khumalo et al, 2016). In a study conducted by Debeila (2012) it was shown that buffalo from the Kruger National Park and Hluhluwe-iMfolozi Park in South Africa were infected with *A. marginale* and *A. centrale*. This would

suggest that wildlife species are natural reservoir hosts of *Anaplasma* infections. It could be that these wildlife species serve a role in the epidemiology and perpetual transmission of anaplasmosis. The tick species thought to be responsible for transmitting *A. marginale* in South Africa are *Rhipicephalus decoloratus*, *R. microplus*, *R. evertsi evertsi* and *R. simus* (Potgieter, 1979), whilst *A. centrale* has only been proven to be transmitted by *R. simus* (Potgieter & van Rensburg, 1987) and *D. andersoni* (Ueti et al., 2009).

Anaplasma centrale is a closely related organism to the type species *A. marginale*, however, the two organisms differ in virulence and localization within infected erythrocytes: *A. centrale* has a low virulence phenotype as compared to *A. marginale*, and rarely causes serious infections; and *A. centrale* is positioned more centrally in the erythrocyte than *A. marginale* (Theiler, 1910; Theiler, 1911). *Anaplasma marginale* and *A. centrale* are said to be antigenically related, with *A. centrale* being capable of cross-protecting against *A. marginale;* therefore it is used as a live vaccine for routine vaccination of cattle in Israel, Australia, Africa and South America (Dreyer et al., 1998; Georges et al., 2001; Inokuma et al., 2005).

However, little is known about the phylogenetic differences between *A. marginale* and *A. centrale,* and the question remains whether they are separate species or whether *A. centrale* is a subspecies of *A. marginale*. Many *Anaplasma* researchers have gradually accepted that *A. centrale* is a separate species as shown by many published papers (Inokuma et al., 2001; Lew et al., 2002; Shkap et al., 2002; Lew et al., 2003; Carelli et al., 2007; Mtshali et al., 2007; Carelli et al., 2008; Decaro et al., 2008; Rymaszewska & Grenda, 2008; Aubry & Geale, 2011; Bell-Sakyi et al., 2015). On the other hand, many still refer to *A. centrale* as a subspecies of *A. marginale* (Kocan et al., 2003; Ueti et al., 2007; Herndon et al., 2010; Herndon et al., 2013; Pierlé et al., 2014). Recent studies have utilized sequence data from 16S rRNA genes, heat-shock protein genes (*groEL*) and major surface protein (*msp*) genes in attempts to differentiate or classify the species within the genus *Anaplasma* (Dumler et al., 2001; Lew et al., 2003). These studies supported Theiler's original description of the organism (i.e. *A. marginale* variety *centrale*) being a variant of *A. marginale* (Theiler, 1911), but there was not sufficient data to determine whether it should be considered a separate species. The paucity of information with regards to *A. centrale* sequences has been a contributing factor to the species having an unclear taxonomic status. The complete genome sequence analysis of *A. centrale* (Herndon et al., 2010) is the most important tool in evolutionary biology and may effectively resolve taxonomic issues. Genome comparative analysis of *A. marginale* and *A. centrale* (Brayton et al., 2005;

Agnes et al., 2010; Herndon et al., 2010) revealed differences in sizes of the two genomes, number of encoding genes, number of pseudogenes and *A. centrale* contains putative genes that are not present in *A. marginale*, while *A. marginale* contains putative genes that are absent from *A. centrale*.

1.2 Problem Statement

Anaplasmosis is endemic in South Africa; meaning there is a high number of ruminants which serve as a reservoir for continuous infection. Buffalo have been documented as the main reservoirs that harbour *Anaplasma* species. This is due to the fact that a lot of the livestock animals are in contact with wildlife species, indicating a complex circulation dynamic of tick-borne pathogens between wild and domestic animals, which then explains the phenomenon of wildlife species as another source of persistent infections (Purnell, 1980; Peter et al., 1998; Tonetti et al., 2009). To date, there is limited information of the role played by wildlife species in the epidemiology of tick-borne pathogens of *Anaplasma* in endemic areas, and this kind of information is important for both domestic and wildlife animal health.

The phylogenetic relationship of *A. centrale* to *A. marginale senso stricto* is not well understood. There is limited information on the molecular epidemiology of *A. centrale*, and only a few strains of *A. centrale* have been reported through molecular characterization (Carelli et al., 2008; Herndon et al., 2010). The low interest in the molecular characterization of *A. centrale* strains has contributed to the uncertain taxonomic status of the species. A molecular characterization study (Dumler et al., 2001), using phylogenetic comparisons of 16S rRNA and *groESL*, revealed that *A. marginale* is divergent from other *Anaplasma* species. Therefore, it should be regarded as a separate species. These authors retained *A. centrale* as a subspecies of *A. marginale*. On the other hand, Inokuma et al. (2001) suggested *A. centrale* to be a separate species from *A. marginale* based on differences in the location of the inclusion bodies in the host erythrocyte, parasite morphology, virulence and geographical distribution. To our knowledge, to date there have not been any studies undertaken to explicitly establish the phylogenetic relationship between these two organisms. There is, therefore, a need for a formal description to resolve the taxonomic status of *A. centrale*. Furthermore, the first report of an anaplasmosis outbreak due to *A. centrale* in Italy indicated that the strains responsible for the outbreak were of African origin. To our knowledge there has not been a study undertaken in Africa to characterize the strains of *A. centrale* in order to determine their genetic diversity.

1.3 Objectives

The main aim of this study was to characterize South African strains of *A. centrale*, in order to determine the genetic diversity of *A. centrale* and infer the phylogenetic relationship between *A. centrale* and *A. marginale.*

In view of the above, the following were the objectives of the study:

(i) To determine the genetic diversity of *A. centrale* strains using the *msp1aS* gene and to establish whether wildlife species are reservoir hosts of *Anaplasma* species.

(ii) To elucidate and infer the phylogenetic relationship between *A. marginale* and *A. centrale* using individual gene sequence analysis.

(iii) To establish the tick species responsible for transmitting *A. centrale* in the apparent absence of *Rhipicephalus simus* in the uThukela district, KwaZulu-Natal, South Africa.

1.4 Thesis overview

Chapter 1: This chapter provides the general introduction and outlines the rationale and objectives of the study.

Chapter 2: This chapter provides a detailed review of the literature of *Anaplasma* species, particularly the taxonomy, transmission, arthropod vectors, geographic distribution, reservoir hosts, diagnosis, treatment and control strategies, and molecular characterization.

Chapter 3: The aim of this chapter was to screen wildlife and cattle for the presence of *Anaplasma* infections and to characterize *A. centrale* strains obtained from different geographical areas within South Africa using an *msp1aS* genotyping tool in order to determine the genetic diversity of *A. centrale* in comparison with the original vaccine strain.

Chapter 4: In this chapter the phylogenetic relationship between *A. marginale* and *A. centrale* was explored to establish whether *A. centrale* is a subspecies of *A. marginale* or a separate species. Characterization of three conserved genes, 16S rRNA, *groEL* and *msp4*, showed separate groupings of *A. marginale* and *A. centrale* in the phylogenetic trees with strong supportive bootstrap values, which was indicative of a separate taxonomic species status for *A. centrale*.

Chapter 5: This chapter focused on examining the possible tick species responsible for transmitting *A. centrale* through detection and characterization of *A. centrale* in different tick species from cattle in the uThukela district, KwaZulu-Natal, South Africa. The *msp1aS* genotype revealed common repeat sequences in DNA isolated from ticks and cattle.

Chapter 6: This chapter provides a general discussion, conclusions and recommendations arising from this study.

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CHAPTER 2

2. Literature Review

Anaplasmosis is a tick-borne disease of cattle and other ruminants caused by Gram-negative intra-erythrocytic rickettsiae of the genus *Anaplasma*, order Rickettsiales, Family Anaplasmataceae (Dumler et al., 2001). Based on location within the erythrocyte there are two forms of *Anaplasma* that infect cattle, *A. marginale* and *Anaplasma marginale* subspecies *centrale* (hereafter referred to as *A. centrale*). Clinical bovine anaplasmosis is usually caused by *A. marginale*. This review is, therefore, particularly focused on the common and unique characteristics of *A. marginale* and *A. centrale,* with an emphasis on the taxonomy, transmission, arthropod vectors, epidemiology, geographic distribution, treatment and control strategies, molecular characterization and phylogeny.

2.1 History and taxonomic position

Anaplasma marginale was first described by Theiler in South Africa in 1910 (Theiler, 1910). He described it as "marginal points" in bovine erythrocytes, and hence the name "*marginale*" derived from its location within the host cell. "*Anaplasma*" refers to the lack of stained cytoplasm. A year later, Theiler isolated and described a very similar parasite which was more centrally located within the erythrocytes of the host cells. He named the parasite *A. marginale* variety *centrale* (Theiler, 1911). The latter was found to be less pathogenic in domestic animals and conferred immunity against infection by *A. marginale* (Theiler, 1911). The following years saw the discovery of other *Anaplasma* species, namely *A. ovis*, *A. platys* (formerly known as *Ehrlichia platys*), *A. bovis* (Kuttler, 1984) and *A. phagocytophilum* (formerly known as *Ehrlichia phagocytophila,* and also includes *E. equi* and the agent of human granulocytic ehrlichiosis (HGE) (Dumler et al., 2001). Recently, *A. capra* was identified and described by Li et al., 2015. This species is zoonotic; infecting humans, sheep and goats and is widespread in China (Li et al., 2015, Yang et al., 2017).

The taxonomy of *A. marginale* has a long history; it has previously been classified as a virus and a protozoan parasite. Although it was the first rickettsial pathogen to be identified, it was only correctly placed within the Order Rickettsiales in the seventh edition of Bergey's manual

in 1957 (Brayton et al., 2009). The subsequent addition of genus or species names (e.g., *Anaplasma caudatum*, *Paranaplasma caudate* and *Paranaplasma discoides*) to describe *A. marginale* strains with apparent different morphological features within infected erythrocytes confused the taxonomy even further (Ristic & Kreier 1984; Smith et al., 1989). It is now known that these features are merely host proteins associated with the vacuole (Brayton et al., 2009). Since then the nomenclature of *A. marginale* and related bacteria has been unified by Dumler et al. (2001); the order Rickettsiales was reorganized and the genera within the families *Rickettsiaceae* and *Anaplasmataceae* were reclassified using phylogenetic analyses of 16S rRNA and *groESL* genes, supported by biological data. *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia* species were assigned to the family *Anaplasmataceae.* Within the genus *Anaplasma*, *A. marginale* is the type species; the other recognized species include the ruminant infecting *A. bovis*, *A. centrale* (originally *Anaplasma marginale* variety *centrale*, also referred to as *Anaplasma marginale* subspecies *centrale*) and *A. ovis*, the canine pathogen *A. platys*, and *A. phagocytophilum* which infects numerous species of mammals including humans (Fig. 2.1; Table 2.1) (Dumler et al. 2001).

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Alpha-proteobacteria
Order:	Rickettsiales
Family:	Anaplasmataceae
Genus:	*Anaplasma*
Species :	*A. marginale* (type species)
	A. marginale subspecies *centrale*
	A. bovis
	A. phagocytophilum
	A. ovis
	A. platys

Figure 2.1 Current taxonomic classification of *Anaplasma* species (Dumler et al., 2001).

Based on the statement by Ristic (1968) that "In 1911, Theiler, who first described *A. centrale*, indicated that it was a separate species and thus distinct from *A. marginale*", the name *A. centrale* was included in List No.15 of Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the International Journal of Systematic Bacteriology. Subsequently the organism was listed as a separate species in Bergey's Manual

of Systematic Bacteriology (Ristic & Kreier, 1984) without a formal species description. In 2001, Dumler et al. (2001) indicated that the 16S rRNA gene sequences of strains of *A. marginale*, *A. ovis* and *A. centrale* are nearly identical with 99.1% similarity, supporting Theiler's original description of *A. centrale* being a variant of *A. marginale* (Theiler, 1911), but they did not resolve this taxonomic fine point. In the study done by Inokuma et al. (2001), the authors have shown that the 16S rRNA sequence of *A. centrale* was closely related to *A. marginale* by both level of similarity (98.08% identical) and distance analysis. They concurred that *A. centrale* is an independent species although closely related to *A. marginale*. To date the taxonomic status of *A. marginale* subspecies *centrale* / *A. centrale* and its relationship to *A. marginale* sensu stricto remain unclear.

Table 2.1 Characteristics of *Anaplasma* species (updated from Rymaszewska & Grenda, 2008)

Aetiological agent	Disease	Vector	Infected organism or host	Infected cell	Distribution
Anaplasma marginale	Bovine anaplasmosis	*Rhipicephalus* spp. *Ixodes* spp. *Dermacentor* spp.	Cattle, wild ruminants	Erythrocytes	Worldwide in tropical and subtropical regions
Anaplasma centrale	Bovine anaplasmosis	*Rhipicephalus* spp. *Ixodes* spp.	Cattle, wild ruminants	Erythrocytes	Africa, Italy, Spain and Brazil
Anaplasma bovis	Bovine anaplasmosis	*Haemophysalis* spp. *Rhipicephalus* spp. *Amblyomma* spp.	Cattle	Monocytes	Africa, North America, South America and Asia
Anaplasma phagocytophilum	Human granulocytic anaplasmosis, tick-borne fever of ruminants, equine granulocytic anaplasmosis, canine granulocytic anaplasmosis	*Ixodes* spp. *Dermacentor* spp.	Ruminants, horses, dogs, humans, rodents	Granulocyte	Worldwide
Anaplasma ovis	Ovine anaplasmosis	*Dermacentor* spp.	Sheep, goats, wild ruminants	Erythrocytes	USA, Europe, Asia and Africa
Anaplasma platys	Canine cyclic thrombocytopenia	*Rhipicephalus sanguineus*	Dogs	Platelets	Worldwide
Anaplasma capra	Anaplasmosis	*Ixodes persulcatu* *Haemophysalis qinghaiensis*	Sheep, goats, humans	Erythrocytes	Asia

2.2 Transmission and arthropod vectors

Anaplasma marginale is transmitted mechanically by biting flies or blood-contaminated fomites (including needles or surgical instruments) and biologically by ticks (Dikmans, 1950; Ewing, 1981; Kocan, 1986); biological transmission by ticks is more efficient than mechanical transmission (Scoles et al., 2005). Mechanical transmission by arthropods has been reported

for bloodsucking diptera of the genera *Tabanus*, *Stomoxys*, and mosquitoes (Ewing, 1981; Foil, 1989; Potgieter et al., 1981). Additionally, to mechanical and biological transmission, *A. marginale* can be transmitted from cow to calf transplacentally during gestation (Norton et al., 1983; Zaugg & Kuttler, 1984; Zaugg, 1985), resulting in healthy but persistently infected calves (Kocan et al., 2015).

Biological transmission of *A. marginale* is effected by ticks and approximately 20 species of ticks have been incriminated as vectors worldwide (Dikmans, 1950; Ewing, 1981; Kocan et al., 2004). In general, tick vectors of *A. marginale* include *Rhipicephalus* spp., selected *Dermacentor* spp. and *Ixodes ricinus*, while *Amblyomma* spp. do not appear to transmit *A. marginale* (Kocan et al., 2004). Pathogen transmission occurs from stage to stage (transstadial) or within a stage (intrastadial), while transovarial transmission from one tick generation to the next does not appear to occur (Stich et al., 1989). In South Africa, five tick species have been implicated as vectors of *A. marginale* (Table 2.2); *Rhipicephalus decoloratus, R. microplus, R. evertsi evertsi*, *R. simus* and *Hyalomma rufipes* (Potgieter & Van Rensburg, 1987; Dreyer et al., 1998). The adults transmit *A. marginale* intrastadially, while *R. microplus* and *R. decoloratus* also transmit *A. marginale* transstadially. *Rhipicephalus simus* transmits *A. marginale* transstadially (De Waal, 2000; Potgieter & Stoltsz, 2004).

Anaplasma centrale was thought not to be tick transmissible (Shkap et al., 2009; Ueti et al., 2007), however, experimental transmission of *A. centrale* by *R. simus* and *D. andersoni* has been demonstrated (Potgieter & van Rensburg 1987; Ueti et al., 2009). Work done in the *D. andersoni* model demonstrated that *A. centrale* infects the midgut and salivary gland at similar rates as *A. marginale*, but *A. centrale* was not transmitted when only a few ticks were used in transmission experiments (Ueti et al., 2007). Further analysis demonstrated that *A. centrale* resides in a different subcellular location in the salivary gland and was secreted into the saliva at a much lower rate than *A. marginale*; when tick numbers were dramatically increased to compensate for the low pathogen load, transmission was achieved (Ueti et al., 2009). These two transmission studies are the only successful transmissions of *A. centrale* on record amongst a myriad of failed transmission attempts.

Table 2.2 Ticks species responsible for transmitting *A. marginale* and *A. centrale* in South Africa (de Waal., 2000).

Tick Species	Common name	Mode of Transmission	General characteristics
Rhipicephalus decoloratus	African Blue tick	Intrastadial by adults (*A. marginale*) Transstadial (*A. marginale*)	One-host tick. Indigenous to Africa (Walker, 1991), historically believed that it might have evolved as parasite of ungulates in East Africa and migrated to South Africa with livestock (Henning, 1956). Infests a variety of livestock and wild animals but cattle are the main domestic hosts.
Rhipicephalus microplus	Asian blue tick	Intrastadial by adults (*A. marginale*) Transstadial (*A. marginale*)	One-host tick. Cattle are the main hosts but it has also been found on other domestic animals such as sheep, goats, dogs and horses (Smith, 1983). It rarely infests wild animals.
Rhipicephalus evertsi evertsi	Red-legged tick	Intrastadial by adults (*A. marginale*)	Two-host tick. It infests cattle, sheep, goats and wildlife (Norval & Horak, 1994). Common in South Africa and occurs extensively in the savanna and grassland biomes.
Hyalomma rufipes	Large, coarse bont-legged tick	Intrastadial by adults (*A. marginale*)	Two-host tick. The adults are hunters and feed on cattle, sheep, goats, horses, and large wild herbivores including rhinoceroses, while the immature stages feed on scrub hares and ground-frequenting birds. Present throughout South Africa. (Norval & Horak 1994).
Rhipicephalus simus	Glossy brown tick	Intrastadial by adults (*A. marginale*) Transstadial (*A. marginale* and *A. centrale*)	Three-host tick. Distributed throughout Southern Africa (Walker et al., 2000). Well established in savanna biome, it is usually not encountered in large numbers. Among domestic animals, the adult ticks primarily parasitize cattle and dogs (Walker et al., 2000), but they have been recovered from many wild animals (Horak et al., 2000). Immature stages feed exclusively on rodents (Spickett, 2013).

The developmental cycle of *A. marginale* in ticks is complex and coordinated with the tick feeding cycle (Kocan, 1986; Kocan et al., 1992) (Fig. 2.2). The developmental stages of *A. centrale* in ticks have not been described, whereas *A. marginale* has been shown to develop in the midgut tissues and in the salivary gland cells of ticks, with the latter stages found to be infective for cattle (Kocan et al., 1992). Ticks ingest infected erythrocytes during the blood meal; the source of *A. marginale* infection for tick gut cells. After development in the tick gut cells, other tick tissues also become infected (including the salivary glands) from where the rickettsiae are transmitted to the vertebrate host during tick feeding. At each infection site in the tick, *A. marginale* develops within membrane-bound vacuoles or colonies. The first form seen within the colony is the reticulated (vegetative) form; it divides by binary fission, forming large colonies that may contain hundreds of organisms. The reticulated form then changes into the dense form; the infective form which can survive outside the host cells. Cattle become infected with *A. marginale* when the dense form is transmitted during tick feeding via the salivary glands (Kocan et al., 2003). *Anaplasma* infect erythrocytes infection of endothelial

cells have only been shown *in vitro* (Munderloh et al., 2004; Carreño et al., 2007; Rodríguez et al., 2009; Wamsley et al., 2011). Once in the bovine erythrocytes, it undergoes cycles of replication, removal of infected erythrocytes by the reticuloendothelial system and subsequent reinvasion of erythrocytes within the ruminant (Aubry & Geale, 2011). Much less is known about the infection process in the endothelial cell (Carreño et al., 2007).

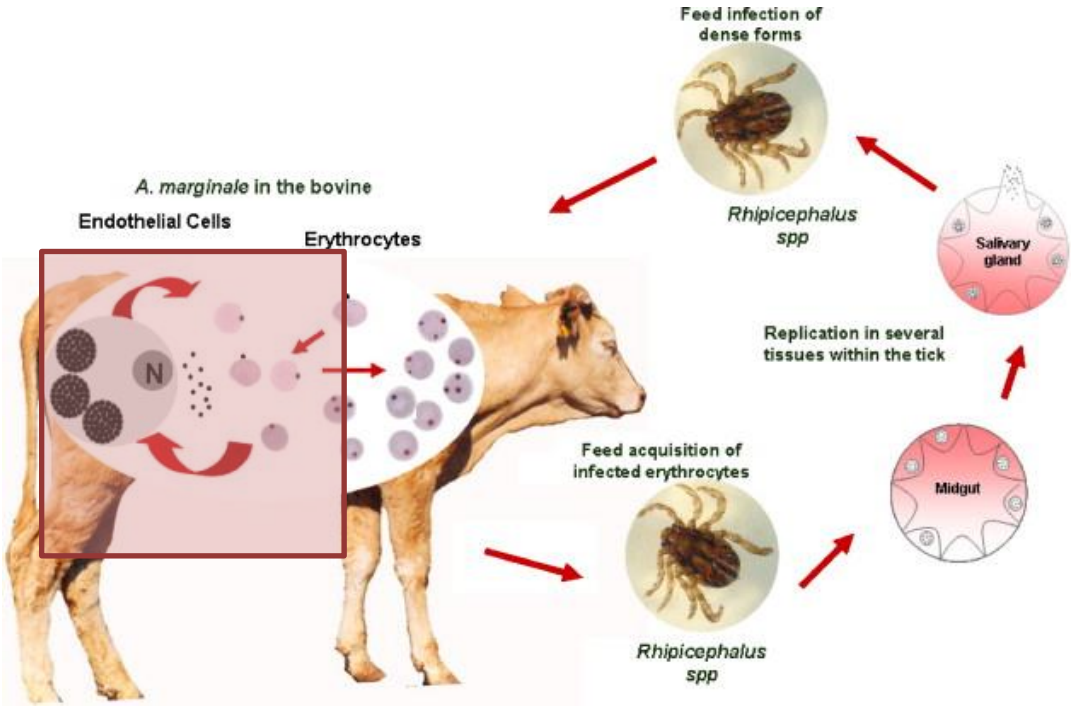

Figure 2.2 The life cycle of *A. marginale*, demonstrating the replication of the rickettsia in endothelial cells and *Rhipicephalus microplus* ticks which transmit different *Anaplasma* species. (from Rodríguez et al., 2009).

2.3 Epidemiology

2.3.1 Geographical distribution

Anaplasmosis occurs in tropical and subtropical areas of the world and is a main constraint to cattle production in many countries (Kocan et al., 2003). Seasonal incidence of anaplasmosis outbreaks occur more frequently during summer and autumn because of the increased abundance of ticks and blood-sucking flies; it is also affected by rainfall and the implementation of tick-control measures (Kocan et al. 2010). In contrast, in areas where vector ticks have been eradicated, mechanical transmission becomes an alternative form of transmission and widened distribution is effected by transportation of cattle (Kocan et al., 2003; Kocan et al., 2010).

In South Africa, anaplasmosis is endemic in most cattle-rearing areas except in the low-rainfall areas where tick populations are low (De Waal, 2000) (Fig. 2.3). More than 99% of the total cattle population is at risk (De Waal, 2000). Only a few studies have been undertaken in South Africa for serological evidence of *A. marginale* endemicity; studies in the Free State (Dreyer et al., 1998; Mbati et al., 2003), Limpopo (Rikhotso et al., 2005) and North West (Ndou et al., 2010) provinces were indicative of an endemically stable situation. Molecular diagnosis of *A. marginale* was determined in the Free State province (Mtshali et al., 2007), and subsequently in Mpumalanga, Gauteng, Eastern Cape, Limpopo, North West, KwaZulu-Natal, Eastern Cape, and Western Cape provinces (Mutshembele et al., 2014). The prevalence of *A. marginale* ranged from 65% to 100%, except in the Northern Cape province where *A. marginale* was not detected. No differences between the prevalence of *A. marginale* in commercial and communal farming systems were observed. The prevalence of *A. marginale* in cattle from South Africa is similar to that of other regions of the world, for example; in Brazil a recent study found 70% prevalence of *A. marginale* in a cattle herd (Pohl et al., 2013), while a study in Italy has shown *A. marginale* prevalences ranging from 25% to 100% in cattle herds (de la Fuente et al., 2005).

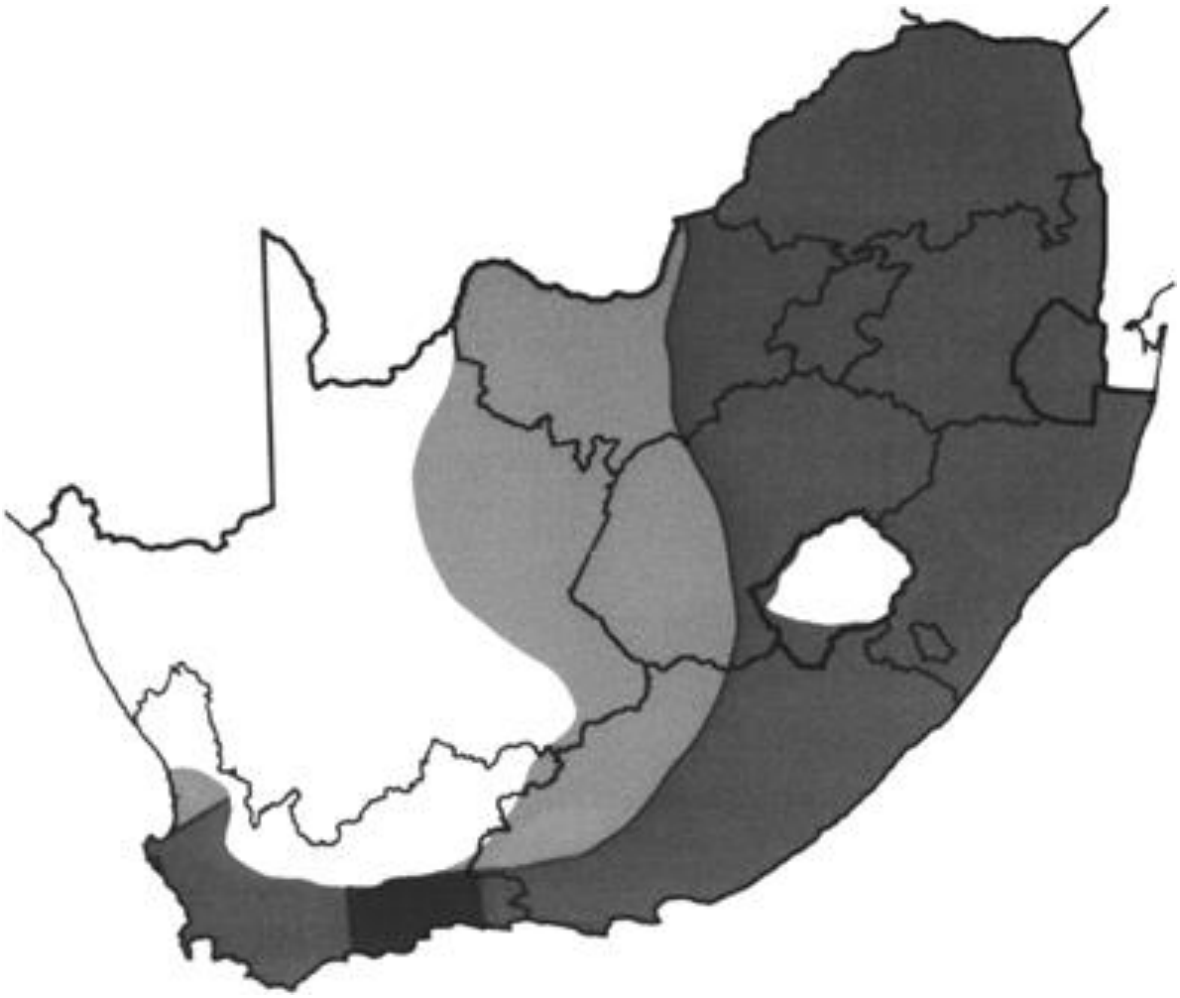

Figure 2.3 Endemic and epidemic areas of anaplasmosis in South Africa: endemic areas are indicated by the darker grey areas, epidemic areas are indicated by the lighter grey areas (from De Waal, 2000).

2.3.2 Reservoir hosts

Anaplasma species infect a wide range of ruminants; however, cattle are naturally susceptible to *A. marginale* and *A. centrale* (Aubry & Geale, 2011). Wild ruminants become persistently infected with *Anaplasma* species and serve as reservoirs for infection of domestic ruminants such as cattle (Kocan et al., 2003); however, there is limited information about the susceptibility of wild ruminants to infection by *A. marginale* and the role played by wild ruminants in the epidemiology of bovine anaplasmosis. Except for two reports of acute anaplasmosis in giraffes, naturally occurring *A. marginale* infections among wild ruminants have not been clinically evident (Davidson & Goff, 2001). The possible subclinical impacts of bovine anaplasmosis on wild ruminants, in terms of effects on survival or reproduction, have also not been assessed (Aubry & Geale, 2011).

Anaplasma marginale has been recovered in a wide range of ruminants including; buffalo (*Bubalus bubalis* and *Syncerus caffer*) (Potgieter, 1979), black wildebeest (*Connochaetes gnou*) (Neitz, 1935; Kuttler, 1984), blue wildebeest (*Connochaetes taurinus*) (Kuttler, 1965; Smith et al., 1982), American bison (*Bison bison*) (Zaugg & Kuttler, 1985), white-tailed deer (*Odocoileus virginianus*) (Smith et al., 1982), mule deer (*Odocoileus hemionus hemionus*) (Kuttler, 1984), black-tailed deer (*Odocoileus hemionus columbianus*) (Kuttler, 1984), Rocky Mountain elk (*Cervus elaphus nelsoni*) (Kuttler, 1984), blesbok (*Damaliscus albifrons*) (Kuttler, 1984), grey duiker (*Sylvicapra grimmia*) (Neitz & Du Toit, 1932), nyala (*Tragelaphus angasii*) (Peirce, 1972) and eland (*Taurotragus oryx*) (Ngeranwa, et al., 1998; Khumalo et al., 2016). Subclinical infections of *A. marginale*, either natural or after artificial infection, have been reported in the African buffalo (Potgieter, 1979), eland, (Peirce, 1972; Ngeranwa, et al., 1998), black wildebeest (Neitz, 1935), blue wildebeest (Smith et al., 1974), grey duiker (Neitz & Du Toit, 1932) and blesbok (Neitz & Du Toit, 1932). *Anaplasma marginale* and *A. centrale* DNA has been detected in buffalo (Debeila, 2012; Eygelaar et al., 2015; Khumalo et al., 2016), eland (Khumalo et al., 2016), blue wildebeest (Khumalo et al., 2016), black wildebeest (Khumalo et al., 2016) and waterbuck (*Kobus ellipsiprymnus*) (Khumalo et al., 2016).

2.4 Clinical signs and diagnosis

Bovine anaplasmosis can affect any age or breed of cattle, however, the severity varies with age of a cattle and the amount of the infective dose of organism. Adult cattle are the most severely affected. Illness is rare in cattle under 6 months of age, while animals between 1 and 2 years of age suffer from acute but rarely fatal disease. In adult cattle over 2 years of age, the disease is acute and often fatal (Richey, 1991; Kocan et al., 2003). Cattle that survive the infection remain persistently infected (Richey, 1991), have lifelong immunity and are resistant to clinical disease on challenge exposure. They also serve as reservoirs for continuous transmission or infection of *A. marginale* (Kocan et al., 2003). *Bos indicus* and *B. taurus* are both susceptible to anaplasmosis infections, althoughbut there might be variation of resistance within individuals of both species (Jonsson et al., 2008). Differences in virulence between *Anaplasma* strains and the level and duration of the parasitaemia also play a role in the severity of clinical manifestations (De Waal, 2000).

The first clinical sign of bovine anaplasmosis is pyrexia which occurs prior to infection of 1% of the erythrocytes, followed by fever of > 40°C that persists as the level of parasitemia

increases; with subnormal temperatures being reported prior to death in terminal cases (Kocan et al., 2010). The most remarkable sign of clinical bovine anaplasmosis is anaemia which is linked with phagocytosis of parasitized erythrocytes. The severity of the clinical signs is associated with the degree of anaemia and includes pallor of the skin and mucous membranes as well as an increase in heart- and respiratory rates. As the parasitemia increases, the packed cell volume declines, resulting in the animal becoming weak, anorexic and lethargic. This leads to a decrease in milk production in lactating cows, while pregnant cows may abort; bulls may develop temporary barrenness. With advanced disease cattle develop gastrointestinal atony, rumen stasis and constipation linked with dehydration and weight loss. Neurological deficits may be seen in some animals which have been attributed to episodes of cerebral anoxia. Icterus usually develops late in the course of sickness and is most commonly seen during early convalescence. Recovery is more common in young animals, mortality rates of 50–60% are reported in adults. Necropsy findings include severe anaemia, icterus, splenomegaly and hepatomegaly. Petechial hemorrhages are frequently observed on serosal surfaces especially over the heart and pericardium; the heart is often pale and flabby (Kocan et al., 2010).

Anaplasma centrale induces a low degree of anaemia, with clinical outbreaks being rare (Ristic & Kreier, 1984).

A tentative diagnosis of bovine anaplasmosis may be made based on geographic position, seasonal period, signalment and presentation of clinical symptoms and/or necropsy findings observed in infected animals (Jones & Brock, 1966). In order to confirm the diagnosis, laboratory tests are required. Light microscopic examination of Giemsa-stained blood smears from clinically infected animals, during the acute phase of the disease, can be performed. Microscopically, *A. marginale* would appear as dense, rounded, intra-erythrocytic bodies approximately 0.3 to 1.0 μm in diameter, mostly situated on or near the margin of the erythrocyte (OIE, 2008). *Anaplasma centrale*, although similar in appearance, will mostly be situated away from the margin of the erythrocyte (i.e. more centrally) (OIE, 2008). It can be difficult to distinguish *A. marginale* from *A. centrale* in a stained smear, particularly with low levels of parasitaemia. Microscopy is, therefore, not reliable for detecting asymptomatic or carrier animals. In these cases, the infection is generally diagnosed by serological assays and confirmed by molecular detection methods (Aubry & Geale, 2011).

Several serological tests are available and have been used extensively for epidemiological studies; these include a competitive enzyme-linked immunosorbent assay (cELISA), indirect fluorescent antibody (IFA) test, complement fixation (CF) test, capillary agglutination assay and card agglutination test (CAT). Of these, the cELISA and CAT are the preferred assays (Kocan, et al., 1992; de la Fuente, 2005; OIE, 2008). The cELISA specifically detects the presence of serum antibodies that target the Msp5 protein of *Anaplasma* spp. (Knowles et al., 1996); it has been proven to be very sensitive and specific for the detection of *Anaplasma*-infected animals. The CAT has also been shown to be sensitive, but nonspecific reactions can occur; subjectivity in interpreting assay reactions can result in variability in test interpretation (OIE, 2008).

Various nucleic-acid-based polymerase chain reaction (PCR) assays have been developed that are able to detect the presence of low-level infection in carrier cattle and tick vectors (Aubry & Geale, 2011; Lew et al., 2002; Shkap et al., 2002). These include the reverse line blot (RLB) hybridization assay (Bekker et al., 2002), restriction fragment length polymorphism (RFLP) assays (Noaman et al., 2009), nested PCR (nPCR) assays (Molad et al. 2009; Decaro et al. 2008) and quantitative real-time PCR (qPCR) assays (Futse et al. 2003; Carelli et al. 2007; Ueti et al. 2007; Decaro et al. 2008; Picoloto et al. 2010; Reinbold et al. 2010). Of the conventional PCR assays that have been developed, assays that target the *msp4* and *msp1α* genes are mostly used for differentiating *A. marginale* strains, which is useful for tracking the origin of an outbreak, but also to differentiate between *A. marginale* and *A. centrale* DNA (Lew et al., 2002; Mtshali et al., 2007; de la Fuente et al., 2007). The *groEL* gene is mostly targeted for detection of *A. centrale* infections (Shkap et al., 2002).

A real-time PCR assay based on the *msp1b* gene has been developed for detection and quantification of *A. marginale* DNA in blood of naturally infected cattle (Carelli et al., 2007). The assay was shown to be sensitive and specific as there were no cross-reactions with other haemoparasites of ruminants (*A. centrale*, *A. bovis*, *A. phagocytophilum*, *Babesia bigemina* and *Theileria buffeli*). A year later Decaro et al. (2008) developed a duplex real-time PCR for simultaneous detection and quantification of *A. marginale* and *A. centrale* based on the *msp1b* and *groEL* genes, respectively. In a recent study (Chaisi et al., 2017), the authors evaluated the ability of the RLB hybridization assay (Bekker et al. 2002), two nPCR assays (Decaro et al. 2008; Molad et al. 2009) and the duplex qPCR assay developed by Decaro et al. (2008) in detecting *A. marginale* and *A. centrale* infections in cattle in South Africa. Results indicated

that the duplex qPCR was more sensitive than the nPCR and RLB assays in detecting carriers of bovine anaplasmosis in South Africa.

2.5 Treatment, prevention and control strategies

Control measures for anaplasmosis vary with geographic location and include control of tick vectors, administration of antibiotics, vaccination and maintenance of anaplasmosis free herds (De Waal, 2000; Aubry & Geale, 2011). The choice of control method is usually influenced by the availability, cost and feasibility of the application.

Dairy and beef cattle farmers rely on dipping for control of tick infestation, however in areas where tick vectors are well established, the exposure to ticks gives a high degree of immunity against anaplasmosis (De Waal, 2000). The repeated application of acaricides can, however, result in the development of acaricide resistant tick populations. Furthermore, environmental pollution is increasingly of concern. Tick (and fly) control remains labour-intensive and expensive.

Antibiotic therapy includes the use of tetracycline drugs (tetracycline hydrochloride, chlortetracycline, oxytetracycline, and doxycycline), gloxazone (alpha-ethoxyethylglyoxal dithiosemicarbazone) and two derivatives of carbanilide, amicarbalide (amicarbalide isethionate) and imidocarb [3, 3'bis-(2-imidazolin-2-yl)-carbanilide dihydrochloride (or dipropionate)]. In addition, a variety of chemotherapeutic agents (including arsenicals, antimalarials, antimony derivatives and dyes) have been used in the past in some parts of the world, but with little to no effect on acute anaplasmosis (Potgieter & Stoltsz, 2004). Although chemotherapy is intended to prevent clinical anaplasmosis, it does not prevent cattle from becoming persistently infected with *A. marginale*; as a result, cattle receiving antibiotic therapy may not be cleared of infection. Tetracyclines, which effectively inhibit the multiplication of *Anaplasma* in erythrocytes, are used extensively; however, it is expensive and the risk exists that resistant *Anaplasma* organisms could develop. To date, the resistance of *A. marginale* to antibiotics has not been reported (De Waal, 2000; Aubry & Geale, 2011).

Vaccination has been shown to be a relatively effective and economical means of controlling bovine anaplasmosis worldwide. Both killed (inactivated) and live vaccines have relied on erythrocyte-derived antigen sources to induce protective immunity or to prevent clinical

disease. However, neither one prevents cattle from becoming persistently infected with *A. marginale* or becoming reservoirs of infections (Kocan et al., 2003; Kocan et al., 2010).

The use of the live *A. centrale* vaccine was introduced by Sir Arnold Theiler in the early 1900s (Theiler, 1911) and this continues to be the vaccine of choice in many parts of the world. Live vaccines involve the infection of cattle via inoculation with erythrocytes infected with *A. centrale* or with less pathogenic, attenuated *A. marginale* (Pipano, 1995; Bock & de Vos, 2001; Bock et al., 2003). The immune response triggered is similar to natural infection; vaccinated animals develop mild and/or asymptomatic infections and become persistently infected with the vaccine strain. Vaccination strategies using live organisms include (i) infection and treatment, (ii) live vaccines containing attenuated strains of *A. marginale*, and (iii) live vaccines containing the less pathogenic *A. centrale* (as reviewed by Kocan et al., 2003).

For the infection and treatment method, calves are infected with *A. marginale* with subsequent treatment with low dosage tetracyclines. Cattle become persistently infected and are subsequently immune to challenge exposure with the same or different *A. marginale* isolates. This vaccination procedure requires close observation of cattle and may not be applicable for use in large herds. The control of post-inoculation reactions is also often unsuccessful in preventing acute infection (Kuttler, 1984).

Vaccination with attenuated *A. marginale* strains produced by irradiation or through passage of the organism in sheep or deer, have been considered for use in live vaccines (Vizcaíno & Betancourt, 1983; Zaugg & Kuttler, 1984; Kocan et al., 2003). Protection provided by these attenuated vaccines has, however, in general been unreliable.

Vaccination using live *A. centrale* has been in use since Theiler observed that *A. centrale* was less pathogenic for cattle than *A. marginale* (Theiler, 1911). He also observed that cattle infected with *A. centrale* developed protective immunity against *A. marginale* infection. *Anaplasma marginale* and *A. centrale* are said to be antigenically related, with *A. centrale* being capable of cross-protecting against *A. marginale* (Shkap et al., 1991); therefore, it is used as a live vaccine for routine vaccination of cattle in Israel, Australia, Africa and South America (Dreyer et al., 1998; Georges et al., 2001; Inokuma et al., 2005). It should, however, be noted that vaccine success can be expected to vary with *A. marginale* genotypes to which vaccinated cattle are exposed (Kocan et al., 2010). Trials which were conducted in South America and

Africa with heterologous strains have shown low effectiveness (Turton et al., 1998; Molloy et al., 2001; Dark et al., 2011). A further drawback of live, blood-derived vaccines is the risk of transmitting other pathogens that persistently infect cattle. It is, therefore, recommended that the use of these types of vaccines be restricted to the area where they were produced (Kocan et al., 2003).

Apart from live vaccines, killed (inactivated) vaccines have also been developed in the U.S.A. in the 1960s. They were, however, only marketed until 1999 after which they were withdrawn from the marketplace (Kocan et al., 2003). Although this vaccine is not formally licensed to for marketing, it appears that it is still made and sold in several states in the US (https://www.anaplasmosisvaccine.com/home.html). The advantages of these killed vaccines included the low risk of contamination with undesirable infectious agents, inexpensive storage and minimal post-inoculation reactions. The disadvantages included the requirement of yearly boosters, expensive purification of *A. marginale* from erythrocytes, lack of cross-protection among isolates from widely separated geographic areas and protective immunity afforded by killed vaccines was usually less than that of live vaccines.

Other vaccine trials have included a variety of subunit vaccines, none of which provided complete protection against heterologous challenge (Palmer & McElwain, 1995). The success of these novel vaccines that use molecular technologies depends on their ability to either mimic or redirect the host response during natural infections or block infection of host cells (Kocan et al., 2003). Major surface proteins have been used experimentally as vaccine candidates against *A. marginale* infections (Turton et al., 1998). The Msp1a protein has the ability to induce a T-cell response and contains conserved B-cell epitopes in the repeated peptides that are recognized by immunized and protected cattle (Kocan et al., 2003; de la Fuente et al., 2003). However, experiments with recombinant Msp1a have only shown partial protection against anaplasmosis in cattle (MacMillan et al., 2006).

Cattle immunized with purified outer membrane preparations (Omps) have been shown to protect against challenge to levels equivalent to those induced by live vaccines (Tebele et al., 1991; Brown et al., 1998; Palmer et al., 1999; Noh et al., 2008; Noh et al., 2013). It was found that approximately 75% of the outer membrane vaccinates are completely protected from infection, and all vaccinates are protected against high-level parasitaemia and clinical disease (Tebele et al., 1991; Brown et al., 1998; Palmer et al., 1999). Although these studies provide

information on the helper T-cell response and its role in protective immunity to *A. marginale*, the immune response is complex. When calves recover from acute infection, they remain infected for a long period of time. Therefore, if persistence results from antigenic variation, characterizing the variant and conserved epitopes recognized by immune T-cells is important for identification of vaccine candidates (Palmer & McElwain, 1995; Brown et al., 1998).

A study by Noh et al. (2008) was conducted to identify Omps associated with the surface proteome of *A. marginale* using cross-linking of adjacent surface proteins, 11 subsets of proteins were found in the cross-linked protein preparation, including: Msp1a, Msp2, Msp3, Msp4, OpAG2, Omp1, Omp7, Omp8, Omp9, Am779, Am854. Brayton et al. (2005) and Noh et al. (2008) used a comparative genomics analysis approach to reveal that the Msp2 protein superfamily (pfam01617) were poor vaccine candidates. From comparative analysis of genomic sequence data, six vaccine candidate genes (Msp2 superfamily genes [Msp4, Omp1, Omp7, and OpAG2] and two non-superfamily members [AM779/ACIS557 and AM854/ACIS486] were found through exclusion of sequences that did not have homologues in the vaccine strain and the highly variable Msp2 and Msp3. Sequence similarity between these candidate genes between the vaccine strain and *A. marginale* was found to range from 63% to 88% (Noh et al., 2008). Through the use of 2D electrophoresis and immunoblotting, along with LC-MS/MS, Agnes et al. (2011) identified *A. marginale* antigens recognized by IgG2 in sera from calves across multiple MHC haplotypes immunized by inoculation with *A. centrale*. A total of 15 proteins were identified and included five house-keeping genes and ten Omps, including Omp7, Omp8, Omp9, Omp11, Omp13, Omp14, AM779, AM854, AM1144, AM1063. Subsequently, Dark et al. (2011) corroborated the validity of the Omps found in the above-mentioned studies, and found that the vaccine candidate genes were conserved amongst *A. marginale senso stricto* strains from the USA, using a comparative genomic sequence analysis. Since then, these Omp candidates have been narrowed to three vaccine candidates with broadly conserved epitopes; Am779, Am854, and a collapsed single Omp7/8/9 (Palmer et al., 2012).

A recombinant DNA vaccine against the vector tick *R. microplus* has been developed by Dayton (1991). The vaccine targets the gut cells of the tick and it destroys the digestive tract of the tick. The vaccine gives an approximately 90% reduction of weight and egg production capacity of the tick. A trial vaccination with this recombinant *R. microplus* gut antigen has been

shown to control tick infestations in South America, however, more field work vaccination needs to be conducted before it is approved for commercial use (de la Fuente et al., 1999).

What prevention and control strategy is recommended in the South African context?

The long term and recommended approach to managing ticks and tick-borne diseases in South Africa is by integrating strategic use of acaricides and application of the live-blood *A. centrale* vaccine (Van Rensburg, 1981; De Waal, 2000). The implementation and long-term maintenance of strict tick eradication programmes to control endemic tick-borne diseases such as; bovine anaplasmosis, heartwater and babesiosis is generally not recommended (Potgieter & Stoltsz, 2004), as it would render the cattle population susceptible to several other tick-borne diseases; which may lead to large disease outbreaks. The repeated application of acaricides can result in the development of acaracide resistant tick populations. Furthermore, populations of wild ruminants sustain tick populations; some of which might be reservoir hosts. It is thus recommended that a stable disease situation be attained by allowing natural exposure of calves to tick-borne diseases, including bovine anaplasmosis, during the period when they are naturally resistant or protected by passively acquired maternal antibody. With regards to bovine anaplasmosis, the administration of the *A. centrale* live-blood vaccine is recommended during periods when calves are not exposed to infected ticks (Potgieter & Stoltsz, 2004). This will ensure that an unstable disease situation (i.e. natural or artificial manipulation of tick populations, and/or any other factor) will generally not result in disease outbreaks.

2.6 *Anaplasma* major surface proteins and their role in host-vector-pathogen interactions

Major surface proteins play a crucial role in the interaction of *A. marginale* with host cells and thus their ability to cause infection. Six major surface proteins (Msps) of *A. marginale* are recognized to date namely, Msp1a (60-105 kDa), Msp1b (100 kDa), Msp2 (36 kDa), Msp3 (86 kDa), Msp4 (31 kDa), and Msp5 (19 kDa). *Anaplasma marginale* Msps have been identified on erythrocyte-derived cells and information about the gene sequences, recombinant protein, monospecific and monoclonal antibodies, isolate variability, and potential value in diagnostic assays and vaccines is available (Meeus & Barbet, 2001; Kocan et al., 2001; Kocan et al., 2003; de la Fuente et al., 2007). The major surface proteins play a major role in interaction of *A. marginale* with host cells; Msp1a, Msp4 and Msp5 are encoded by single copy genes, while Msp1b, Msp2 and Msp3 are encoded by multigene families (Palmer et al., 1994; Brayton et al.,

2005). Previous studies have focused mainly on Msp1, as it is primarily involved in adhesion to bovine erythrocytes and tick cells, and it has been widely used as a stable genetic marker for identification of *A. marginale* geographical isolates (Kocan et al., 2001; de la Fuente et al., 2007; Rodriguez et al., 2009). Major surface protein 1 is a heterodimer composed of two structurally unrelated polypeptides, Msp1a which is encoded by a single gene *msp1α* (Lew et al., 2002) and Msp1b which is encoded by at least two genes, *msp1β1* and *msp1β2* (Bowie et al. 2002; MacMillan et al., 2006). Msp1a is variable in molecular weight among geographic isolates because of the different numbers of tandem 28 or 29 amino acid repeats located in the amino-terminal portion of the protein (de la Fuente et al., 2001; de la Fuente et al., 2003). Major surface protein 1b is polymorphic between geographic isolates of *A. marginale* and there are only small variations in the protein sequences of Msp1b1 and Msp1b2 during the life cycle of the rickettsia in cattle and ticks, (Palmer et al., 1994; Bowie et al., 2002).

Anaplasma centrale was thought not to have a homolog of *msp1α*, however, complete genome sequencing of the Israel vaccine strain revealed that there is a gene that resides in a syntenic position to *A. marginale msp1α* (Herndon et al., 2010). This gene is *msp1aS* encoding Msp1aS. This gene has structural similarities to *msp1α*, including repeats near the amino terminus and two sets of transmembrane domains near the carboxy-terminus. The repeats in *A. centrale* strain Israel Msp1aS are longer (47 amino acids in length) than the *A. marginale* Msp1a repeats (Khumalo et al., 2016). It is not known whether Msp1aS is involved in the interaction of *A. centrale* with host cells.

Major surface protein 2 is the most studied Msp because it is a significant contributor towards antigenic variation and immunologic evasion by *A. marginale* (French et al., 1998; French et al., 1999; Palmer et al., 2000; Brayton et al., 2001; Brayton et al., 2002). Variants of Msp2 are involved in the cyclic rickettsemia during *A. marginale* persistent infection (French et al., 1998; French et al., 1999; Palmer et al., 2000). Analysis of variants in sequential rickettsemic cycles revealed that Msp2 sequence heterogeneity increases over time during persistent infection (French et al., 1999; Rurangirwa et al., 1999). Ticks that feed on cattle with persistent infections ingest a heterogeneous population of variants that differ over time and within different cattle in a herd. However, the heterogeneity of the variants is lost as *A. marginale* passes intrastadially within the tick (Rurangirwa et al., 2000). The Msp2 multigene family includes five or more variable genes widely dispersed throughout the genome. One operon with four open reading frames (ORFs) containing the *msp2* gene at the 3' terminus has been identified (Barbet et al.

2000). Five to seven pseudogenes for *msp2* have been identified within the genome and these pseudogenes recombine into the operon expression site to generate new hypervariable sequences (Brayton et al. 2001; Brayton et al., 2002; Brayton et al., 2005). Antigenic variants arise from a mechanism in which a single population of *A. marginale* expresses multiple forms of *msp2* pseudogenes, each encoding conserved amino- and carboxyl termini, but a central hypervariable region (HVR) of about 100 amino acids (Brayton et al., 2001). There are at least four different variants of Msp2 in each rickettsemic cycle of persistent infection; these variants differ from one another by a combination of substitutions, deletions, and insertions. The HVR contains exposed surface epitopes that induce antibody after the rickettsemia cycle causing a delay in immunity to the new variant (Palmer et al., 1994; French et al., 1999; Rurangirwa et al., 2000). The immunodominant protein Msp3 has shown potential as a diagnostic test antigen; when used experimentally in immunoblot assays, it revealed good sensitivity and specificity in detection of *A. marginale* infections in carrier cattle (McGuire et al., 1991; Alleman & Barbet, 1996). Major surface protein 4 is highly conserved, and it is said to be a useful tool in phylogenetic analyses of *A. marginale* strains (de la Fuente et al., 2004; de la Fuente et al., 2005; de la Fuente et al., 2007; Mtshali et al., 2007). Major surface protein 5 is also a highly conserved surface protein that has been proven effective as a diagnostic antigen and is used in a commercially available competitive enzyme-linked immunosorbent assay (cELISA) (Torioni et al., 1998).

Phylogenetic analyses of *A. marginale* strains using Msps has been reviewed (de la Fuente et al., 2004; de la Fuente et al., 2005; de la Fuente et al., 2007). These analyses suggest that Msps are not good markers for biogeographical studies on a global scale. However, they may be useful for strain comparison in given areas and could provide information about the evolution of host–pathogen and vector–pathogen relationships. A study by de la Fuente et al. (2007) characterized 131 strains of *A. marginale* from North and South America, Europe, Asia, Africa and Australia; 79 Msp1a repeat sequences in these strains were described. Thereafter, new *A. marginale* strains were characterized in Mexico (Alamzán et al., 2008). These results corroborated the genetic heterogeneity of *A. marginale* strains from widely separated endemic regions worldwide. Multiple introductions of different strains into geographic regions may have contributed to the genetic diversity observed within *A. marginale*.

Detection and phylogenetic charecterisation of *Anaplasma* species using 16SrRNA and *groEL* gene

The use of 16SrRNA gene sequences for phylogenetic and taxonomy purposes, dates back to the 1970's in the bacterial-research community (Woese et al., 1975; Woese, 1987). The usefulness of this gene is due to the fact that 16SrRNA is present in most bacteria, and the slow rate of change over time enables studying evolution. However, though this gene is useful for bacterial classification, it is hard to conclusively use it to classify some bacteria to the species level because of poor discriminatory power (Dumler et al., 2001; Mignard & Flandrois, 2006; Bosshard et al., 2006; Janda & Abott, 2007). When used for classification it is usually used along with additional genes, such as *msp4, groEL* to support the findings (Dumler et al., 2001; Lew et al., 2003; Yang et al., 2017).

GroEL is a bacterial antigen related to the chaperonin/heat shock protein 60 (HSP60) family of heat shock proteins (Dasch et al., 1990). It has been shown to be useful for characterization and phylogenetic analysis of *Anaplasma* species (Dumler et al., 2001, Lew et al., 2003, Yang et al., 2016). Recently Sisson and coworkers (2017) used *groEL* gene sequences to show that *A. centrale* from South African buffalo clades separately from other isolates.

There have been many characterization and phylogenetic studies of *A. marginale* based on *msp1α* and *msp4* gene sequences (de la Fuente et al., 2004, de la Fuente et al., 2005; Mtshali et al., 2007; Cabezas-Cruz et al., 2013; Mutshembele et al., 2015). These studies have enabled researchers to determine the occurrence, genetic diversity and phylogenetic relationships of *A. marginale* strains around the world. However there are few *A. centrale* characterization and phylogenetic studies, due to a scarcity of *A. centrale* gene sequence data. There is thus a need to characterize *A. centrale* strains in order to determine their genetic diversity and phylogenetic relationships with other *Anaplasma* strains.

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CHAPTER 3

3. Characterization of *Anaplasma marginale* subspecies *centrale* strains using Msp1aS genotyping reveals a wildlife reservoir

3.1 Abstract

Bovine anaplasmosis caused by the intraerythrocytic rickettsial pathogen *Anaplasma marginale* is endemic in South Africa. *Anaplasma marginale* subspecies *centrale* also infects cattle, however, it causes a milder form of anaplasmosis and is used as a live vaccine against *A. marginale.* There has been less interest in the epidemiology of *A. marginale* subsp. *centrale,* and, as a result, there are few reports detecting natural infections of this organism. When detected in cattle, it is often assumed that it is due to vaccination, and in most cases it is reported as co-infection with *A. marginale* without characterization of the strain. In this study a total of 380 blood samples from wild ruminant species and cattle collected from Biobanks, National Parks, and other regions of South Africa were used in duplex real-time PCR assays to simultaneously detect *A. marginale* and *A. marginale* subsp. *centrale.* PCR results indicated high occurrence of *A. marginale* subsp. *centrale* infections ranging from 25-100% in National Parks. Samples positive for *A. marginale* subsp. *centrale* were further characterized using the *msp1aS* gene, a homolog of *msp1α* of *A. marginale* which contains repeats at the 5' end that are useful for genotyping strains. A total of 47 Msp1aS repeats were identified which corresponded to 32 *A. marginale* subsp. *centrale* genotypes detected in cattle, buffalo and wildebeest. RepeatAnalyzer was used to examine strain diversity. Our results demonstrate a diversity of *A. marginale* subsp. *centrale* strains from cattle and wildlife hosts from South Africa and indicate the utility of *msp1aS* as a genotypic marker for *A. marginale* subsp. *centrale* strain diversity.

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3.2 Introduction

Bovine anaplasmosis (gallsickness) is a tick-borne disease caused by the intra-erythrocytic rickettsial pathogen *Anaplasma marginale* (Theiler, 1910). *Anaplasma marginale* is globally prevalent and results in anemia, with mortality rates of up to 30% (Losos, 1986). *Anaplasma marginale* subspecies *centrale*, is a less virulent subspecies detected by Sir Arnold Theiler, who recognized its potential as a vaccine against anaplasmosis; 100 years later this live vaccine is still in use in South Africa, Israel, South America and Australia (Theiler, 1911; Aubry & Geale, 2011). The strain that is used as a vaccine originated from Theiler's original isolation and was exported at various times to other countries where it has been propagated in the laboratory; the strain known as the "Israel strain" or the "vaccine strain" was sent to Israel in the 1950s, and was used to generate the complete genome sequence for *A. marginale* subsp. *centrale* in 2010 (Herndon et al., 2010). *Anaplasma marginale* subsp. *centrale* does not provide complete protection against *A. marginale* infection, but does protect against severe anaplasmosis (Kuttler, 1984; Anziani et al., 1987).

Anaplasma marginale infects a wide range of ruminants including buffalo (*Bubalus bubalis* and *Syncerus caffer*), wildebeest (*Connochaetes gnou* and *Connochaetes taurinus*), American bison (*Bison bison*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus hemionus*), black-tailed deer (*Odocoileus hemionus columbianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*) (Neitz, 1935; Potgieter, 1979; Smith et al., 1982; Potgieter & Stoltsz, 2004). Cattle are naturally susceptible to *A. marginale* (Aubry & Geale, 2011). There has not been much interest in the epidemiology of *A. marginale* subsp. *centrale*, with few reports detecting natural infections of this organism; most often, when detected in cattle it is assumed that it is due to vaccination and is reported as co-infection with *A. marginale* without characterization of the strain. Georges et al. (2001) reported *A. marginale* subsp. *centrale* single infections detected by the reverse line blot (RLB) hybridization assay in Italy without characterizing the strain. More recently, the first known case of bovine anaplasmosis caused by *A. marginale* subsp. *centrale* in Europe was reported (Carelli et al., 2008). While this study described genetic heterogeneity of *A. marginale* subsp. *centrale* strains from different geographic areas in Italy, it is not clear how these are related to the vaccine strain.

For *A. marginale*, the Msp1a protein/gene (*msp1α*) has been used as a genotypic marker to differentiate strains (Allred et al., 1990). Msp1a is encoded by the single copy gene, *msp1α*,

and differs among strains due to variable sequence and numbers of a 28 or 29 amino acid (84/87-bp) sequence repeat located near the amino-terminus of the protein (Allred et al., 1990). A number of studies have examined Msp1a repeats in the USA, South America, Australia, the Philippines, Europe, Israel, China and Mexico resulting in identification of over 200 repeats (Allred et al., 1990; Bowie et al., 2002; de la Fuente et al., 2007). In South Africa, two studies have been conducted to genetically characterize strains using *msp1α* (Mutshembele et al., 2014; Mtshali et al., 2007), revealing that the repeat structure is common between South African, American and European strains of *A. marginale*; in fact, some of the repeat sequences that were detected were identical to ones that were detected in the USA. Not surprisingly, there were also new repeat sequences detected that are, thus far, unique to South Africa.

A. marginale subsp. *centrale* was thought not to have a homolog of *msp1α*, however, complete genome sequencing of the Israel vaccine strain revealed that there is a gene that resides in a syntenic position to *A. marginale msp1α* (Herndon et al., 2010). This gene was named *msp1aS* (S for syntenic; a gene flanked by the same set of genes in two genomes), and has 31-36% amino acid sequence identity depending on the *A. marginale* strain compared. Importantly, there are structural similarities, including repeats near the amino terminus and two sets of transmembrane domains near the carboxy-terminus that indicate that these proteins are likely homologs (Fig. 3.1). The repeats in *A. marginale* subsp. *centrale* strain Israel Msp1aS are longer (47 amino acids in length) than the *A. marginale* Msp1a repeats and there is no sequence identity between the repeats in the two organisms. The vaccine strain (abbreviated as "Ac") has four repeats with an *msp1aS* genotype of Ac1 Ac1 Ac1 Ac2 (with the number indicating the tandem repeat type).

In the present study, we have used a duplex qPCR assay to screen for the presence of *A. marginale* subsp. *centrale* and *A. marginale* in vaccinated and unvaccinated cattle and wildlife indicating that these infections are common and often occur as mixed infections. Samples that tested positive using this screen were then further analyzed for *msp1aS* genotype, demonstrating that the vaccine strain genotype is prevalent in cattle herds that practice vaccination while other more divergent genotypes are present in wildlife species.

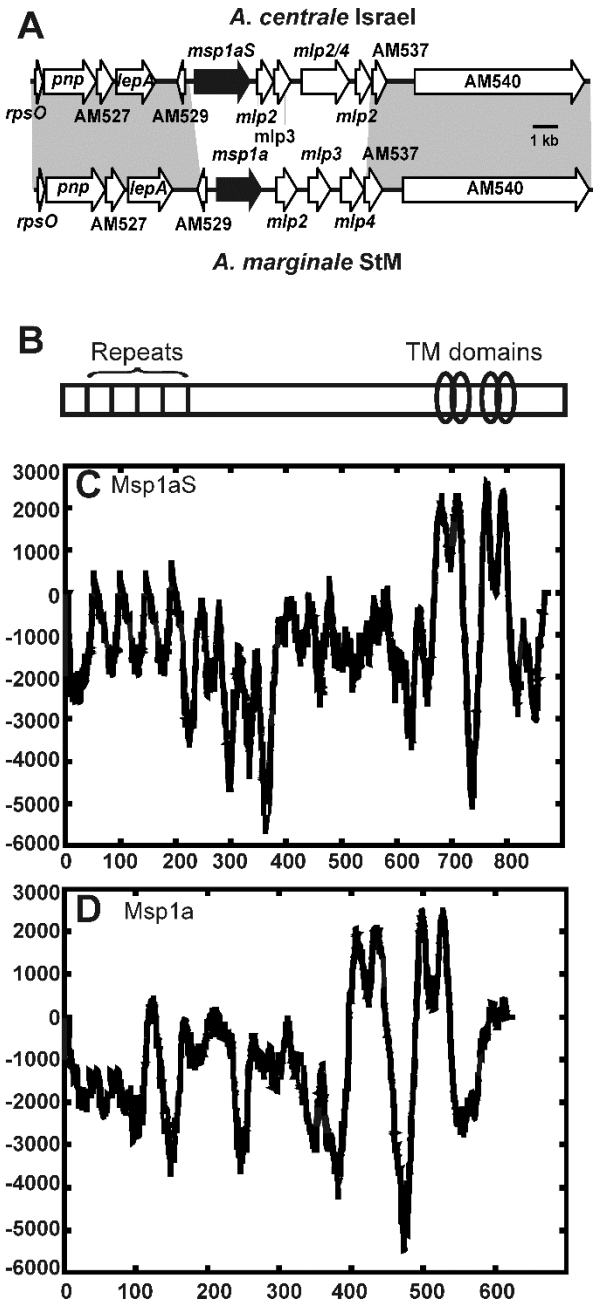

Figure 3.1 Schematic representation (Panels A and B) and TMpred plots of Msp1aS (Panel C). Msp1aS of *A. marginale* subsp. *centrale* sits in a syntenic position to Msp1a of *A. marginale* suggesting that these proteins are homologs (Panel A). While there is little sequence conservation, these proteins have similar structures (Panels B and C). Panel B shows how both proteins have a set of repeats near the amino terminus and two sets of transmembrane domains towards the carboxy-terminus. The TMpred plots (Panels C and D) show the transmembrane prediction profile for both molecules (Msp1aS from the fully sequenced Israel strain of *A. marginale subsp. centale* and Msp1a from the fully sequenced St. Maries strain of *A. marginale*). Values greater than 500 (Y axis) indicate transmembrane domains. The repeats of Msp1aS are almost twice as long as those of Msp1a.

3.3 Materials and Methods

3.3.1 Blood collection and DNA extraction

A total of 380 blood samples from wild ruminant species including African buffalo (n=97) (*Syncerus caffer*), waterbuck (n=14) (*Kobus ellipsipyrymnus*), eland (n=23) (*Taurotragus oryx*), black wildebeest (n=54) (*Connochaetes gnou*) and blue wildebeest (n=23) (*Connochaetes taurinus*) together with 86 cattle samples were obtained from the Wildlife Biological Resource Center (WBRC) and Biobank South Africa under the auspices of the National Zoological Gardens of South Africa (NZG) as well as from the South African National Parks (SANParks) Biobank. The remaining buffalo blood samples (n=41), were made available to us by Dr. Dave Cooper from Hluhluwe-iMfolozi Park. Additionally, 42 blood samples from vaccinated cattle were obtained from two commercial farms in Bergville, KwaZulu-Natal, South Africa (Table 3.1). Standard techniques were followed in collecting blood samples for laboratory examination. Genomic DNA was extracted using the QIAmp DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 100 µl elution buffer and stored at -20°C.

The study was approved by the Animal Ethics Committee of the University of Pretoria, South Africa (V085-14) and permission to use wildlife samples was given by SANParks Biobank under reference number "LARBJ1118 Conservation Genetics", the WBRC, and Biobank SA under the auspices of the NZG of South Africa and the Johannesburg Zoo with project number NZG/P13/05. Collection of cattle samples was approved by the Department of Agriculture Forestry and Fisheries under section 20 of the Animal Diseases Act of 1984 with reference 12/11/1/1.

Table 3.1 Host samples used in this study.

Sample number	Species	Number of samples	Sample type	Collection	Origin[1]	Province
565-614	Buffalo	50	EDTA-Blood	SANParks[2]	KNP	Mpumalanga
974-987	Buffalo	14	EDTA-Blood	SANParks	CNP	Eastern Cape
1002-1016	Buffalo	15	EDTA-Blood	SANParks	AEP	Eastern Cape
988-995 & 66/13	Buffalo	9	EDTA-Blood	SANParks	GNP	Northern Cape
998-1001 & 1017-1021	Buffalo	9	EDTA-Blood	SANParks	MNP	Northern Cape
1-41	Buffalo	41	EDTA-Blood	HiP	HiP	KwaZulu-Natal
924-937 &947-955	Black wildebeest	23	EDTA-Blood	SANParks	MTNZNP	Eastern Cape
938-939	Black wildebeest	2	EDTA-Blood	SANParks	TMNP	Western Cape
942, 944-953 & 955-972	Black wildebeest	29	EDTA-Blood	SANParks	MNP	Northern Cape
1036-1056	Blue Wildebeest	21	EDTA-Blood	SANParks	MNP	Northern Cape
1057-1058	Blue Wildebeest	2	EDTA-Blood	SANParks	WCNP	Western Cape
1022-1031	Eland	10	EDTA-Blood	SANParks	MNP	Northern Cape
1032-1035	Eland	4	EDTA-Blood	SANParks	AEP	Eastern Cape
459-467	Eland	9	FTA filter paper	WBRC, SA, NZG[3]	NZG	Gauteng
1059-1062	Waterbuck	4	EDTA-Blood	SANParks	MNP	Northern Cape
468-470	Waterbuck	3	FTA filter paper	WBRC, SA, NZG	Rietvlei NR, JHB Zoological Gardens, Mohale Gate (Gauteng area)	Gauteng
543, 549	Waterbuck	2	EDTA-Blood	WBRC, SA, NZG	KNP	Mpumalanga
544-548	Waterbuck	5	EDTA-Blood	WBRC, SA, NZG	MaNP	Limpopo
WC103-WC128	Cattle	26	EDTA-Blood	NZG collection	WC [4]F3	Western Cape
KZN129-KZN158	Cattle	30	EDTA-Blood	NZG collection	KZN [4]F4	KwaZulu-Natal
FS1-FS30	Cattle	30	EDTA-Blood	NZG collection	FS [4]F5	Free State
Berg 1-Berg 21	Cattle	21	EDTA-Blood	Bergville farm	Bergville [4]F1	KwaZulu-Natal
Berg 22-Berg 42	Cattle	21	EDTA-Blood	Bergville farm	Bergville [4]F2	KwaZulu-Natal

[1]Origin = the park/farm from where the sample originates; Kruger National Park (KNP), Camdeboo National Park (CNP), Graspan National Park (GNP), Mokala National Park (MNP) Addo Elephant National Park (AEP), Hluhluwe-iMfolozi Park (HiP), Mountain Zebra National Park (MTNZNP), Table Mountain National Park (TMNP), West Coast National Park (WCNP), Marakele National Park (MaNP), [2]SANParks = South African National Parks, [3]WBRC, NZG = Wildlife Biological Research Center, National Zoological Gardens, South Africa, [4] F = farm.

3.3.2 Duplex real-time PCR assay

Quantitative real-time PCR (qPCR) for simultaneous detection and quantification of *A. marginale* and *A. marginale* subsp. *centrale* DNA was performed as described previously (Decaro et al., 2008) with some modifications for use on a Light Cycler real-time machine (Chaisi et al., 2017) (Roche Diagnostics, Mannheim, Germany). The qPCR was performed in a final reaction volume of 20 µl, containing 2 µl of DNA template (100-200 ng of DNA), 12.5 µl of FastStart DNA Master Hybridization kit (Roche Diagnostics, Mannheim, Germany), 600 nM of *A. marginale*-specific primers AM-For (5' TTG GCA AGG CAG CAG CTT 3') and AM-Rev (5' TTC CGC GAG CAT GTG CAT 3'), 900 nM of *A. marginale* subsp. *centrale*-specific primers AC-For (5' CTA TAC ACG CTT GCA TCT C 3') and AC-Rev (5' CGC TTT ATG ATG TTG ATG C 3') and 200 nM of probes AM-Pb (5' 6FAM-TCG GTC TTA ACA

TCT CCA GGC TTT CAT-BHQ1 3') and AC-Pb (5' LC610-ATC ATC ATT CTT CCC CTT TAC CTC GT-BHQ2 3'). Thermal cycling conditions were: UDG activation at 40°C for 10 min, pre-incubation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 1 min and annealing-extension at 60°C for 1 min, and a final cooling step at 40°C for 30 sec. The results were analyzed using the Lightcycler Software version 4.0 (Roche Diagnostics, Mannheim, Germany). The software indicates a positive result by a Cq value (quantification cycle, synonymous with the Cp, crossing point, value given by the Lightcycler instrument), at which fluorescence from amplification exceeds the background fluorescence, and a score of 1 to 5. Negative samples have a score of -1 to -5 and no Cp values. A lower Cq correlates with a higher starting concentration of target DNA in a sample, which then indicates a positive infection. FAM fluorescence (530 nm) was generated in *A. marginale* positive samples and LC-610 (610 nm) signals were generated in *A. marginale* subsp. *centrale* positive samples. Deoxyribonucleic acid extracted from the *A. marginale* subsp. *centrale* vaccine strain (Onderstepoort Biological Products, Pretoria) was used as a positive control, and samples C14, C57 or F48 (originating from cattle in the Mnisi Community area, Mpumalanga, South Africa) were used as positive controls for *A. marginale.* The presence of *A. marginale* in these samples was confirmed by sequencing of the *msp1β* genes. A negative and positive control was included in each set of PCR reactions that was performed. The analytical specificity of the assay was determined by analyzing DNA from closely related species such as *Anaplasma* sp. Omatjenne and *A. phagocytophilum* (Carelli et al., 2007). The efficiency of the assay was determined from 10-fold serial dilutions of plasmid DNA from clones 9410c (*A. marginale* subsp. *centrale*) and F48a (*A. marginale*).

3.3.3 Analysis of the msp1aS gene

Anaplasma marginale subsp. *centrale*-positive samples which had low Cq values as detected by qPCR were selected for analysis of the *msp1aS* gene. Primers MSP1asFZ (5' CAA GGT CAA GAG TCA GCA TCA TCA GAT G 3') and MSP1asRZ (5' CTC CGC GCA CAA TAC TTT CAA CCT CC 3') were designed based on the *A. marginale* subsp. *centrale* genome sequence (Genbank accession # CP001759) to target tandem repeats within the *msp1aS* gene. PCR was performed in a final reaction volume of 25 µl containing Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 10 pM of each primer and genomic DNA. Thermal cycling was carried out in a Veriti thermal cycler (Thermo Fisher Scientific), and consisted of an initial denaturation at 98°C for 10 sec, followed by 30 cycles of denaturation at

98°C for 1 sec, annealing at 67°C for 30 sec and extension at 72°C for 15 sec, and a final extension at 72°C for 1 min. DNA extracted from the *A. marginale* subsp. *centrale* vaccine obtained from Onderstepoort Biological Products (OBP, Pretoria, South Africa) was used as a positive control.

Purified PCR amplicons were cloned into the pJET vector (Thermo Fisher Scientific). Recombinant plasmids were isolated using a High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany) and sequenced using 1 µl of 2 µM M13 primers with ABI Big Dye V3.1 Kit on an ABI 3500XL genetic analyzer at Inqaba Biotec (Pretoria, South Africa).

Sequences were assembled, edited, and translated to amino acids using CLC Main Workbench 7.0.3 (Qiagen, Denmark). Tandem repeats were identified using Tandem Repeats Finder (https://tandem.bu.edu/trf/trf.html) (Benson, 1999). The repeats were named Ac#, to distinguish them from *A. marginale* Msp1a repeats. Truncated repeats were designated with a T at the end of the name. Repeats were curated and analyzed using RepeatAnalyzer (Catanese et al., 2016). Repeat sequences were aligned using the AlignX module of Vector NTI (Invitrogen).

3.3.4 Diversity Measures

RepeatAnalyzer calculates four genetic diversity metrics, each of which captures the diversity of repeats in a geographic region in a different way. Broadly, they fall into two groups, those that measure the amount of different repeats and those that measure the distribution of those repeats. Within each of these categories, there is a global and a local formulation. The local version of a metric calculates the score independently on each genotype and averages these together to get the final score, while the global version looks at all genotypes together. Specifically, the GDM1L score can be interpreted as the percent of unique repeats in each genotype in the region, while the GDM1G score is the percent of unique repeats across all genotypes in the region. The GDM2L score can be interpreted as the amount of variation (measured as standard deviation) in the number of occurrences of the repeats in a genotype, while the GDM2G score is the amount of variation in the number of occurrences of all the repeats in all genotypes in the region. A high GDM1 score means that there are more unique repeats, with 0 as the minimum (when all repeats are the same) and 1 being the maximum

(when each repeat is unique). A high GDM2 score means that the repeats are distributed more unevenly, with a minimum of 0 (when all repeats occur the same number of times) and values ranging up to but not including 0.5 as the unevenness of repeat distribution increases.

3.4 Results

3.4.1 Occurrence of Anaplasma species in wild ruminants and cattle in South Africa

Duplex qPCR results indicated that *A. marginale* subsp. *centrale* single infections are common among black wildebeest (MNP: 79.3%), blue wildebeest (WCNP: 50%), waterbuck (MNP: 25%) and eland (MNP: 100%). Wildebeest did not harbor any *A. marginale* infections. Mixed infections were frequently found in both buffalo and cattle, ranging from 28% to 100% of animals from a given area being positive for both *A. marginale* and *A. marginale* subsp. *centrale* infections. Buffalo samples had high rates of mixed infections, and also had lower rates of single infections with *A. marginale* subsp. *centrale* than *A. marginale*. Interestingly, single infections of both species predominated in sets of animals from specific parks (see eland and waterbuck in Fig 3.2), indicating that environment plays a role in exposure to the two pathogens.

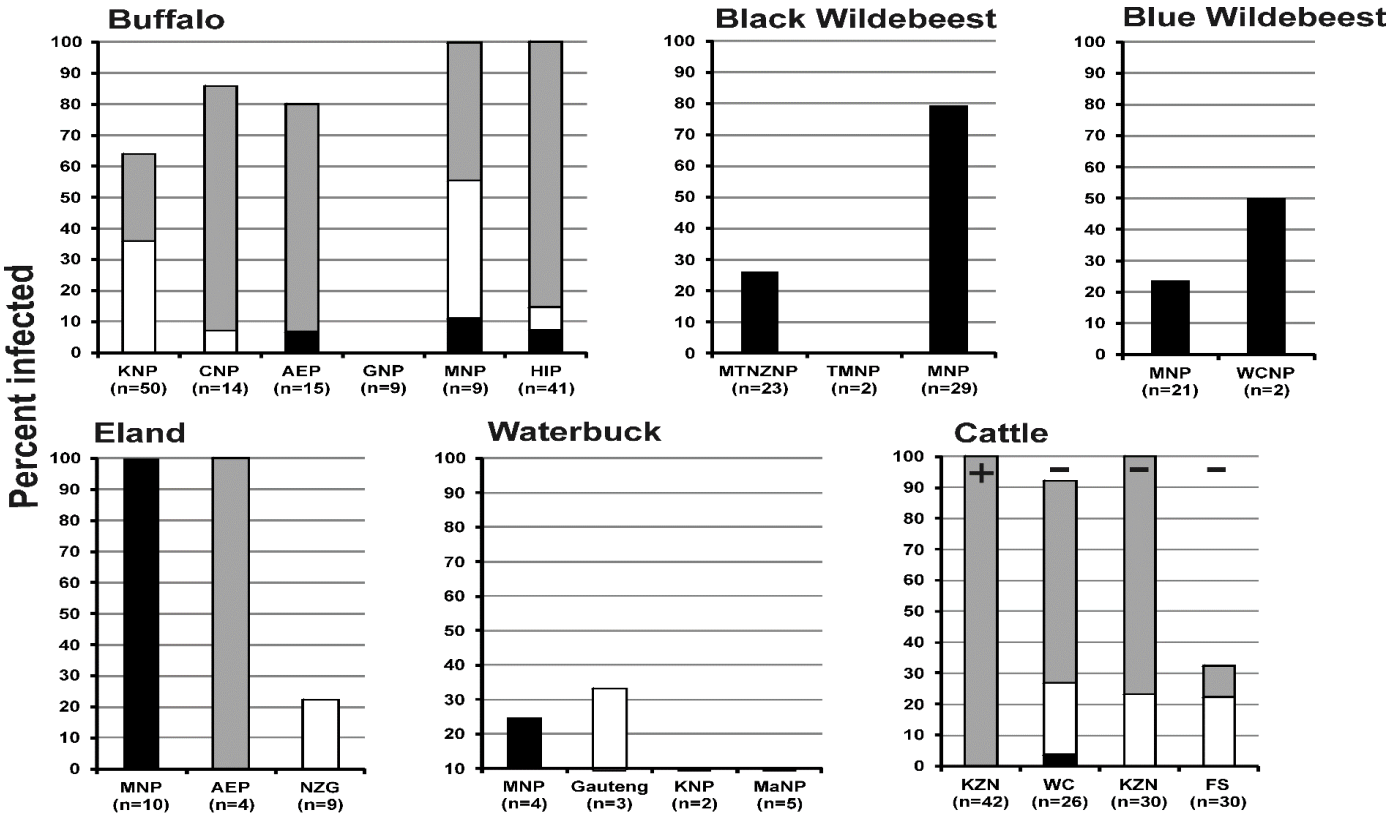

Figure 3.2 Stacked bar graphs showing occurrence of *Anaplasma* species in wild ruminants and cattle. Buffalo, black and blue wildebeest, eland, waterbuck, and cattle were analyzed by duplex real-time PCR. Animals were sampled from the following National Parks: Kruger National Park (KNP), Camdeboo National Park (CNP), Addo Elephant National Park (AEP), Graspan National Park (GNP), Mokala National Park (MNP), Hluhluwe-iMfolozi Park (HiP), Mountain Zebra National Park (MTNZNP), Table Mountain National Park (TMNP), West Coast National Park (WCNP), National Zoological Gardens of South Africa (NZG), Marakele National Park (MaNP), KwaZulu-Natal (KZN), Western Cape (WC) and the Free State (FS) provinces. Numbers in parentheses indicate the total number of animals sampled from. Samples were collected from vaccinated (+) and unvaccinated (-) cattle. Black indicates animals positive for *A. marginale* subsp. *centrale*, gray indicates animals with mixed infections, and white indicates animals positive for *A. marginale*.

3.4.2 Characterization of Msp1aS

Because the sequenced Israel vaccine strain was removed from South Africa more than 60 years ago, we obtained a batch of the vaccine currently produced at OBP in Pretoria, South Africa and sequenced the *msp1aS* gene. The sequence of the OBP vaccine strain Msp1aS tandem repeat from 2014 was identical to that of the Israel strain (Herndon et al., 2010) with four tandem repeats: Ac1 Ac1 Ac1 Ac2.

Based on the duplex qPCR results, *A. marginale* subsp. *centrale*-positive samples (n=25) were selected for further analysis. *Msp1aS* primers amplified at least one single strong product from all samples tested. Some samples exhibited multiple bands which demonstrated mixed infection (Fig. 3.3). The *msp1aS* PCR products were cloned and sequenced, and sequence analyses confirmed the presence of tandem repeats similar to the vaccine strain (Table 3.2). The first five columns of Table 2 would combine to provide the full strain and sample designation as suggested by Catanese et al. (2016), i.e. Ac11 Ac8_ZA, EC_2007_CNP_986, however, we have used shorter names for some of the genotypes for ease of discussion. The strains tested in this study yielded one to five repeat units as predicted from the PCR product sizes; however there were strains that did not correspond with their PCR products (data not shown). Altogether, 47 different Msp1aS tandem repeats were identified. The repeats ranged from 45-51 amino acids with seven truncated repeats ranging from 31-33 amino acids (Fig. 3.4). The most common repeat length was 46 amino acids (Fig. 3.5 A). The Ac1 and Ac2 tandem repeats, contained in the Vaccine strain, were detected in cattle, buffalo and wildebeest.

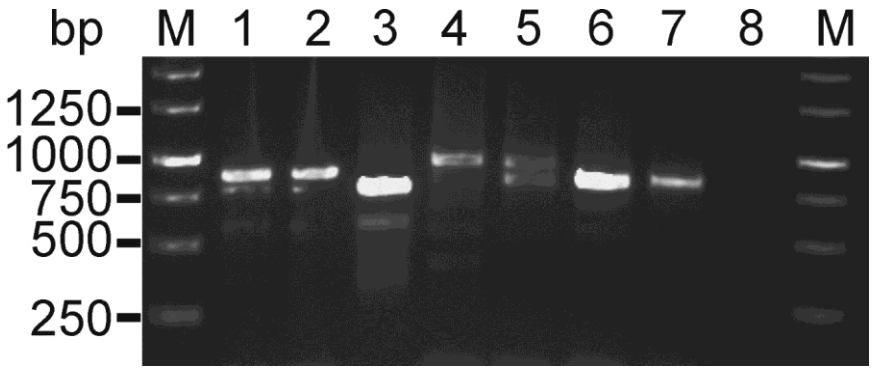

Figure 3.3 Gel image showing amplicons of *msp1aS*. Lanes 1 and 2 = Vaccine strain (814 bp), 3 = animal # FS 383 (790 and 637 bp), 4 = animal # Berg10 (922 and 814 bp), 5 = animal # Berg12 (937 and 814 bp), 6 = animal # Berg20 (814 bp), 7= animal # WC_108 (799 bp), 8 = negative control. Note that for some samples only a subset of the amplicons were successfully sequenced, while for others, clones with different sequences were obtained from what appeared as a single band.

Table 3.2 *Anaplasma marginale* subsp. *centrale* genotypes detected from South African bovine hosts (cattle, buffalo and black wildebeest).

Genotype	Country code[1]	Province code[1]	Year	Animal I.D	clone number	Host species	Origin	Vaccine status	Size (bp)	Number of repeats	Short Name
Ac1 Ac1 Ac1 Ac2	IL	M	2010	Genome	CP001759	Cattle	Israel 2010	+	814	4	Vaccine
Ac1 Ac1 Ac1 Ac2	ZA	GP	2014	-	OBP	Cattle	OBP 2014[2]	+	814	4	Vaccine
SANParks Biobanked samples[3]											
Ac11 Ac8	ZA	EC	2007	CNP_986	G	Buffalo	Camdeboo	-	525	2	
Ac9 Ac8	ZA	EC	2007	CNP_986	C	Buffalo	Camdeboo	-	526	2	
Ac11 Ac11 Ac11 Ac11 Ac8	ZA	EC	2007	CNP_986	C2	Buffalo	Camdeboo	-	940	5	
Ac3 Ac4 Ac5 Ac6	ZA	EC	2007	CNP_987	J2	Buffalo	Camdeboo	-	823	3	
Ac7 Ac8	ZA	EC	2007	CNP_979	D	Buffalo	Camdeboo	-	526	2	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2008	HiP_6	1	Buffalo	Hluhluwe	-	815	4	Vaccine
Ac30 Ac24 Ac25	ZA	NL	2008	HiP_6	A	Buffalo	Hluhluwe	-	940	3	
Ac29 Ac29 Ac29	ZA	NL	2008	HiP_6	B	Buffalo	Hluhluwe	-	703	3	
Ac33 Ac3 Ac6	ZA	NL	2008	HiP_6	L	Buffalo	Hluhluwe	-	691	3	
Ac6 Ac35 Ac36T Ac37T	ZA	NC	2013	MNP_999	L	Buffalo	Mokala	-	889	5	
Ac38Ac39T Ac34 Ac40T	ZA	NC	2013	MNP_999	N	Buffalo	Mokala	-	759	4	
Ac38Ac41T Ac42 Ac40T	ZA	NC	2013	MNP_1000	A	Buffalo	Mokala	-	733	4	
Ac6Ac6	ZA	NC	2013	MNP_1000	G	Buffalo	Mokala	-	790	2	
Ac1 Ac1 Ac1 Ac2	ZA	EC	2013	AEP_1003	D	Buffalo	Addo	-	814	4	Vaccine
Ac7 Ac8	ZA	EC	2013	AEP_1006	D	Buffalo	Addo	-	525	2	
Ac38 Ac44T Ac43	ZA	EC	2013	AEP_1006	N	Buffalo	Addo	-	628	3	
Ac31 Ac8	ZA	EC	2013	AEP_1006	S	Buffalo	Addo	-	526	2	
Ac1 Ac1 Ac1 Ac1	ZA	LP	2008	KNP_586	A	Buffalo	Kruger	-	814	4	VV1
Ac26 Ac26 Ac26 Ac2	ZA	NC	2011	MNP_958	F_w	black	Mokala	-	862	4	
NZG Biobanked samples[3]											
Ac20 Ac32 Ac21 Ac10	ZA	WC	2011	WC _107	E	Cattle	WC	-	700	4	
Ac1 Ac1 Ac1 Ac2	ZA	WC	2011	WC _108	A	Cattle	WC	-	799	4	Vaccine
Ac12 Ac12 Ac13 Ac13	ZA	NL	2011	KZN_138	B	Cattle	NL	-	919	5	
Ac12Ac12Ac13 Ac13Ac14	ZA	NL	2011	KZN_132	A	Cattle	NL	-	941	4	
Ac12 Ac12 Ac13 Ac13	ZA	NL	2011	KZN_130	B	Cattle	NL	-	980	5	
Ac15 Ac16 Ac16 Ac16	ZA	FS	2011	FS_56	B	Cattle	FS	-	821	4	
Ac16 Ac16 Ac16	ZA	FS	2011	FS_383	B	Cattle	FS	-	637	3	
Farm 1											
Ac33 Ac3 Ac6	ZA	NL	2015	Berg 10	A	Cattle	Bergville	+	691	3	
Ac19 Ac19 Ac3 Ac6	ZA	NL	2015	Berg 10	G	Cattle	Bergville	+	814	4	
Ac17 Ac18 Ac45 Ac46T	ZA	NL	2015	Berg 10	J	Cattle	Bergville	+	922	5	
Ac1 Ac1 Ac1 Ac22	ZA	NL	2015	Berg 12	B	Cattle	Bergville	+	811	4	VV3
Ac20 Ac21 Ac21 Ac20	ZA	NL	2015	Berg 12	E	Cattle	Bergville	+	937	5	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 12	N	Cattle	Bergville	+	814	4	Vaccine
Ac23 Ac24 Ac25 Ac34	ZA	NL	2015	Berg 19	A	Cattle	Bergville	+	940	5	
Ac26 Ac12 Ac12 Ac27	ZA	NL	2015	Berg 19	A_2	Cattle	Bergville	+	946	5	
Ac1 Ac1 Ac1 Ac22	ZA	NL	2015	Berg 19	B	Cattle	Bergville	+	811	4	VV3
Ac19 Ac3 Ac6 Ac6	ZA	NL	2015	Berg 19	I	Cattle	Bergville	+	826	4	
Farm 2											
Ac1 Ac1 Ac1 Ac22	ZA	NL	2015	Berg 25	A	Cattle	Bergville	+	814	4	VV3
Ac1	ZA	NL	2015	Berg 25	E	Cattle	Bergville	+	391	1	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 25	B	Cattle	Bergville	+	814	4	Vaccine
Ac1 Ac1 Ac1 Ac1	ZA	NL	2015	Berg 25	E_2	Cattle	Bergville	+	814	4	VV1
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 25	X	Cattle	Bergville	+	914	5	VV2
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 27	D	Cattle	Bergville	+	814	4	Vaccine
Ac1 Ac1 Ac1 Ac1	ZA	NL	2015	Berg 27	E	Cattle	Bergville	+	956	5	VV1
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 27	B	Cattle	Bergville	+	955	5	VV2
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 17	A	Cattle	Bergville	+	943	5	Vaccine
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 24	A	Cattle	Bergville	+	814	5	Vaccine
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 24	C	Cattle	Bergville	+	955	5	VV2
Ac1 Ac28 Ac2 Ac28	ZA	NL	2015	Berg 24	V	Cattle	Bergville	+	814	4	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 30	G	Cattle	Bergville	+	811	4	Vaccine
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 30	I	Cattle	Bergville	+	954	5	VV2
Ac1 Ac1 Ac1 Ac1	ZA	NL	2015	Berg 20	H3	Cattle	Bergville	+	814	4	VV1

[1] Country and Province abbreviations follow ISO 3166-2. [2] OBP = Onderstepoort Biological Products (Pretoria, South Africa) which produces *A. marginale* subsp. *centrale* vaccine for sale. [3] SANParks = South African National Parks, NZG = National Zoological Gardens of South Africa Biobanks.

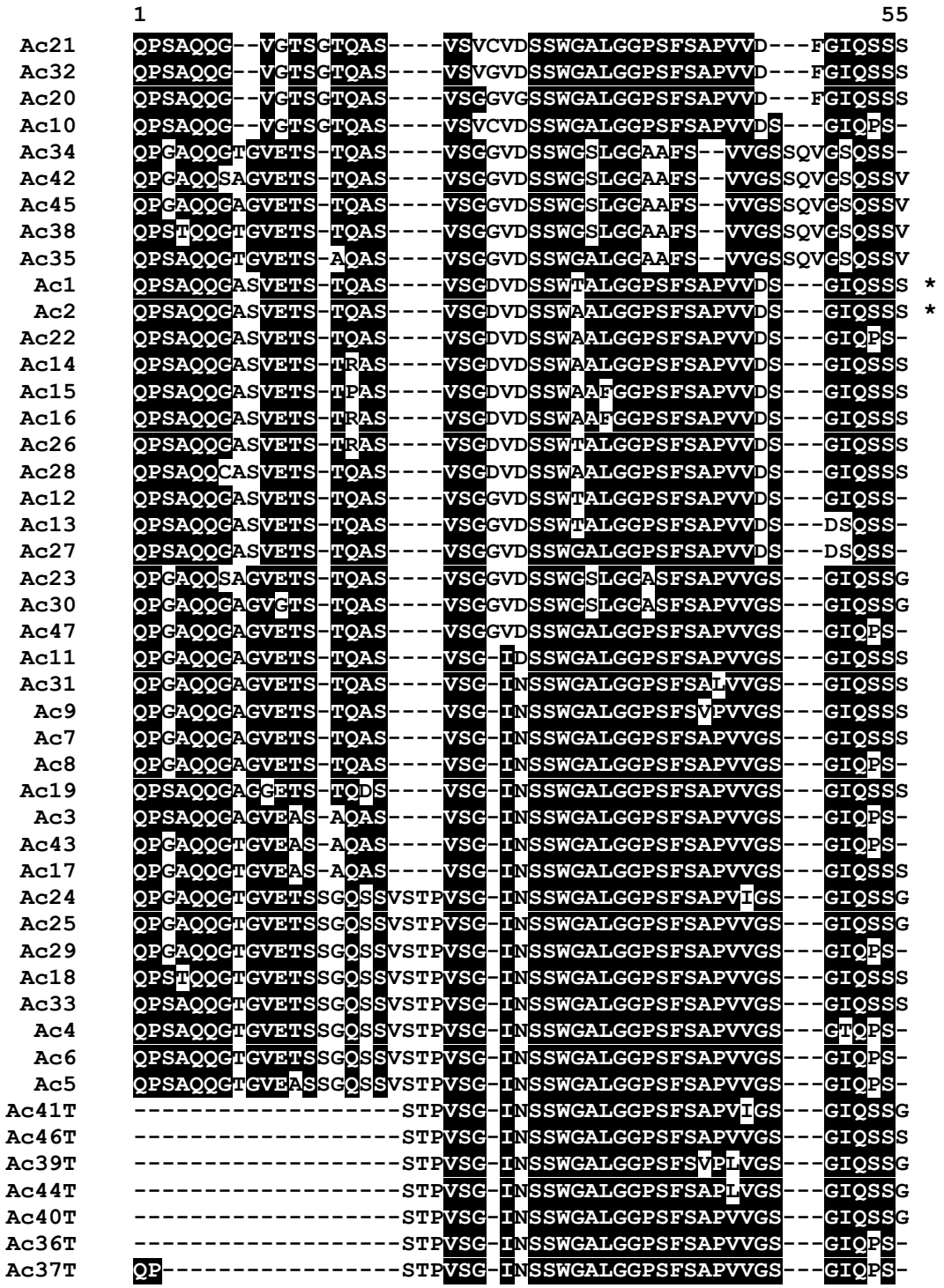

Figure 3.4 Alignment of *A. marginale* subsp. *centrale* Msp1aS tandem repeats detected from South African cattle, buffalo and black wildebeest. The 47 repeat types were aligned using the AlignX module of Vector NTI and groups of identical amino acids are highlighted on a black background. Ac1 and Ac2, the repeats present inthe vaccine strain indicated with an asterisk.

The vaccine strain was detected in cattle from Bergville which were previously vaccinated with *A. marginale* subsp. *centrale* vaccine. We tested six cattle from Bergville farm 2 which yielded 15 *msp1aS* sequences. The vaccine genotype was detected in five of the six cattle (Table 3.2). Interestingly, two "vaccine variants (VV)" genotypes were detected that were closely related to the vaccine strain genotype, and differed by only a single amino acid (VV1 and VV3). Another vaccine variant genotype, VV2 (Ac1 Ac1 Ac1 Ac2 Ac2), was noted that had one additional Ac2 repeat but was otherwise identical to the vaccine strain genotype. Two additional genotypes were detected that were less obviously related to the vaccine strain. Three cattle were tested on Bergville farm 1 resulting in 10 *msp1aS* sequences. Interestingly, the vaccine genotype was only detected in one of these animals despite the fact that these animals were reported as being vaccinated, while two animals contained the related genotype VV3. Seven additional genotypes were detected on farm 1 that were not closely related to the vaccine genotype.

Interestingly, the vaccine genotype as well as one of the vaccine variant genotypes were also detected in unvaccinated animals, including buffalo (HiP_6, AEP_1003 and KNP_586) and cattle (WC_108). Genotype Ac33 Ac3 Ac6 was detected in a buffalo from Hluhluwe National Park as well as in a cow from Bergville farm 1. Several truncated repeats were detected (i.e. Ac36T), and although these predominated in the buffalo samples, a genotype containing a truncated repeat was also detected on Bergville farm 1.

RepeatAnalyzer, is a program we developed recently to house, curate and provide metrics for repeat sequences used to characterize bacteria (Catanese et al., 2016). In the present study, we applied it to the analysis of *msp1aS* repeats. The most common genotype structure we detected contained four repeats, with genotypes having from one to five repeats (Fig. 3.5B). Most repeats occurred only once with two repeats being detected in six different genotypes (Ac1 and Ac6) (Fig. 3.5C). The Ac1 repeat is not only detected in the vaccine strain, but in several "vaccine variant" genotypes that were detected on Bergville farm 2. The Ac6 repeat was prevalent in genotypes detected in wildlife, and interestingly, was also detected in genotypes found on Bergville farm 1. In general, we found that the average number of amino acid changes (edit distance) between any two *A. marginale* subsp. *centrale* repeats was high (13.7) and was normally distributed, with 97.8% of data falling within two standard deviations. There was a mean of 0.9 and 1.4 repeats at an edit distance of 1 and 2, respectively, from any given repeat. Despite the high level of variation between repeats, we found five repeats within an edit

distance of two from Ac1 (Ac2, Ac26, Ac12, Ac20 and Ac48) and seven repeats within two edits of Ac2 (Ac1, Ac14, Ac28, Ac15, Ac16, Ac22 and Ac26).

Table 3.3 Diversity scores for cattle and wildlife hosts by province and host.

Location	GDM1-L	GDM1-G	GDM2-L	GDM2-G
All	0.747	0.420	0.065	0.022
Eastern Cape	0.863	0.583	0.069	0.060
Gauteng	0.500	0.500	0.250	0.250
KwaZulu-Natal	0.696	0.419	0.071	0.044
Mpumalanga	0.250	0.250	0	0
Northern Cape	0.760	0.632	0.067	0.050
Free State	0.417	0.286	0.125	0.357
Western Cape	0.750	0.750	0.125	0.093
Buffalo	0.781	0.500	0.051	0.030
Cattle	0.684	0.418	0.081	0.041

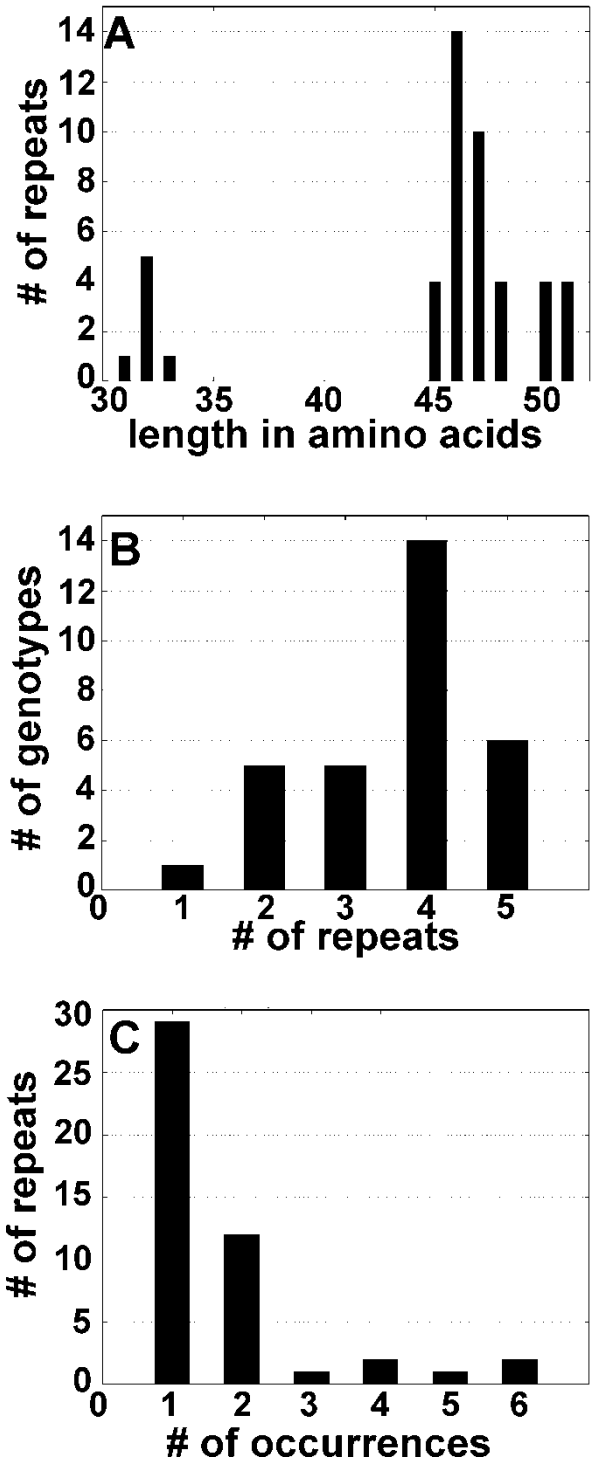

Figure 3.5 Metrics for *A. marginale* subsp. *centrale* Msp1aS repeats. (A) Number of repeats with a given number of amino acids; i.e. there are four repeats with a length of 45 amino acids. (B) Number of genotypes having a given number of repeats; i.e. 14 genotypes contain four repeats. (C) Number of times a given repeat occurs in our genotype dataset; i.e. two repeats occur in six different genotypes.

3.4.3 Diversity analysis and repeat distribution

Using RepeatAnalyzer we see that South Africa has a large number of unique *A. marginale* subsp. *centrale* repeats (GDM1-Local; Table 3.3), while having an intermediate amount of repeat diversity in general (GDM1-Global). There is a higher diversity of repeats among the samples isolated from buffalo hosts than those from cattle hosts, although this would be expected as many of the cattle were vaccinated, and would be expected to exhibit the same repeat structure as the vaccine strain. GDM2 measures how uniformly the repeat occurrences in the strains in a region (local) or the region as a whole (global) are distributed. For both GDM2 metrics, the South African values are low, indicating that the repeats are dispersed; i.e., there is not a preponderance of a single repeat type in individual strains or for the country as a whole. The GDM2 values are higher for cattle than for buffalo derived samples, reflecting more uniformity in the repeats detected in samples from cattle than from buffalo. When examining whether repeats and strains occur in multiple provinces, we have *msp1aS* data from seven of South Africa's nine provinces (Fig. 3.6). The repeats and strains are mapped according to GPS coordinates, so multiple locations within a province can be visualized and distinguished. Several repeats were detected in multiple locations (Fig. 3.6A). Repeats Ac1 and Ac2 were found in Mpumalanga, Gauteng, KwaZulu-Natal, Eastern Cape and Western Cape provinces. The vaccine strain is detected in cattle from KwaZulu-Natal, the Eastern Cape, and the Western Cape (Fig. 3.6B), which is interesting as we tested vaccinated animals only in KwaZulu-Natal. Gauteng also shows positive for the vaccine strain, but this is due to the purchased vaccine itself.

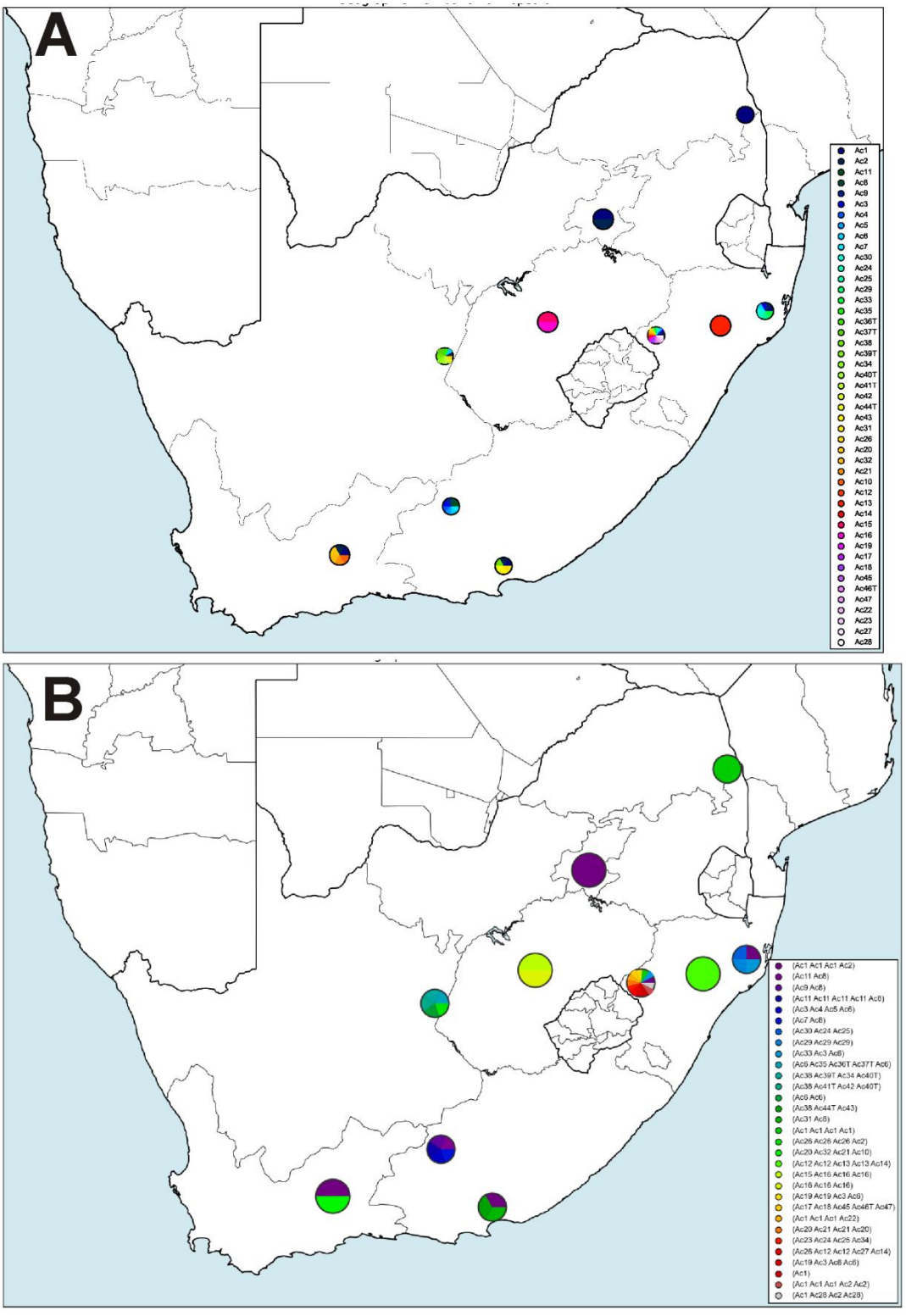

Figure 3.6 Maps of repeat and strain distribution. (A) Repeats mapped to the provinces of South Africa by GPS coordinates. (B) Strain genotypes mapped to the provinces of South Africa by GPS coordinates. The size of the circle indicates the precision of the location report, with three sizes being possible, corresponding to country, province, and precise GPS location. In these maps, there are no reports that are simply to the country level; i.e., allocations are at the provincial level or more specific. Therefore, there are only two sizes of circles shown. The samples collected from the Free State and Western Cape are marked at the provincial level and, thus, have larger markers.

3.5 Discussion

We tested animals from a several different parks and farms and showed that *A. marginale* subsp. *centrale* infection is prevalent in black and blue wildebeest, eland, buffalo, waterbuck and cattle. *Anaplasma marginale* subsp. *centrale* has rarely been examined on its own, as typically researchers/ranchers are interested in *A. marginale* infection, and the cELISA often used for detection does not discriminate between *A. marginale* and *A. marginale* subsp. *centrale* infection. One study using the cELISA showed high seroprevalence of *Anaplasma* spp. in wildlife from Kenya with eland and blue wildebeest testing at 100% and 96%, respectively. Using a reverse line blot assay it was shown that *Anaplasma* spp. are prevalent in buffalo in northern Botswana with *A. marginale* subsp. *centrale* being the most prevalent (Eygelaar et al., 2015). This suggests that wildlife species are reservoirs of *A. marginale* subsp. *centrale*.

We examined positive samples for *msp1aS* genotype, a genotyping scheme that has not previously been employed for *A. marginale* subsp. *centrale*. We identified 47 Msp1aS repeats which corresponded to 32 *A. marginale* subsp. *centrale* genotypes detected in cattle, buffalo and wildebeest. The most common *A. marginale* subsp. *centrale* genotype amongst cattle samples was the vaccine genotype. This is not surprising as both farms that we sampled previously vaccinated with *A. marginale* subsp. *centrale* vaccine purchased from OBP. It is worth noting that cattle from farm 1 graze together with goats, sheep and reedbuck, which might explain the diversity of *A. marginale* subsp. *centrale* strains detected on farm 1. We speculate that there is circulation of *A. marginale* subsp. *centrale* strains among different hosts, which led to the variety of genotypes detected on this farm. Cattle from farm 2 are confined within a grazing area with no interaction with other ruminants. The vaccine genotype was detected in all but one of the animals tested on this farm. In addition to the vaccine genotype, several closely related genotypes were detected, which suggests that the vaccine genotype is changing under selection pressure. This is interesting as we do not see these types of changes in *msp1α* genotype in *A. marginale* infected cattle. All repeats detected on farm 2 had an edit distance of two or less from one of the vaccine strain repeats, indicating that these repeats were closely related to the vaccine strain repeats. However, we cannot be sure that the vaccine strain is changing rather than there being an introduction of these new, related genotypes.

The unvaccinated cattle samples from Western Cape and Free State each had different *A. marginale* subsp. *centrale* genotypes, while unvaccinated cattle from KwaZulu-Natal all had the same *A. marginale* subsp. *centrale* genotype. The vaccine strain was detected in one of the unvaccinated cattle in the Western Cape. The *A. marginale* subsp. *centrale* genotypes obtained from wild ruminants were diverse, demonstrating geographic segregation of National Parks. The repeat Ac8 was common in the *msp1aS* genotypes found in buffalo, even though the buffalo were sourced from parks distributed around South Africa. Ac8 has an edit distance of nine to both repeats Ac1 and Ac2, indicating that it is not closely related to the vaccine strain repeats.

While we have presented diversity metrics broken down by province, we think that the sample size is too small for this to be really meaningful in most cases, i.e., in Mpumalanga and Gauteng, there is an n=1. More importantly, these metrics show us that for South Africa, as a whole, there is a high degree of repeat diversity within genotypes (Table 3.3, GDM1-Local) and a moderate degree of novel genotypes across the country (Table 3.3, GDM1-Global). The low GDM2 values indicate the repeats are dispersed, which is what is expected when the numbers of unique repeats and genotypes are high. This high degree of novel repeats indicates that the repeats have likely been circulating in nature and undergoing selection and change separate from the vaccine strain. As more data is collected it will be interesting to see if these metrics shift and how these metrics compare with those collected in other countries.

While the *A. marginale* subsp. *centrale* vaccine strain was thought for a long time not to be transmitted by most ticks, it was shown that, in fact, it colonized the tick well, but was not secreted into the tick saliva in sufficient quantities for robust transmission (Ueti et al., 2007, Shkap et al., 2009; Ueti et al., 2009). Dramatically increasing tick numbers in transmission experiments overcame the transmission barrier (Ueti et al., 2009). Is the reduced ability of the *A. marginale* subsp. *centrale* vaccine strain to be tick transmitted due to long serial needle passage through cattle? Or, is there, perhaps, a specific vector-pathogen adaptation? There is a report of apparently efficient tick transmission of *A. marginale* subsp. *centrale* vaccine strain from *Rhipicephalus simus* ticks (Potgieter & van Rensburg, 1987). Although *R. simus* is a proven vector in laboratory conditions, this tick is not found on cattle in large numbers, and the immature stages do not normally infest cattle (Potgieter, 1981). It would appear that the strains that we have detected circulating in wild animals today are maintained in nature via a natural tick-transmission cycle, however, this remains speculation at this stage, as we have not tested

ticks or performed transmission studies due to the complexities of working with the ecosystem of infections present in South Africa. If, in fact, *A. marginale* subsp. *centrale* is being spread through natural transmission to cattle, it is likely mitigating some of the disease burden of anaplasmosis caused by *A. marginale*.

3.6 Conclusion

This paper presents a novel genetic test based on *msp1aS* to discriminate strains of *A. marginale* subsp. *centrale* and shows that the vaccine strain is found widely distributed across South Africa and in animals that do not have a history of vaccination. Further, we present metrics indicating a high degree of Msp1aS repeat diversity in South Africa. Our results indicate the significance of wildlife as reservoir host for *A. marginale* subsp. *centrale.*

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Chapter 4

4. Evidence confirming the phylogenetic position of *Anaplasma centrale* (ex Theiler1911) Ristic & Kreier 1984

4.1 Abstract

In 1911, Sir Arnold Theiler isolated and described a parasite that was very similar to *A. marginale* but which was more centrally located within the erythrocytes of the host cells, and was much less pathogenic than *A. marginale*. He named the parasite *A. marginale* variety *centrale*. The name *A. centrale*, referring to the same organism, was published in Validation List no. 15 in 1984, but the publication was based on an erroneous assumption that Theiler had indicated that it was a separate species. Many authors have subsequently accepted this organism as a separate species, but evidence to indicate that it is a distinct species has never been presented. The near full length 16S rRNA gene sequence, and the deduced amino acid sequences for *groEL* and *msp4* from several isolates of *A. marginale* and *A. centrale* from around South Africa were compared with those of the *A. marginale* type strain, St Maries, and the *A. centrale* Israel strain and other reference sequences. Phylogenetic analyses of these sequences demonstrated that *A. centrale* consistently forms a separate clade from *A. marginale*, supported by high bootstrap values (≥90%), revealing that there is divergence between these two organisms. In addition, we discuss distinctive characteristics which have been published recently, such as differences in Msp1a/Msp1aS gene structure, as well as genome architecture that provide further evidence to suggest that *A. centrale* is, in fact, a separate species. Our results, therefore, provide evidence to support the existing nomenclature, and confirm that *A. centrale* (ex Theiler, 1911) sp. nov., comb. nov (Ristic & Kreier, 1984) is, indeed, a valid species.

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4.2 Introduction

In 1896, a point-like pathogen in blood smears of cattle was reported and described as a "very minute roundish body which is stained blue to bring it into view. The body as a rule is situated near the edge of the corpuscle" (Salmon & Smith, 1896). Fourteen years later, after extensive microscopic examination of infected red blood cells, Sir Arnold Theiler described this minute roundish body as *Anaplasma marginale*; referring to the pathogen as having "marginal points" in bovine erythrocytes, and being the causative agent of gallsickness or bovine anaplasmosis (Theiler, 1910). A year later, Theiler isolated and described a very similar parasite which was more centrally located within the erythrocytes of the host cells. He named the parasite *A. marginale* variety *centrale* (Theiler, 1911). The latter was found to be less pathogenic in domestic animals and conferred immunity against infection by *A. marginale* (Theiler, 1911).

Anaplasma marginale variety *centrale* is often referred to as a separate *Anaplasma* species (Inokuma et al., 2001; Shkap et al., 2002; Lew et al., 2002; Kocan et al., 2003; Carelli et al., 2007; Mtshali et al., 2007; Carelli et al., 2008; Decaro et al., 2008; Rymaszewska & Grenda, 2008; Aubry & Geale, 2011; Bell-Sakyi et al., 2015). Ristic (1968) erroneously stated that "In 1911, Theiler, who first described *A. centrale*, indicated that it was a separate species and thus distinct from *A. marginale*". This resulted in the inclusion of the name *A. centrale* in List No.15 of Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the International Journal of Systematic Bacteriology (1984). In conjunction, the organism was listed as a separate species in Bergey's Manual of Systematic Bacteriology (Ristic & Kreier, 1984). Thus, many authors refer to *A. marginale* variety *centrale* as a separate species. However, others have recognized that the description by Ristic and Kreier (1984) was flawed, and that, since a formal species description is lacking, the official taxonomic classification should revert to its original designation as a variety of *A. marginale* (Dumler et al., 2001; Brayton et al., 2009).

In 2001, Dumler et al. (2001) reorganized the order Rickettsiales, based on phylogenetic analyses of the 16S rRNA and *groEL* genes. These authors indicated that the 16S rRNA gene sequences of strains of *A. marginale, A. ovis* and *A. centrale* are nearly identical with 99.1% similarity, supporting Theiler's original description of *A. centrale* being a variant of *A. marginale* (Theiler, 1911). However, they note the existence of a strain of *A. centrale* with a 16S rRNA gene sequence that has 1.8% nucleotide difference from other phenotypically

characterized strains of *A. centrale* (the Aomori strain). Sequence data from other genes was not available at the time to enable these authors to resolve this taxonomic fine point.

To date, the taxonomic status of *A. centrale* and its relationship to *A. marginale* sensu stricto remains unclear. In this study, a comparative phylogenetic analysis was performed for three conserved genes of *A. marginale* and *A. centrale* obtained from cattle, buffalo, and black wildebeest samples originating from different geographical areas of South Africa. The full length 16S rRNA (coding for the small subunit ribosomal RNA gene), *groEL* (encoding a chaperone) and *msp4* (encoding major surface protein 4) genes were amplified and sequenced. Phylogenetic analysis was employed to resolve the appropriate phylogenetic position of *A. centrale* and coupled with other retrospective genomic analyses to provide evidence that *A. centrale* is indeed a separate species.

4.3 Materials and Methods

A total of 380 blood samples from African buffalo, waterbuck, eland, black wildebeest, blue wildebeest and cattle were obtained from the Wildlife Biological Resource Center and Biobank South Africa under the auspices of the National Zoological Gardens of South Africa, from the South African National Parks Biobank and from two commercial farms in Bergville, KwaZulu-Natal. The samples were screened for the presence of *A. marginale* and *A. centrale* as previously described (Chapter 3)

4.3.1 Selection of samples for amplification, cloning and sequencing

At least three samples were selected from each National Park and the two Bergville farms for targeted gene sequencing (Table 4.1). National Parks included: Mokala National Park (MNP), Camdeboo National Park (CNP), Kruger National Park (KNP), Hluhluwe iMfolozi Park (HiP) and Addo Elephant National Park (AEP). Only samples that were co-infected with *A. marginale* and *A. centrale* (Chapter 3), were chosen for further analysis.

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Table 4.1 Origin of samples used for sequencing and phylogenetic analysis.

#	Sample	Origin	Province	Host
1	MNP958	Mokala National Park	Northern Cape	Black wildebeest
2	MNP999			Buffalo
3	MNP1000			Buffalo
4	MNP1021			Buffalo
5	CNP976	Camdeboo National Park	Eastern Cape	Buffalo
6	CNP978			Buffalo
7	CNP979			Buffalo
8	CNP985			Buffalo
9	KNP581	Kruger National Park	Mpumalanga	Buffalo
10	KNP582			Buffalo
11	KNP584			Buffalo
12	HiP2	Hluhluwe iMfolozi Park	KwaZulu-Natal	Buffalo
13	HiP3			Buffalo
14	HiP4			Buffalo
15	HiP5			Buffalo
16	HiP6			Buffalo
17	HiP7			Buffalo
18	AEP1002	Addo Elephant National Park	Eastern Cape	Buffalo
19	AEP1003			Buffalo
20	AEP1007			Buffalo
21	AEP1013			Buffalo
22	Berg19	Bergville Farm 1	KwaZulu-Natal	Cattle
23	Berg25	Bergville Farm 2	KwaZulu-Natal	Cattle
24	Berg27	Bergville Farm 2		Cattle

4.3.2 Amplification of the 16S rRNA, groEL and msp4 genes

The near full-length genes were amplified using the primers described in Table 4.2. The primer sets; GroELF, GroELR and MSP4ACF, MSP4ACR were designed based on the published sequences of *Anaplasma* species, targeting the conserved regions between *A. centrale* and *A. marginale* using CLC Main Workbench version 7.5.1 software. The other primer sets were obtained from previous studies as cited on Table 4.2. The PCR was performed in a final reaction volume of 25 µl, containing 1X Phusion Flash High-Fidelity PCR Master Mix (includes Phusion Flash II DNA Polymerase, reaction buffer, dNTPs, and $MgCl_2$) (Thermo Fisher Scientific, South Africa), 0.1 µM of each primer and 10 to 25 ng total genomic DNA. The thermal cycling programme was as follows: an initial denaturation at 98ºC for 10 s, 30 cycles of denaturation at 98ºC for 1 s, annealing temperature as shown in Table 2 for 5 s, and extension at 72ºC for 15 s, followed by a final extension at 72ºC for 1 min and a hold at 4ºC.

Table 4.2 Oligonucleotide primers used in this study.

Gene	Primer name	Sequence (5'-3')	Annealing temperature	Amplicon size (bp)	Reference
16S rRNA	**fD1** **rP2**	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	55˚C	1470	Weisburg et al., 1991
groEL	**GroELF** **GroELR**	GCGCATTCTGGAGGCTG GCGTTTGACTTGGCTGTGTC	64˚C	1482	This study
A. marginale msp4	**MSP45** **MSP43**	GGGAGCTCCTAATTACAGAGAATTGTTTAC GCAAGATTCCTGTTCAGCTAAGGATCCGG	60˚C	848	de la Fuente et al., 2005
A. centrale msp4	**MSP4ACF** **MSP4ACR**	GCTCCCTACTTGTCAGTGGGCCTG GATTACGGCTTTAACCTCGGAGC	67˚C	800	This study

4.3.3 Sequencing and phylogenetic analysis of 16S rRNA, groEL and msp4 genes

Amplicons of the correct sizes were purified, ligated into the pJET vector (Thermo Fisher Scientific) and transformed into *E. coli* JM109 High Efficiency Competent cells (Promega, Madison, WI). Recombinant plasmids were purified using a High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany) and sequenced using 0.2 µM M13 primers and ABI Big Dye V3.1 on an ABI 3500XL Genetic Analyzer at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). Sequences were assembled and edited using CLC Main Workbench 7 (Qiagen, http://www.clcbio.com). Searches of databases for homologous sequences were performed using BLASTn (Altschul et al., 1990). A multiple sequence alignment was performed for each gene or deduced amino acid sequence, along with sequences of related genera available in GenBank (Table S1), using MAFFT (multiple sequence alignment programme) v6 employing the FFT-NS-1 algorithm (Katoh et al., 2002). The alignments were truncated to the size of the shortest sequence using BioEdit v7 (Hall, 1999).

Phylogenetic trees were constructed by the neighbour-joining, maximum likelihood and maximum parsimony methods as implemented by the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software package (Kumar et al., 2016). The maximum likelihood tree was inferred based on the Poisson correction model for Msp4 and GroEL, while the 16S rDNA tree was based on the Jukes-Cantor model and GTR model; this was in combination with the bootstrap method (Felsenstein, 1985) using 1000 replicates/tree for each method. The genetic distances between the sequences were estimated by determining the number of nucleotide/amino acid differences between sequences using MEGA7 (Kumar et al., 2016). Bayesian phylogenetic trees were constructed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). The WAG+G+F and JTT+G+F model were determined to be the best-fit for the Msp4 and GroEL amino acid sequence data, respectively. This was determined by the ProtTest (Abascal et al., 2005), while the 16S rRNA nucleotide data best-fit model was determined to be invgamma using the Modeltest v3.7 software package (Posada & Crandall, 1998). All consensus trees were edited using MEGA7. The GenBank accession numbers of reference sequences used in this study are reported in Appendix 1, while the 16S rRNA, *groEL* and *msp4* gene sequences used to construct the phylogenetic trees were submitted to GenBank, and these accession numbers are reported in Appendix 2.

4.4 Results

4.4.1 16S rRNA, groEL and msp4 gene sequence and phylogenetic analysis

We analyzed the 16S rRNA, *groEL* and *msp4* gene sequences from 24 animals co-infected with *A. marginale* and *A. centrale*; these included three cattle, 20 African buffalo and one black wildebeest. We found four (Ac1, Ac2, Ac3 and Ac4) 16S rRNA gene sequence types for *A. centrale* and three (Am1, Am2 and Am3) for *A. marginale*. Except for Ac4, which was identical to the *A. centrale* Israel strain, the *A. centrale* 16S rRNA sequences obtained in this study differed from the *A. centrale* Israel strain by 1 to 2 nucleotides. The *A. marginale* 16S rRNA sequences differed from the *A. marginale* St Maries strain by 1 to 2 nucleotides (Appendix 3).

The *A. centrale* (Ac1) GroEL deduced amino acid sequence was completely conserved and identical to the *A. centrale* Israel vaccine strain and the recently published *groEL* sequences from KNP (Sisson et al., 2017) [data not shown], but differed from other previously published South African and Italian isolates by 1 and 2 amino acids, respectively (Appendix 4). Two GroEL sequence types were identified for *A. marginale* (Am1 was identical to the St Maries sequence, while Am2 differed from it by 3 amino acids). We found four (Ac1, Ac2, Ac3 and Ac4) *A. centrale* Msp4 deduced amino acid sequence types that differed by 1 to 4 amino acid residues from the *A. centrale* Israel strain, and three (Am1, Am2 and Am3) sequence types for *A. marginale* that differed by 1 to 2 amino acid from the *A. marginale* St Maries strain (Table 4.3) (Appendix 5).

Table 4.3 *Anaplasma centrale* and *A. marginale* 16S rRNA, GroEL and Msp4 genotypes.

Sample	16S rRNA							GroEL			Msp4						
	Ac*				Am**			Ac	Am		Ac				Am		
	1	**2**	**3**	**4**	**1**	**2**	**3**	**1**	**1**	**2**	**1**	**2**	**3**	**4**	**1**	**2**	**3**
MNP958										X							
MNP999			X	X	X		X	X					X				X
MNP1000			X				X	X		X				X			X
MNP1021							X			X							X
CNP976				X			X	X	X					X	X		X
CNP978				X				X						X			
CNP985				X				X									
CNP979														X			
KNP581		X		X			X	X			X						X
KNP582										X						X	X
KNP584		X		X			X	X		X			X				X
HiP2										X				X			X
HiP3		X						X		X				X			
HiP4	X									X				X			
HiP5												X	X				
HiP6			X	X			X	X		X				X			X
HiP7										X				X			X
AEP1002										X							
AEP1003								X									
AEP1007				X		X	X	X	X					X			X
AEP1013			X	X			X	X									
Berg19												X		X			
Berg25													X	X			
Berg27			X	X			X	X		X			X	X			X

NB. It was not possible to obtain clones for all genes from some of the samples, e.g only *A. marginale* GroEL clones could be obtained for sample MNP985.
*Ac = *A. centrale*
**Am = *A. marginale*

The *A. centrale* and *A. marginale* genotypes obtained did not show any specific geographic distribution pattern. The most common *A. centrale* 16S rRNA sequence genotypes were Ac3 and Ac4; Ac4 was common to all of the study sites. The sequence of the Ac4 genotype was identical to that of the *A. centrale* Israel vaccine strain over a 1116 bp region of the 16S rRNA gene. The *A. marginale* 16S Am3 genotype was identical to the St Maries strain and was found in all the study sites. Notably, a sequence named *A. centrale* Aomori strain that was described by Inokuma et al. (2001) grouped with *A. capra* sequences Fig. 4.2. The *groEL* gene was

completely conserved, yielding only one *A. centrale* GroEL genotype (Ac1). *A. marginale* had two GroEL genotypes of which Am2 was more common than Am1 and was distributed in all of the study sites except CNP. The *A. centrale* Msp4 Ac4 genotype was found to be present in all study sites except in KNP where Ac1 and Ac3 were present. The *A. centrale* Israel vaccine strain Msp4 sequence differed by 1 amino acid to that of Ac4 over the 212 amino acid region that was sequenced. As for *A. marginale*, Msp4 genotype Am3 was found to be the most common and was present in all the study sites (Fig. 4.1).

All of the phylogenetic tree topologies obtained using all four tree algorithms were similar, and the maximum likelihood tree was chosen as a representative tree. The trees inferred using 16S rDNA (Fig. 4.2), GroEL (Fig. 4.3) and Msp4 (Fig. 4.4) sequences always grouped *A. marginale* and *A. centrale* into two distinct clades, indicative of a divergence between the two organisms. This was supported by high bootstrap values of 90% (16S rRNA), 92% (GroEL) and 99% (Msp4).

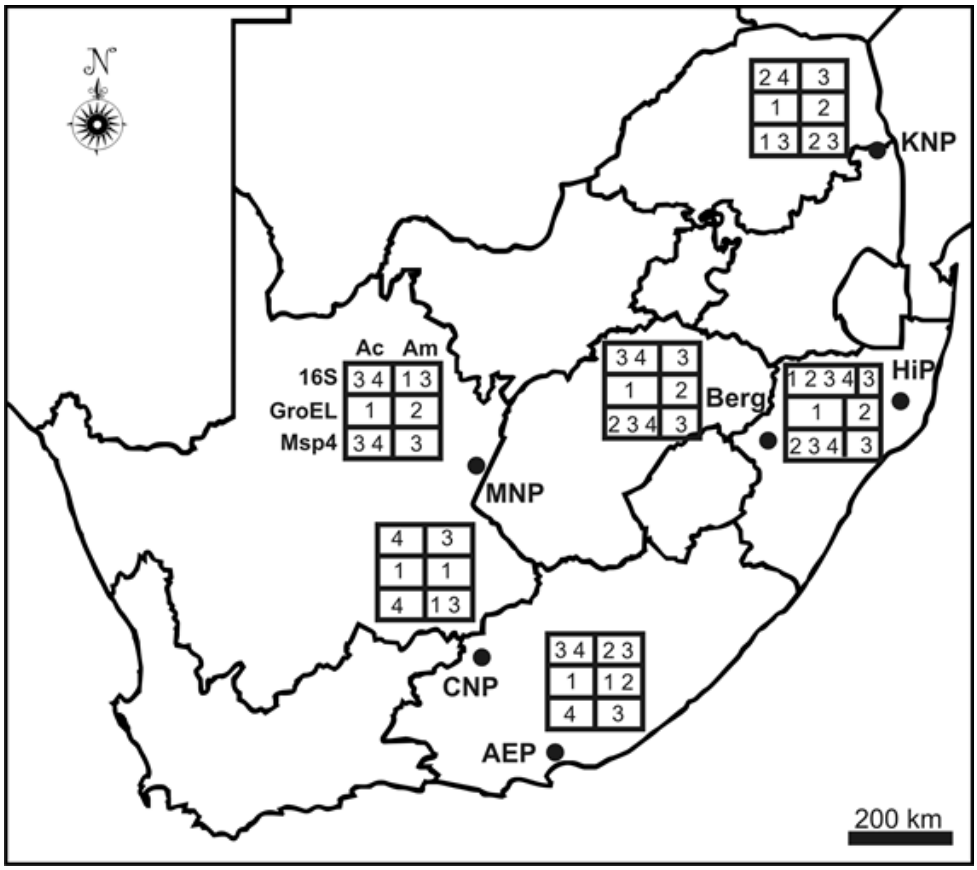

Figure 4.1 Map of South Africa showing the *A. centrale* and *A. marginale* 16S rRNA, GroEL and Msp4 genotypes in selected study areas.

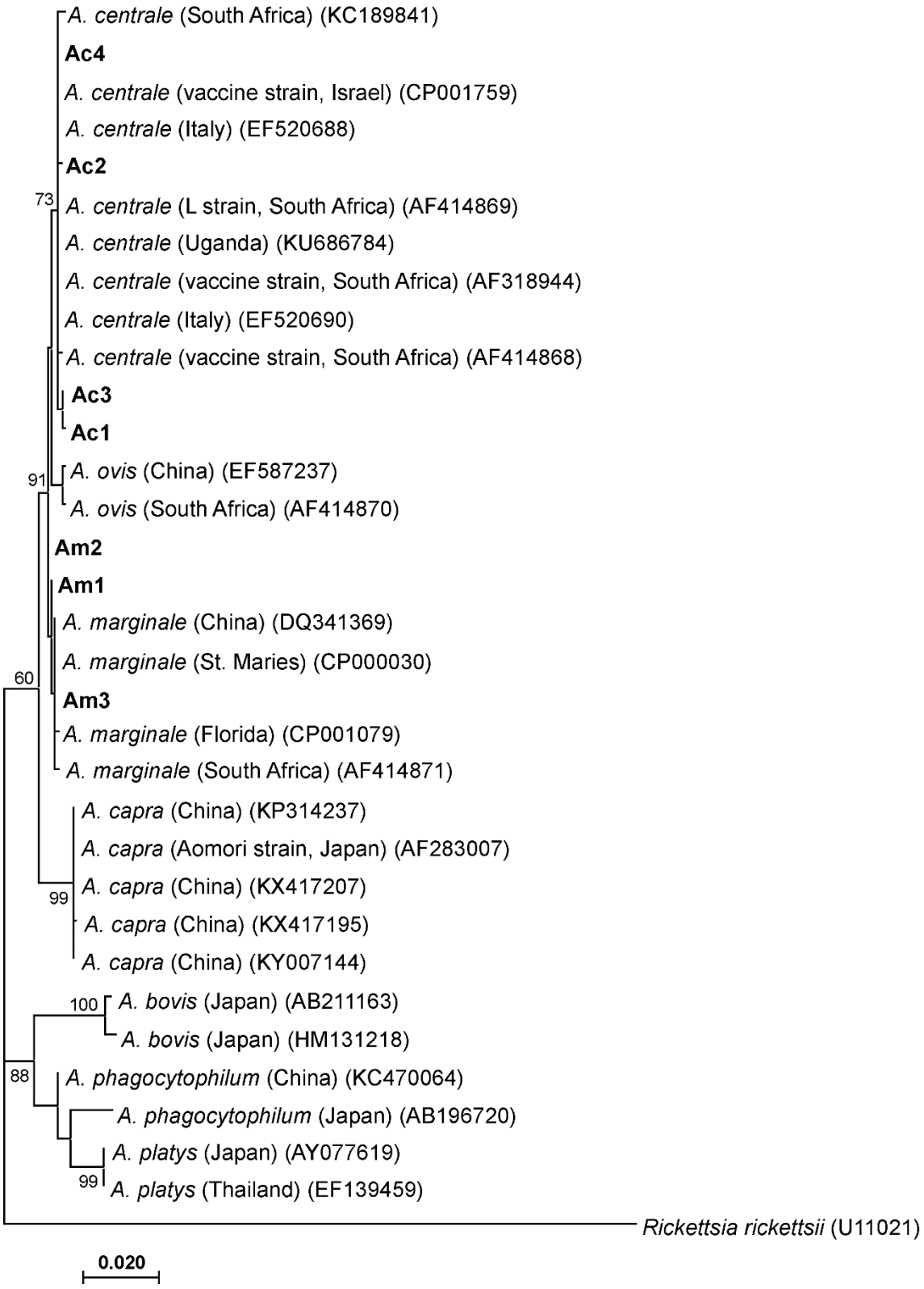

Figure 4.2 Maximum likelihood tree based on 16S rRNA nucleotide sequences. The tree shows the phylogenetic relationship between *A. centrale, A. marginale* and other *Anaplasma* species. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. There were a total of 1108 positions in the final dataset. As some sequences obtained in this study were identical to each other we have coded them with a genotype name (i.e. "Am1") and used a single representative of the genotype to construct the tree. Genotype representation is provided in Table 4.3.

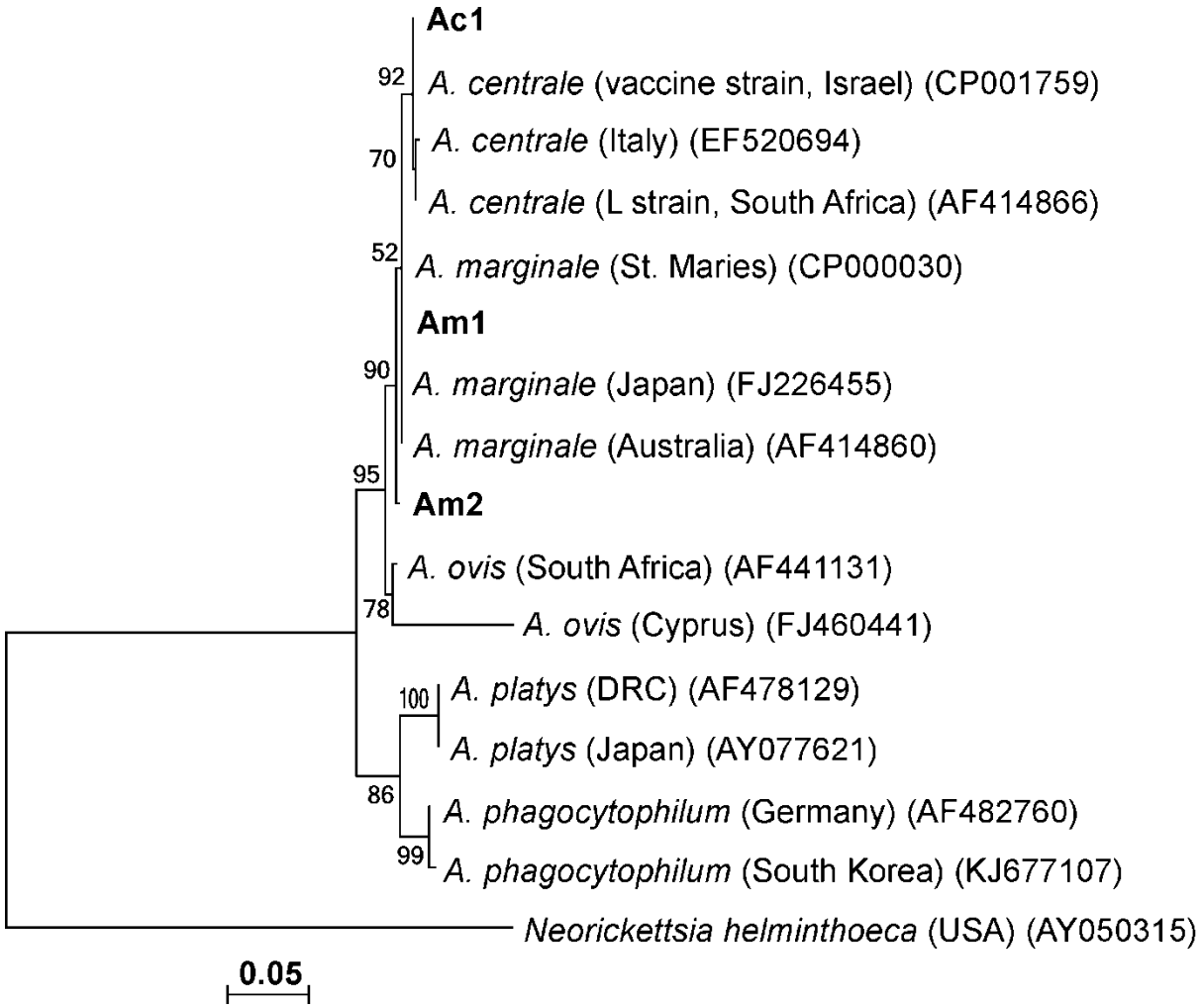

Figure 4.3 Maximum likelihood tree based on GroEL deduced amino acid sequences. The tree shows the phylogenetic relationship between *A. centrale*, *A. marginale* and other *Anaplasma* species. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. There were a total of 488 positions in the final dataset. As some sequences obtained in this study were identical to each other we have coded them with a genotype name (i.e. "Am1") and used a single representative of the genotype to construct the tree. Genotype representation is provided in Table 4.3.

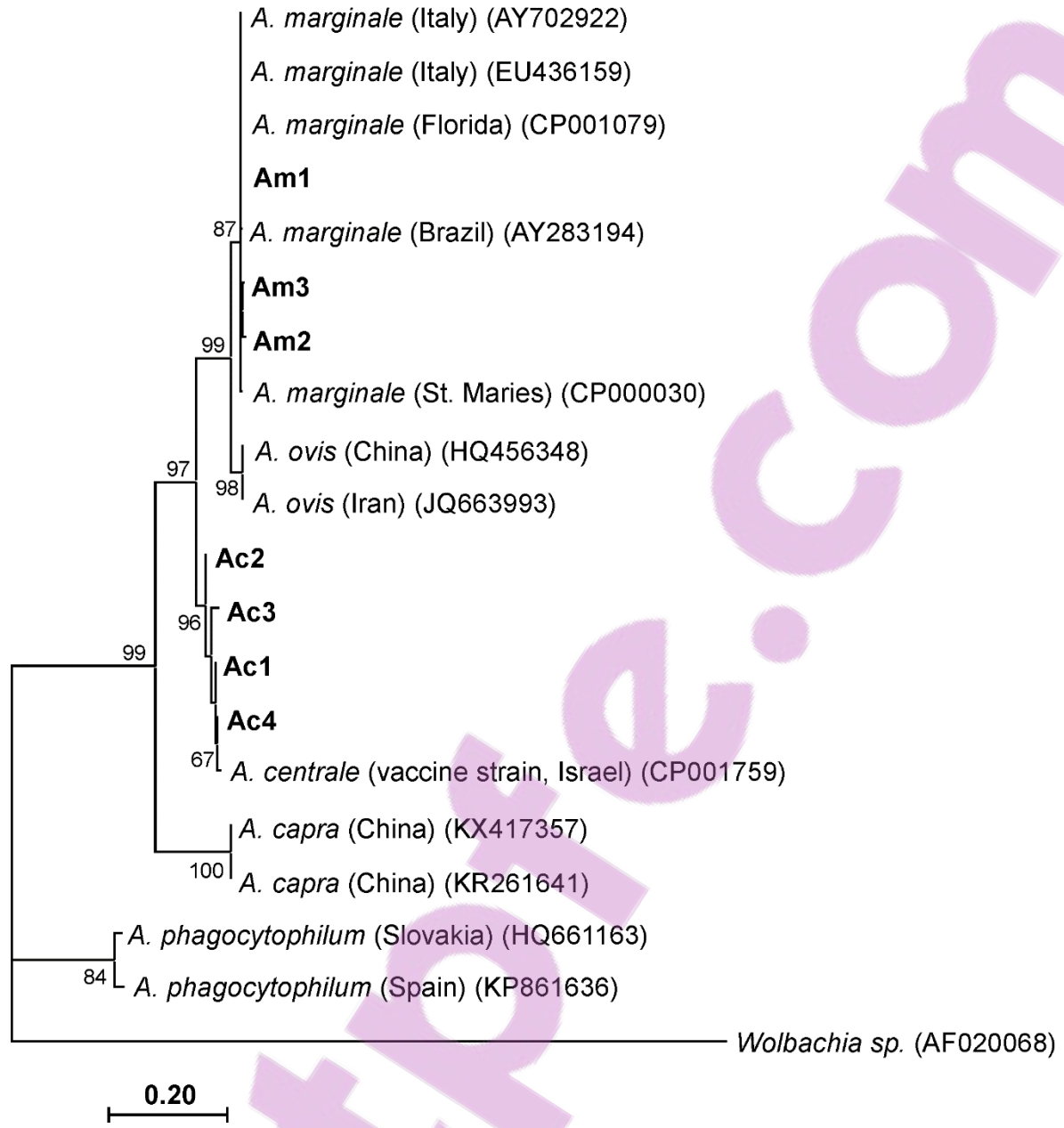

Figure 4.4 Maximum likelihood tree based on Msp4 deduced amino acid sequences. The tree shows the phylogenetic relationship between *A. centrale, A. marginale* and other *Anaplasma* species. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. There were a total of 198 positions in the final dataset. All positions containing gaps and missing data were eliminated. As some sequences obtained in this study were identical to each other we have coded them with a genotype name (i.e. "Am1") and used a single representative of the genotype to construct the tree. Genotype representation is provided Table 4.3.

4.5 Discussion

The *A. centrale* Israel vaccine strain 16S rRNA, GroEL and Msp4 genotype sequences (Ac4, Ac1 and Ac4, respectively) were found in all of the study sites, with the exception of Msp4 Ac4 that was absent in the KNP. This is in concordance with our previous findings based on *msp1aS* that the *A. centrale* vaccine strain is found widely distributed across South Africa and in animals that do not have a history of vaccination (Khumalo et al., 2016) (Chapter 3).

The pattern of two distinct clades for *A. marginale* and *A. centrale* that was observed from the phylogenetic trees was in concordance with previous findings: Inokuma et al. (2001), Lew et al. (2003), Liu et al. (2005), Carelli et al. (2008), Yang et al., (2017) and Sisson et al. (2017), to name a few. In a study done by Inokuma et al. (2001), the authors showed that the 16S rRNA sequence of "*A. centrale* Aomori strain" was related to *A. marginale* by both level-of-similarity (98.08% identical) and distance analysis. They concurred that this "*A. centrale*" is an independent species although closely related to *A. marginale*. These findings were based on the Aomori strain, a Japanese isolate which actually appears to be a novel *Anaplasma* species, *A. capra* (Li et al., 2015). Phylogenetic analysis of 16S rRNA revealed that *A. capra* sequences clustered together in a clade but grouped separately from other *Anaplasma* species (Li et al., 2015). Interestingly, *A. capra* is not a formally recognized species, and is not on the List of Prokaryotic Names with Standing in Nomenclature (www.bacterio.net/anaplasma.html). However, it should be noted that when the Inokuma study was done, there were relatively few *A. centrale* 16S rRNA gene sequences available, which led to the misclassification of the Aomori strain as *A. centrale*; and thus some confusion when others have compared their data with this sequence, as relatively speaking, it is somewhat distant from other *A. centrale* 16S rRNA gene sequences.

Carelli and coworkers (2008) compared *A. ovis* isolates from China with *A. marginale* and *A. centrale* using 16S rRNA and GroEL peptide sequences; the phylogenetic trees revealed two distinct clades representative of *A. marginale* and *A. centrale*. Since their study was focused on the taxonomic placement of the Chinese *A. ovis* isolates, no further taxonomic classification or mention of *A. centrale* was made. In the study done by Lew et al. (2003), 16S rDNA analysis grouped *A. marginale* and *A. centrale* separately; however, it could not delineate *A. ovis* isolates from either *A. marginale* or *A. centrale*. In contrast, *A. ovis* grouped separately in our study, although with very low bootstrap support (28%). Our results were in concordance with

the studies of Liu et al. (2005) and Carelli et al. (2008) who also found that phylogenetic analyses based on 16S RNA sequences resolved *A. ovis*, *A. marginale* and *A. centrale* into separate groups. Furthermore, Lew et al. (2003) concluded that GroEL sequences were more reliable for phylogenetic inferences of the species of the erythrocytic *Anaplasma* species (*A. centrale, A. ovis* and *A. marginale*). Our GroEL results were in concordance with those of Lew et al. (2003) who showed that the differences between *A. marginale* and *A. ovis* were more prominent than those between *A. marginale* and *A. centrale.* This was also consistent with differences previously demonstrated by RFLP and monoclonal antibody studies (Palmer et al., 1988; Visser et al., 1991; Ngeranwa et al., 1998). Recently Sisson et al. (2017), used *groEL* to characterize *A. centrale* strains, amplifying *A. centrale groEL* nucleotide sequences from DNA of buffalo collected in KNP. This study confirmed that the *groEL* gene is conserved and can discriminate *A. centrale* strains from *A. marginale* strains; however, phylogenetic analysis of *A. centrale groEL* sequences grouped South African sequences separately from other *A. centrale* strain sequences from Italy and Australia – this is likely due to the very limited number of samples represented in their analysis. The authors used an 881 bp fragement of the *groEL* gene sequence for their phylogenetic analysis. When these nucleotide sequences were translated and added to our GroEL deduced amino acid sequences, they formed one clade with our sequences and those from other countries (i.e. the GroEL Ac1 genotype).

As for phylogenetic trees inferred from Msp4 sequence data, most publications focus on the description of *A. phagocytophilum* isolates (de la Fuente et al., 2005; Zhan et al., 2010) with no statements made about the taxonomic position of *A. centrale*. However, it remains clear from these published phylogenetic trees that *A. marginale* and *A. centrale* always group into two distinct clades.

A. centrale is known to be closely related to *A. marginale* based on morphological, protein structural and immunological studies. The size and location of *A. marginale* and *A. centrale* organisms within red-blood cells have traditionally been used to differentiate morphologically between these two organisms. *Anaplasma marginale* is situated on the margins of the red blood cells, appearing as deeply stained "points", round in shape ranging from 0.3 to 1.0 µm in diameter (Bruner & Gillespie**,** 1966), while *A. centrale* is situated towards the centre of the red blood cells, with size variation from 0.4 - 0.95 µm in diameter (Henning, 1949; Waddel, 1964). *A. marginale* and *A. centrale* are antigenically related, sharing immunodominant epitopes that play a role in the protection induced by *A. centrale* (Shkap et al., 1991). However, the

protection is partial and varies with *A. marginale* genotype. This is because there is a lower degree of conservation between the deduced amino acid sequences of surface proteins of *A. centrale* and *A. marginale* strains (72.4%) than between any two *A. marginale* strains (95.1%) (Agnes et al., 2011).

Although *A. marginale* and *A. centrale* are detected in similar hosts (i.e. buffalo, black wildebeest, blue wildebeest, eland, waterbuck and cattle) (Khumalo et al., 2016) (Chapter 3), the tick vector and/or the biology of transmission of *A. centrale* appears to differ from that of *A. marginale* sensu stricto. Biological transmission of *A. marginale* is effected by 20 tick species around the world (Kocan et al., 2010), and five tick species have been implicated in the transmission *A. marginale* in South Africa: *Rhipicephalus microplus, R. decoloratus, R. evertsi evertsi, R. simus* and *Hyalomma rufipes* (de Waal, 2000). *Anaplasma centrale* was thought not to be tick transmissible (Shkap et al., 2009), however, experimental transmission of *A. centrale* by *R. simus* and *D. andersoni* has been demonstrated (Potgieter & van Rensburg 1987; Ueti et al., 2009). Work done in the *D. andersoni* model demonstrated that *A. centrale* infects the midgut and salivary gland at similar rates to *A. marginale*, but *A. centrale* was not transmitted when only a few ticks were used in transmission experiments. Further analysis demonstrated that *A. centrale* resided in a different subcellular location in the salivary gland and was secreted into the saliva at a much lower rate than *A. marginale*; when tick numbers were dramatically increased to compensate for the low pathogen load, transmission was achieved (Ueti et al., 2009). These two transmission studies are the only successful transmissions of *A. centrale* on record amongst a myriad of failed transmission attempts.

Genomic comparisons of the two organisms have also revealed that the two organisms are divergent (Herndon et al., 2010; Brayton et al., 2005): *A. marginale* sensu stricto strains have closed core, highly syntenic genomes (Dark et al., 2009), while the *A. centrale* genome exhibits a marked lack of syteny with sensu stricto strains. The genome of *A. marginale* is comprised of 949 protein encoding genes and 16 pseudogenes, while that of *A. centrale* is comprised of 925 protein encoding genes and 19 pseudogenes. The genome of *A. marginale* contains 18 putative genes that are absent in *A. centrale*, while the *A. centrale* genome contains 10 putative genes that are absent in *A. marginale* (Agnes et al., 2011). The *A. centrale* genome also revealed the presence of a homolog of *msp1α*, a gene that was thought to be absent from *A. centrale*. The *A. centrale* homolog, *msp1aS*, was used in a recent study to genotype strains of *A. centrale* in the same manner that *msp1α* is used to genotype strains of *A. marginale* (Khumalo et al.,

2016) (Chapter 3). The *A. centrale* Msp1aS repeats are longer (~51 amino acids in length) than the *A. marginale* Msp1a repeats (28-29 amino acids in length) and there is no sequence similarity in the repeat regions of these proteins, although the carboxy-terminus of the protein has approximately 30% amino acid identity. This genotyping analysis provides clear distinction between *A. marginale* and *A. centrale*. The results further demonstrate the diversity of *A. centrale* strains from cattle and wildlife hosts from South Africa, also highlighting the significance of wildlife as reservoir hosts for *A. centrale*.

In conclusion, the phylogenetic analysis presented here, together with differences in genome architecture, *msp1α*/*msp1aS* gene sequence, and the biology of tick transmissibility, provide sufficient divergence between *A. centrale* and *A. marginale* to classify them as separate species. Therefore, there are seven officially recognized species of *Anaplasma*: *A. marginale, A. centrale, A. ovis, A. phagocytophilum, A. platys, A. bovis,* and *A. caudatum. A. capra* represents a potential eighth species and is discussed in this article as a novel *Anaplasma* species whose name has not yet been officially recognized.

4.5.1 Description of Anaplasma centrale

***Anaplasma centrale* (ex Theiler, 1911) sp. nov., comb. nov. (Ristic & Kreier, 1984)**

Etymology: *cen. tra' le* L. neut. adj. centrale, in the middle, central, referring to the location of the organism within erythrocytes.

Synonym: "*Anaplasma marginale* variety *centrale*" Theiler, 1911. Other names that have been used in the literature include: *Anaplasma marginale centrale*; *Anaplasma marginale* subspecies *centrale*; *Anaplasma marginale* vaccine strain.

Anaplasma centrale is a separate species from *A. marginale*. It is centrally located in the red blood cell. It has been detected in some African countries; i.e. South Africa (Mtshali et al., 2007; Debeila, 2012; Mutshembele et al., 2014), Uganda (Oura et al., 2011) and Botswana (Eygelaar et al., 2015). Also in Italy (Carelli et al., 2008), Spain (Palomar et al., 2015) and Brazil (Joazeiro et al., 2015), usually causing mild disease in cattle; death may result depending on the virulence of the strain. It is prevalent in wildlife, especially in the African buffalo (*Syncerus caffer*). The genome of *A. centrale* contains a homolog of *A. marginale msp1α*, *msp1aS*, which serves as a genetic marker for *A. centrale* strains. *A. centrale* is antigenically

related to *A. marginale*, they both share specific immunological epitopes that enables *A. centrale* to provide limited protection against *A. marginale*.

The mol % G +C of DNA: 50% (Herndon *et al*., 2010)
Type strain: Israel
Genbank accession number *A. centrale* (genome): CP0001759

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Chapter 5

5. *Anaplasma centrale* Msp1aS genotyping: Can it shed light on the possible tick vector(s) of *A. centrale* that circulate in the cattle populations in uThukela district, South Africa?

5.1 Abstract

Ticks are known vectors of *Anaplasma marginale* infections in South Africa. However, since 1987, only two ticks have been experimentally demonstrated to transmit *A. centrale* in cattle, and only one of these tick species, *Rhipicephalus simus*, occurs in South Africa. Data presented in the preceding chapters, detecting *A. centrale* in unvaccinated cattle and several ruminant species, suggests that the parasite is naturally circulating, most likely through a tick vector. However, it is not known how many tick species are involved in the natural transmission of *A. centrale* in South Africa. Therefore this study was designed to screen *A. centrale* from infections in cattle and characterize the genotypes present/common in the cattle and in ticks infesting the cattle. In total, 458 ticks were collected from 109 cattle from three dip tanks in uThukela district in KwaZulu-Natal, South Africa between June 2015 and February 2016. Based on morphological characteristics, two genera and four species of ticks were identified: *Rhipicephalus evertsi evertsi* (50.0%), *R. appendiculatus* (30.1%), *R. microplus* (19.0%), and *Hyalomma rufipes* (0.9%). The screening of tick salivary glands and midguts using a duplex real-time PCR (qPCR) assay revealed the presence of *Anaplasma marginale* and *A. centrale* infections in 53 to 100% of the samples. All 109 cattle tested positive for the presence of both *A. marginale* and *A. centrale* DNA. Samples from five cattle infested with *R. evertsi evertsi* and *R. appendiculatus*, were selected for *A. centrale* strain characterization using *msp1aS* genotyping. The *msp1aS* genotyping revealed that genotypes Ac8 and Ac20 were present in DNA samples from one of the cattle and *Rhipicephalus evertsi evertsi* ticks that had fed on that animal. The findings from this study suggest that *R. evertsi evertsi* may be responsible for transmission of *A. centrale* infections in uThukela district, and *R. appendiculatus* and *R. microplus* may also be implicated.

5.2 Introduction

Ticks are good vectors for pathogen transmission; they are second only to mosquitoes as vectors of arthropod-borne infectious diseases (Andreotti et al., 2011). Tick infestations and the pathogen they transmit occur widely in South Africa (Rikhotso et al., 2005; Mtshali et al., 2007; Andreotti et al., 2011; Spickett et al., 2011; Nyangiwe et al., 2013; Mutshembele et al., 2014). The most important tick-borne diseases of cattle in South Africa are theileriosis, babesiosis, heartwater and anaplasmosis (De Vos, 1979; De Waal, 2000; Regassa et al., 2003; Marufu et al., 2010; Spickett et al., 2011).

Anaplasmosis is caused by an intra-erythrocytic rickettsia, *Anaplasma marginale*. This rickettsia parasitizes and destroys the red blood cells of the host, which leads to progressive anaemia. The clinical signs of anaplasmosis are most notable in cattle, but not in wildlife species (Dumler et al., 2001; Kocan et al., 2010). The susceptibility of wildlife species to *A. marginale* infections is not well understood. However, *A. marginale* and *A. centrale* infections have been found occurring in African buffalo (*Syncerus caffer*), waterbuck (*Kobus ellipsipyrymnus*), eland (*Taurotragus oryx*), black wildebeest (*Connochaetes gnou*) and blue wildebeest (*Connochaetes taurinus*) (Khumalo et al., 2016) (Chapter 3). *Anaplasma centrale* has been confirmed as a separate species from *A. marginale* based on 16S rRNA, *groEL* and *msp4* gene data analysis (Chapter 4). *Anaplasma centrale* is prevalent in wildlife species and is used as a live blood vaccine against *A. marginale* infections. In the recent study by Khumalo et al. (2016) (Chapter 3), *msp1aS* genotyping of *A. centrale* revealed high genetic diversity of *A. centrale* strains. The genotypes detected indicated the presence of the vaccine strain in cattle and wildlife species with no history of vaccination.

Biological transmission of *A. marginale* is effected by 20 tick species worldwide (Kocan et al., 2010). However, five tick species are implicated as vectors in South Africa, namely: *Rhipicephalus microplus, R. decoloratus R. evertsi evertsi, R. simus* and *Hyalomma rufipes*. *Anaplasma centrale* is experimentally proven to be transmitted by only *R. simus* (Potgieter, 1979, Potgieter & van Rensburg, 1987) and *Dermacentor andersoni* (Ueti et al., 2009). A laboratory transmission study using the live blood vaccine strain from Onderstepoort, South Africa and *R. simus* resulted in a successful transstadial transmission by adult ticks (Potgieter & van Rensburg, 1987) *Rhipicephalus simus* is extensively distributed throughout Southern Africa (Walker et al., 2000). Among domestic animals, the adult ticks primarily parasitize cattle

and dogs (Walker et al., 2000), but they have been recovered from many wild animals including felids (Horak et al., 2000; Golezardy et al., 2016). The immature stages prefer murid rodents as hosts, some species of which may be burrow-dwelling (Golezardy et al., 2016). Work done in the *D. andersoni* model showed that *A. centrale* colonized the tick well, but was not secreted into the tick saliva in sufficient quantities for robust transmission (Ueti et al., 2007). Dramatically increasing tick numbers in transmission experiments overcame the transmission barrier (Ueti et al., 2009). Interestingly, *A. centrale* does not occur in areas where the *D. andersoni* tick occurs.

Recently, a study by Berggoetz et al. (2014) revealed the presence of *A. centrale* in the salivary glands of *R. gertrudae*, suggesting that this tick species may be a possible vector of *A. centrale*. The presence of pathogens in tick salivary glands suggests that the pathogen may be transmitted to the host; however, this is not sufficient to prove its vectoral role/capacity, as demonstrated by the work in the *D. andersoni* model. Factors such as ability of a tick to acquire the infection and successful pathogen replication within the tick salivary glands are considered as good markers of vector ticks (Futse et al., 2003).

UThukela district municipality is approximately 11 500km^2. It is a predominately rural area located on the western boundary of KwaZulu-Natal. This municipality is characterized by the socio-economic indicators such as low revenue base, poor infrastructure, limited access to services and low economic base (http://www.uthukeladm.co.za). Bergville and Ladysmith are small towns that fall under the uThukela district municipality. Bergville is said to be one of the major beef production in Kwazulu-Natal (Okhahlamba Local Municipality, 2012). Previous studies have shown the presence of tick vectors responsible for transmission of anaplasmosis at Ladysmith (Mtshali et al., 2015). A molecular study conducted by Khumalo et al. (2016) (Chapter 3) in Bergville indicated the presence of *A. centrale* infections in cattle. Although these cattle (Farm 1) were previously vaccinated with the *A. centrale* vaccine strain, the genotypes obtained differed from that of the vaccine strain (Ac1 Ac1 Ac1 Ac2). It could be that the cattle were naturally infected with a dissimilar strain of *A. centrale* prior or post vaccination. The study by Khumalo et al. (2016) (Chapter 3) focused on *A. centrale* infections found in cattle only, no ticks were collected or screened.

The objectives of this study were to identify and characterize *A. centrale msp1aS* genotypes in cattle blood samples and their associated ticks and determine whether the ticks were carrying

A. centrale strains with identical genotypes to the cattle. While this would not provide definitive proof that the ticks were transmitting this pathogen, it would be an indication that they were capable of this transmission.

5.3 Materials and Methods

5.3.1 Ethical approval

The study was approved by the Animal Ethics Committee at the University of Pretoria (Protocol number V088-16). Permission was obtained to do research in terms of Section 20 of the Animal Diseases Act, 1984 (Department of Agriculture, Forestry and Fisheries, Pretoria, South Africa; reference number, 12/11/1/1). Standard techniques were followed during the collection of blood samples and ticks. All participating farmers gave informed verbal consent before the study commenced.

5.3.2 Study area

The study was conducted at three dip tanks in two towns, Bergville and Ladysmith, in uThukela district, South Africa. These study areas were chosen due to previous evidence of the presence of *Anaplasma* infections in cattle (Mtshali et al., 2015; Khumalo et al., 2016).

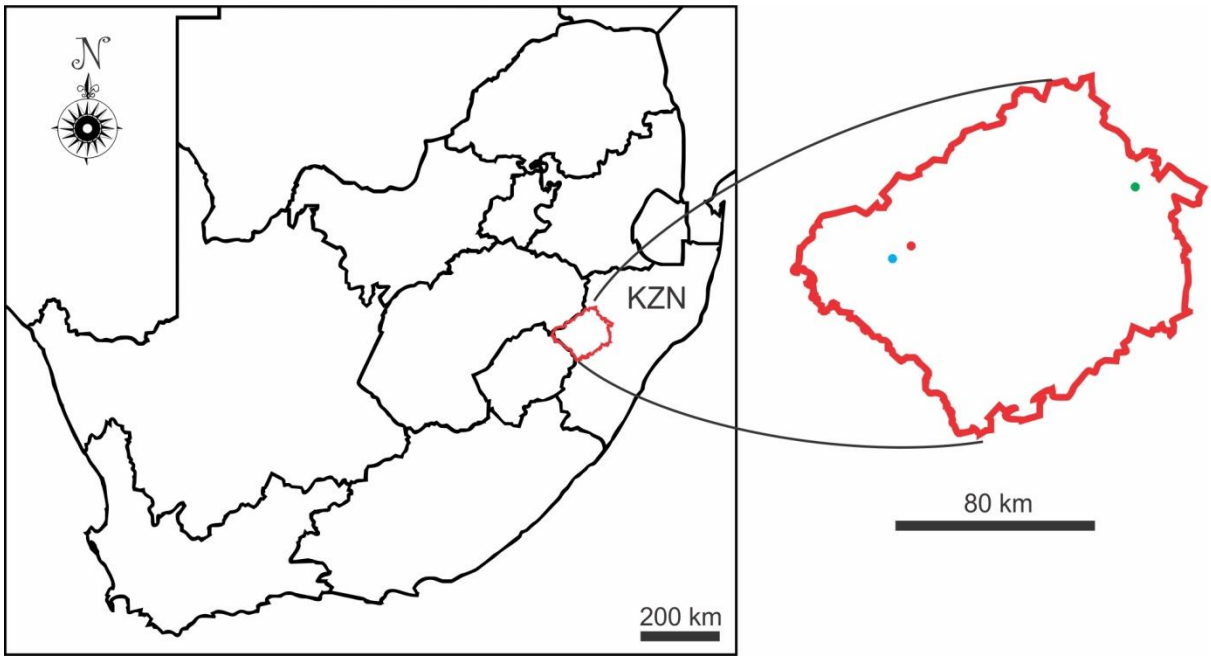

Figure 5.1 Map of South Africa showing the three dip tanks in the uThukela district, Kwa-Zulu Natal. The dip tanks are shown in coloured dots (Blue = Rookdale, Red = Woodford, Green = Mabhekazi). Rookdale and Woodford are dip tanks in Bergville, while Mabhekazi is a dip tank in Ladysmith.

5.3.3 Collection of ticks and blood samples from cattle

Sampling was based on availability of the cattle herds, the historical known distribution of tick species (i.e. *R. evertsi evertsi*, *Rhipicephalus* species, *Amblyomma hebraeum* and *R. decoloratus*) (Mtshali et al., 2015) and the occurrence of *Anaplasma* infections (Chapter 3). The cattle were at least one-year-old. During March 2015, a pilot study was done at the Woodford dip tank to establish which tick species were present in the area; ticks (n=113) were collected for species identification only. Subsequently, ticks were collected from cattle at the Woodford (June 2015 and February 2016) (n=164), Rookdale (February 2016) (n=111) and Mabhekazi (June 2015) (n=183) dip tanks. Cattle blood samples (n=109) were collected in June 2015 and February 2016 from the caudal vein into Vacutainer EDTA tubes. Ticks were collected from the ears, bodies, bellies, feet, with particular focus on the tail and tufts with the aim to specifically find *R. simus* ticks. To minimise stress, cattle were kept in a crush pen until the end of the tick collection. Tick collection focused mainly on adult stages, but nymphs were also collected where possible. Ticks were placed in labelled tick collection bottles with cotton wool and a drop of water, and then maintained in the bottles for 2 to 4 weeks for completion of blood meal digestion. Ticks were identified to species level under a dissecting microscope according to Walker et al. (2003). Prof Luis Neves, Department of Veterinary Tropical Diseases, University of Pretoria, South Africa provided technical assistance during tick identification.

5.3.4 DNA extraction

Ticks were grouped according to species in groups of 10 per sample. These samples of 10 ticks were grouped according to which cattle they infested, and thereafter symmetrically dissected ventrally to collect the salivary glands and midguts. In total there were 51 pairs of salivary glands and midgut samples. The tick salivary gland or midgut samples were pooled according to groups of five cattle they infested [i.e. Pools 4 (*R. appendiculatus*) and 5 (*R. evertsi evertsi*) came from ticks that infested cattle R17, R18, R19, R20 and R21]. The salivary gland and midgut pools were stored in PBS. The tick tissues were homogenised using a Tissue Lyser (Qiagen, USA). DNA was extracted from homogenised tick tissues using the QIAamp DNA Blood Mini Kit (Qiagen, USA), with slight changes as suggested by Crowder et al. (2010) and eluted in 100 µl elution buffer. Genomic DNA was extracted from blood samples of individual cattle using a QIAamp DNA Blood Mini Kit and was eluted in 100 µl elution buffer according to the manufacturer's instructions.

5.3.5 Duplex qPCR for simultaneous detection of A. marginale *and A.* centrale

Extracted DNA from cattle blood samples (n=109) and pooled tick tissue samples [(n=51); salivary gland and midgut pairs)] was analyzed using the duplex qPCR assay as reported by Decaro et al. (2008) for simultaneous detection of *A. marginale* (based on the *msp1β* gene) and *A. centrale* (based on the *groEL* gene), with minor modifications as described by Chaisi et al. (2017). Each PCR of 25 µl contained 0.32 U of FastStart Taqman mix (Roche Diagnostics), 1 U of UDG, 0.6 µM of *A. marginale* specific primers AM-For (5′-TTG GCA AGG CAG CAG CTT- 3′) and AM-Rev (5′-TTC CGC GAG CAT GTG CAT-3′), 0.9 µM of *A. centrale*-specific primers AC-For (5′-CTA TAC ACG CTT GCA TCT C-3′) and AC-Rev (5′-CGC TTT ATG ATG TTG ATG C-3′), 0.2 µM of probes AM-Pb (5′-6FAM-TCG GTC TTA ACA TCT CCA GGC TTT CAT-BHQ1-3′) and AC-Pb (5′-LC610-ATC ATC ATT CTT CCC CTT TAC CTC GT-BHQ2-3′) and 2.5 µl of template DNA (approximately 200 ng). DNA samples extracted from the *A. centrale* vaccine strain obtained from Onderstepoort Biological Products (Pretoria, South Africa) and field sample 9410 (obtained from Dr. Helena Steyn, Onderstepoort Veterinary Institute, Pretoria, South Africa) were used as positive controls for *A. centrale*. Field samples C14 and F48 (originating from bovines in the Mnisi Community area, Mpumalanga, South Africa) were used as positive controls for *A. marginale*. The positive control samples had low Cq values for *A. centrale* and *A. marginale*, and their sequences were confirmed by analysis of the *groEL* (for *A. centrale*) and *msp1b* (for *A. marginale*) genes (Chaisi et al., 2017). Nuclease free water was used as a negative control. Thermal cycling was performed in a LightCycler® version 4.1 (Roche Diagnostics, Mannheim, Germany), UDG was activated at 40°C for 10 min, followed by pre-incubation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 1 min and annealing-extension at 60°C for 1 min, and a final cooling step at 40°C for 30 sec. The results were analyzed using the Lightcycler Software version 4.0 (Roche Diagnostics, Mannheim, Germany) as described by Chaisi et al. (2017).

5.3.6 PCR amplification of the msp1aS *gene*

Based on the *A. centrale*-positive duplex qPCR results, the *msp1aS* gene was amplified from samples that had lower Cq values (tick DNA samples corresponding with the cattle DNA samples) using primers MSP1asFZ (5'-CAA GGT CAA GAG TCA GCA TCA TCA GAT G-3') and MSP1asRZ (5'-CTC CGC GCA CAA TAC TTT CAA CCT CC-3') (Chapter 3). This primer pair targets repeat structures of the gene and are useful for genotyping of *A. centrale*. The PCR consisted of 1x (final concentration) Phusion™ Flash High-Fidelity PCR Master Mix

(includes Phusion Flash II DNA Polymerase, reaction buffer, dNTPs, and $MgCl_2$) (Thermo Scientific™), 0.5 µM of each primer, 2.5 µl of DNA and nuclease free water to a total volume of 25 µl. DNA samples extracted from the *A. centrale* vaccine strain obtained from the Onderstepoort Biological Products and sample 9410 (obtained from Dr. Helena Steyn, Onderstepoort Veterinary Institute, Pretoria, South Africa) were used as positive controls for *A. centrale*. Nuclease free water was used as a negative control. The amplification cycles, following an initial denaturation of 98°C for 10 sec, consisted of 40 cycles of 98°C for 1 sec, 67°C for 5 sec and 72°C for 18 sec, followed by a final extension step at 72°C for 1 min. The PCR products were analyzed by electrophoresis on a 2.0% TAE agarose gel (stained with ethidium bromide) and visualised under UV light, using a 1 kb ladder as a DNA size marker (Thermo Scientific™).

5.3.7 Cloning and sequencing of PCR products

The *msp1aS* amplicons from two tick DNA sample pools (Pool 4 & Pool 5) and five DNA samples of cattle (R17, R18, R19, R20 and R21) were subsequently cloned and sequenced. The PCR products from quadruplicate reactions of each sample were pooled, to obtain a total volume of 100 µl. The amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN), eluted in 10 µl elution buffer, ligated into the pJET vector (CloneJET® PCR Cloning Kit, Thermo Scientific™) and transformed into *E. coli* JM 109 High Efficiency Competent cells (Promega, Madison, USA), according to the manufacturers' instructions. At least 10 colonies per sample were picked and screened by colony PCR using primers pJET 1.2F (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJET 1.2R (5'-GAA GAA CAT CGA TTT TCC ATG GCA G-3'). The colony PCR mixture contained 0.2 µM of each primer, 1x (final concentration) DreamTaq Green PCR Master Mix (Thermo Fisher Scientific™) (containing DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, 4 mM $MgCl_2$ and nuclease-free water), one colony as template and nuclease free water to a total volume of 20 µl. The amplification cycle, following an initial denaturation of 95°C for 3 min, consisted of 25 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by a final extension step at 72°C for 7 min. Recombinant plasmids were isolated using a High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany) and sequenced using 1 µl of 2 µM pJET1.2 primers with ABI Big Dye V3.1 Kit on an ABI 3500XL genetic analyzer at Inqaba Biotec (South Africa).

5.3.8 Sequence analysis

Sequence data was analyzed and assembled using CLC Genomics Workbench version 7.5.1 (CLC Bio, Qiagen, MA, USA). Consensus sequences were obtained from 37 clones, these clones corresponded to 9 *msp1aS* sequences. The Basic Local Alignment Search Tool (BLAST) was used to search for homologous sequences from GenBank using BLASTn (Altschul, et al., 1990). The Tandem Repeat finder programme (Benson, 1999) was used to identify the number of repeats in each consensus sequence. The repeats were then translated to amino acids using the CLC Genomics Workbench version 7.5.1 software. The translated repeats were analyzed using RepeatAnalyzer (Catanese et al., 2016). Repeat sequences were aligned using the AlignX module of Vector NTI (Invitrogen).

5.4 Results

5.4.1 Identification of tick species

During the pilot study, 113 ticks were collected at the Woodford dip tank and identified as *R. microplus* (n=98; 86.73%), *R. evertsi evertsi* (n=5; 4.42%) and *R. appendiculatus* (n=10; 8.85%) (Appendix 6). Thereafter, a total of 458 ticks belonging to four species, *R. evertsi evertsi*, *R. appendiculatus*, *R. microplus* and *H. rufipes* were collected from 109 cattle at three dip tanks (Woodford, Rookdale and Mabhekazi) (Table 5.1). Collections took place at the Woodford and Mabhekazi dip tanks in June 2015, and at Woodford and Rookdale dip tanks in February 2016. No *R. microplus* was found during the collection in February 2016. Overall, the highest number of ticks collected was *R. evertsi evertsi* (n=229; 50%) (Table 5.1). The lowest proportion of ticks collected was *H. rufipes* (n=4; 0.9%). The Rookdale dip tank had the highest number of ticks collected throughout the study (n=183) (Table 5.1).

Table 5.1 Number and proportion of four tick species collected from cattle at three dip tanks in KwaZulu-Natal, South Africa, in June 2015 and February 2016. (R. microplus (n=87) ticks were collected in June 2015 only).

Dip tanks	*R. evertsi evertsi*	*R. appendiculatus*	*R. microplus*	*H. rufipes*	TOTAL
Woodford (Jun 2015, Feb 2016)	127 (55.5%)	21 (15.2%)	16 (18.4%)	0 (0%)	**164**
Mabhekazi (Jun 2015)	0 (0%)	39 (28.3%)	71 (81.6%)	1 (25.0%)	**111**
Rookdale (Feb 2016)	102 (44.5%)	78 (56.5%)	0 (0%)	3 (75.0%)	**183**
TOTAL	**229 (50.0%)**	**138 (30.1%)**	**87(19.0%)**	**4 (0.9%)**	**458**

5.4.2 Duplex qPCR for simultaneous detection of A. marginale *and* A. centrale *in tick tissues and cattle DNA*

All of the cattle DNA from blood samples (n=109) analyzed by the qPCR assay tested positive for both *A. marginale* (100%) and *A. centrale* (100%). A high proportion of the tissues from *R. microplus*, *R. evertsi evertsi* and *R. appendiculatus*, ranging from 53 to 100%, tested positive for *A. marginale* and/or *A. centrale* (Figure 5.2). The tick species, *H. rufipes*, was not screened for presence of *Anaplasma* infections due to the low numbers collected. The midgut and salivary glands from the ticks mostly had mixed infections of *A. centrale* and *A. marginale*, ranging from 53 to 93%. The tick tissues from *R. microplus* revealed the highest mixed infection for *A. centrale* and *A. marginale* (midguts: 93%; salivary glands: 57%). Single infections for *A. centrale* (7%) and *A. marginale* (14%) were demonstrated only in the salivary glands and not in the midguts of *R. microplus*. The *R. evertsi evertsi* tick tissues revealed mixed infections with *A. centrale* and *A. marginale* (midguts: 80%: salivary glands: 55%). Single infections with *A. centrale* (15%) and *A. marginale* (5%) were demonstrated only in the midguts of *R. evertsi evertsi* and not in the salivary glands. There were fewer mixed infections from the tick tissues of *R. appendiculatus* (midgut: 53%; salivary gland: 53%), and no single infections (Figure 5.2).

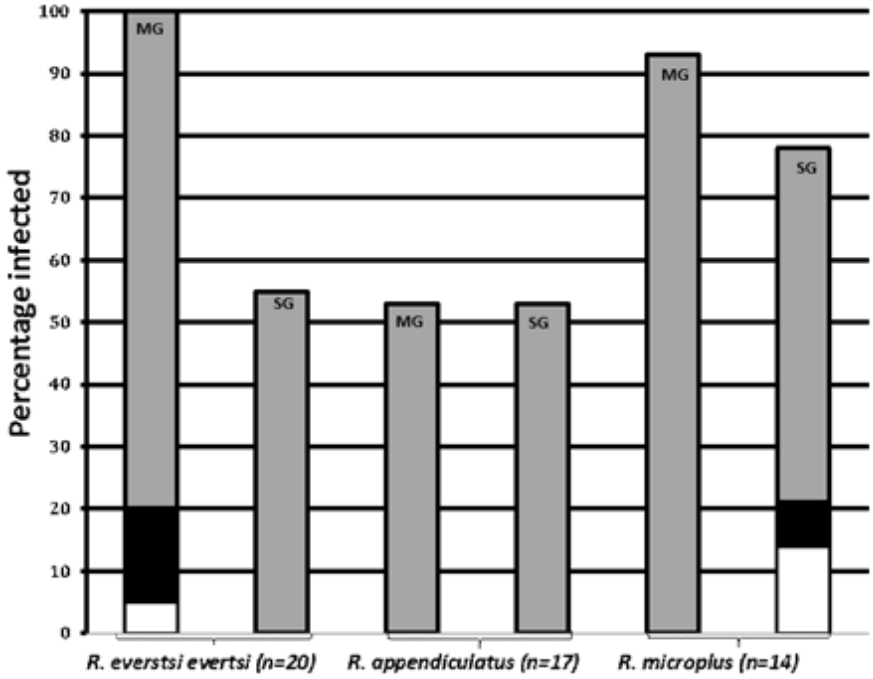

Figure 5.2 Stacked bar graphs showing occurrence of *Anaplasma* species in tick midguts (MG) and salivary glands (SG) as detected by duplex real-time PCR. The ticks were collected at three dip tanks in KwaZulu-Natal, South Africa in June 2015 and February 2016. The numbers in parenthesis represent the number of pools per tick species. Black indicates samples positive for *A. centrale*, gray indicates those with mixed infections, and white indicates those positive for *A. marginale*.

5.4.3 PCR amplification of msp1aS gene

The *msp1aS* PCR generated more than one amplicon with sizes of the sequences varying from 382–1 000 bp (Table 5.2). There was a presence of more than one *msp1aS* PCR product in some samples, indicating the presence of multiple *A. centrale* strains infecting individual cattle or ticks. Out of the 109 blood samples from cattle and 51 tick samples, three DNA samples (R18, R19 and R21) from cattle blood, one DNA sample pool of *R. appendiculatus* tick pools (MG4 and SG4) and one DNA sample pool of *R. evertsi evertsi* tick pools (MG5 and SG5) successfully yielded clones which were subsequently sequenced.

5.4.4 **Anaplasma centrale** ***msp1aS tandem repeats***

The 37 clones analyzed yielded 10 *msp1aS* genotype sequences. The *msp1aS* genotypes of *A. centrale* found in this study corresponded to various combinations of Msp1aS tandem repeats as shown in Table 5.2. The *msp1aS* genotypes of *A. centrale* were defined following the nomenclature by Catanese et al. (2016). Five *A. centrale* Msp1aS tandem repeats were found in this study; Ac1, Ac2, Ac8, Ac20 and Ac48. Of these, one new tandem repeat was described: Ac48 (Figure 5.3).

Figure 5.3 Alignment of amino acid sequences of a new Msp1aS tandem repeat (Ac48) found in *A. centrale* isolates from tick samples collected from uThukela district, KwaZulu-Natal province, South Africa. Amino acids shown in black font on a clear background depict the differences found in Msp1aS repeats. * indicates the repeat forms Ac1 and Ac2 that are in the vaccine strain and are used as a model against which to compare the new repeat.

Twelve clones from cattle R18 and R19 contained the *msp1aS* genotype Ac1 Ac1 Ac1, with an amplicon size of 686 bp. Eight clones from bovine R21 revealed a mixed *msp1aS* genotype in this animal, each with a single repeat, Ac20 (382 bp) or Ac8 (535 bp). Ten clones from the *R. evertsi evertsi* DNA sample contained multiple *msp1aS* genotypes with different combination of repeats; the genotype Ac1 Ac1 Ac2 occurred in the midguts and salivary glands of this tick, with an amplicon size of 667 bp. The genotype Ac1 Ac2 was detected only in the salivary gland, with an amplicon of 526 bp. The genotypes Ac1 (385 bp), Ac8 (526 bp) and Ac20 (382 bp) each were detected once in the midgut or salivary gland. Seven clones from the *R. appendiculatus* DNA samples contained two *msp1aS* genotypes: Ac1 Ac48 with an amplicon size of 526 bp and Ac2 with an amplicon size of 383 bp. Other *msp1aS* genotypes were not detected in the *R. appendiculatus* pools, however this may be due to low numbers of sequences. The Ac8 and Ac20 *msp1aS* genotypes were found to be common between bovine R21 and the *R. evertsi evertsi* tick pool from this animal, although the Ac8 sequences in bovine R21 and in the tick pool differed in size by 9 bp. This was due to a 9 bp nucleotide deletion in the 5’ region flanking the repeats in the sequence from the pooled ticks.

Table 5.2 The *msp1aS* genotypes detected from the cattle and tick samples collected from uThukela district, KwaZulu-Natal province, South Africa.

Genotype	Country code	Province code	Year	Animal I.D	Clone number	Host species	Origin	Vaccine status	Size (bp)	Number of repeats
Ac1 Ac1 Ac1 Ac2	IL	M	2010	Genome sequence	CP001759	Cattle	Israel 2010	+	814	4
Ac1Ac1 Ac1	ZA	NL	2016	R_18	A	Cattle	Bergville	-	686	3
Ac1 Ac1 Ac1	ZA	NL	2016	R_19	B	Cattle	Bergville	-	686	3
Ac8	ZA	NL	2016	R_21	G	Cattle	Bergville	-	535	1
Ac20	ZA	NL	2016	R_21	C	Cattle	Bergville	-	382	1
Ac1 Ac48	ZA	NL	2016	MG4	1A	*R.appendiculatus*	Bergville	-	526	2
Ac2	ZA	NL	2016	SG4	1	*R.appendiculatus*	Bergville	-	383	1
Ac1 Ac48	ZA	NL	2016	SG4	2	*R.appendiculatus*	Bergville	-	526	2
Ac1	ZA	NL	2016	MG5	11	*R.evertsi evertsi*	Bergville	-	385	1
Ac20	ZA	NL	2016	MG5	9	*R.evertsi evertsi*	Bergville	-	382	1
Ac1 Ac1 Ac2	ZA	NL	2016	MG5	B1	*R.evertsi evertsi*	Bergville	-	667	3
Ac1 Ac2	ZA	NL	2016	SG5	7	*R.evertsi evertsi*	Bergville	-	526	2
Ac8	ZA	NL	2016	SG5	10	*R.evertsi evertsi*	Bergville	-	526	1
Ac1 Ac1 Ac2	ZA	NL	2016	SG5	A6	*R.evertsi evertsi*	Bergville	-	667	3

5.5 Discussion

This study provides information on the tick species present in cattle herds at the Woodford, Mabhekazi and Rookdale dip tanks in the uThukela district, KwaZulu-Natal, South Africa. The tick species found were also correlated with the presence of *A. centrale* and *A. marginale* infections in the cattle. Altogether four tick species were collected and identified; *Rhipicephalus evertsi evertsi*, *R. microplus*, *R. appendiculatus* and *Hyalomma rufipes.*

The highest number of a given species of ticks collected was *R. evertsi evertsi*; this was expected as previous studies have shown that it is the most prevalent tick species present in different parts of South Africa, including KwaZulu-Natal (Mbati et al., 2003; Spickett et al., 2011; Mtshali et al., 2015). This tick has also previously been shown to be widely distributed throughout sub-Saharan Africa (Walker, 2000). *Rhipicephalus appendiculatus* was also present in high numbers in the study area. It has previously been shown to be present in KwaZulu-Natal (Mtshali et al., 2015). In this study, *R. microplus* (19.0%) was present on cattle at the Woodford and Mabhekazi dip tanks in June 2015, but could not be detected during the collection at Woodford and Rookdale dip tanks in February 2016. *Rhipicephalus microplus* has reportedly become well-established in KwaZulu-Natal because of warm temperatures and high moisture (Mbati et al., 2003). The absence of this tick during the second collection may be attributed to the drought experienced during this time period (October 2015 to February 2016) in KwaZulu-Natal. The drought was so severe that animals started to die due to lack of forage;

also, cattle were not presented at the dip tanks for dipping to prevent injuries and/or death to immunocompromised animals. Our findings are in agreement with those of Norval et al. (1992), who reported the absence of *R. microplus* in Zimbabwe following the 1980 to 1983 drought.

The lowest number of ticks collected was for *H. rufipes*. This tick species is the most widely distributed species of the genus *Hyalomma* in South Africa (Norval & Horak 1994). In order to obtain more data about the distribution of this tick in this study area, collection should be carried out throughout the year.

Most salivary glands and midguts from *R. evertsi evertsi*, *R. microplus* and *R. appendiculatus* revealed mixed infections of *A. marginale* and *A. centrale*. *Rhipicephalus appendiculatus* is not known to transmit *A. marginale* or *A. centrale*, while *R. evertsi evertsi* and *R. microplus* ticks are not known to transmit *A. centrale*. At this stage, we cannot confirm the vectorial capacity and/or competence to transmit *A. marginale* and/or *A. centrale* by the mere presence of *Anaplasma* infections. However, *msp1aS* genotyping established the presence of *A. centrale* infection in midguts and salivary glands of *R. appendiculatus* and *R. evertsi evertsi*, moreover, the presence of common *msp1aS* genotypes between bovine R21 and *R. evertsi evertsi* is suggestive that this tick is a transmission vector. A study conducted by Futse et al. (2003), demonstrated successful transmission of *A. marginale* Puerto Rico and St. Maries strains by *R. microplus* and *D. andersoni*, the strain identities in the infected cattle were confirmed using *msp1α* genotyping.

Based on the work done by Ueti et al. (2009), *A. centrale* is capable of infecting *D. andersoni* ticks at rates similar to *A. marginale*. However, it was not transmitted in a similar manner. These authors revealed that *A. centrale* resided in a different subcellular location within the salivary gland and was not secreted into the saliva at rates equivalent to *A. marginale*. When the numbers of ticks were increased to compensate for the lower amount of organism in the saliva, transmission was achieved. These experiments were conducted with the Israel vaccine strain, which has been removed from "the wild" for >100 years, and it is unknown if this strain has lost the ability to be transmitted, or if all *A. centrale* would behave in the same manner. Therefore, two possible scenarios exist: 1) current circulating strains of *A. centrale* are capable of being transmitted by one or more tick species, and we have not tested with the right combination of tick and strain of *A. centrale*; or 2) all *A. centrale* strains behave like the tested

vaccine strain, and when tick burden is high, inefficient vectors can transmit this organism, similar to the experimental transmission effected by Ueti et al. (2009).

In regards to the latter scenario, it is possible that successful transmission of *A. centrale* infections may be attributed to the high tick numbers of *R. evertsi evertsi* and *R. appendiculatus* in the apparent absence of the implicated vector *R. simus*. In addition, it has been shown that it is possible to propagate *A. centrale* in tick cell lines of *R. appendiculatus* (Bell-Sayki et al., 2015). This is notable because culture was attempted in 25 cell lines, and only one cell line was able to support the growth of *A. centrale*.

Therefore, based on our findings there is a necessity to undertake a tick transmission study in cattle, to establish the vectorial capacity of these tick species. This will aid in a better understanding of the vector-pathogen-host interaction.

5.6 Conclusion

We determined and characterized *A. centrale msp1aS* genotypes in cattle blood samples and their associated ticks in an attempt to gain knowledge on the possible tick vector(s) of *A. centrale* that circulate in the cattle populations in uThukela district, South Africa. Salivary glands and midguts from *R. evertsi evertsi*, *R. microplus* and *R. appendiculatus* revealed mixed infections of *A. marginale* and *A. centrale*. We found common *msp1aS* genotypes (Ac8 and Ac20) in cattle blood and *R. evertsi evertsi* ticks; this may suggest that these ticks are responsible for *A. centrale* infections. In order to establish whether these ticks are indeed vectors of *A. centrale*, a tick transmission study must be undertaken to clearly demonstrate the *A. centrale* transmission cycle from tick to host.

5.7 References

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Chapter 6

6. General Discussion and Conclusions

This study was conducted to understand the occurrence of *Anaplasma* species, particularly *A. centrale*, in wildlife species in South Africa. In this study, we examined genetic diversity of *A. centrale* strains based on *msp1aS* genotyping and the phylogenetic position of *A. marginale* and *A. centrale* based on three genes (16S rRNA, *groEL* and *msp4*) assayed from wildlife species and cattle samples. The results obtained from this study are useful for determining the infection status of *Anaplasma* in wildlife and livestock, cataloguing *A. centrale* strains and inferring of phylogenetic relationships between *A. marginale* and *A. centrale*.

We used duplex real-time PCR for screening the occurrences of *A. marginale* and *A. centrale* infections in wildlife species (buffalo, black wildebeest, blue wildebeest, eland and waterbuck) and cattle samples. This was to determine the anaplasmosis status in these hosts; results indicated that *A. centrale* single infections are most common among black wildebeest, blue wildebeest, waterbuck and eland while *A. marginale* single infections are common in buffalo, eland, waterbuck and cattle samples; co-infections with both spp. were common in cattle and in buffalo.

Previous studies have shown seroprevalence of *Anaplasma* species in eland and blue wildebeest from Kenya (Ngeranwa et al., 2008) using cELISA. A study conducted by Eygelaar et al. (2015), using a RLB assay, showed that *Anaplasma* species infections are prevalent in buffalo in northern Botswana with *A. centrale* (30%) being the most prevalent. Most recently, Sisson et al. (2017), using species-specific PCR markers, found a similar prevalence of *A. centrale* (28.2%) and *A. marginale* (32.4%) in African buffalo samples collected from KNP, with 17.3 % single *A. marginale* infections, 13.1% single *A.centrale* infections, and 15.1 % of samples being co-infected with both species. The duplex qPCR assay used in our study also revealed a large proportion of mixed infections of *A. centrale* and *A. marginale* in buffalo from KNP (28%), although we found a higher prevalence of *A. marginale* single infections (37%) and no *A. centrale* single infections. We also observed high numbers of mixed infections (28-85%) in buffalo samples from other national parks in South Africa which correlates with other

studies. For example, in East Africa, a study done in four national parks in Uganda found buffalo to be co-infected with *A. marginale* and *A. centrale* (22 to 84%) (Oura et al., 2011).

Anaplasmosis is endemic in South Africa (Dreyer et al., 1998; De Waal, 2000; Rikhotso et al., 2005; Mtshali et al., 2007; Ndou et al., 2010, Spickett et al., 2011; Mutshembele et al., 2014), although the Northern Cape is considered free of this disease due to the absence of the tick vector (De Waal, 2000). Buffalo from Eastern Cape are considered "disease-free" due to absence of foot-and-mouth, brucellosis, bovine tuberculosis and Corridor disease (Smith & Parker, 2010); however, they are not free from anaplasmosis as buffalo samples from Eastern Cape National Parks (Addo Elephant National Park and Camdeboo National Park) were revealed to be carriers of *A. marginale* and *A. centrale* infections. Wildlife species serve as reservoir hosts for many of the tick-borne diseases (Sibeko et al., 2008; Chaisi et al., 2011; Debeila, 2012; Eygelaar et al., 2015), therefore the presence of *Anaplasma* infections in wildlife species pose a threat for anaplasmosis outbreaks at the wildlife/livestock interface. According to Jori & Etter (2016), interactions between cattle and buffalo are more likely to occur inside the KNP when cattle herds gather to share water sources with buffalo herds than when small groups of buffalo escape from the KNP.

Susceptibility of wildlife species to anaplasmosis is not well documented except in blesbok which were shown to be susceptible to *A. centrale* infections (Neitz & Du Toit, 1932). On the other hand, it is well known that cattle may die of anaplasmosis, depending on the virulence of the *A. marginale* strain and the age of the cattle (Kocan et al., 2003). Given that *A. centrale* is used as a vaccine against virulent *A. marginale* infection, finding this organism, albeit a different strain, naturally co-infected with the pathogen, begs the question of a natural cycle of infection with *A. centrale* that could mitigate *A. marginale* disease severity and contribute to endemic stability. Therefore, it would be interesting to show whether natural infection with "wild-type" *A. centrale* provides protection against *A. marginale*. Furthermore, identification of potential virulence genes associated with *A. centrale* may aid in understanding the *A. centrale*-associated anaplasmosis outbreak in Italy (Carelli et al., 2008). Given the diversity of *A. centrale* strains identified, it may even be possible that *A. centrale* strains are responsible for clinical cases of anaplasmosis in some instances in South Africa.

The taxonomic status of *A. centrale* has caused confusion in the past years: this organism is interchangeably referred to as a subspecies of *A. marginale* or a species closely related to *A.*

marginale. The taxonomic uncertainty was perpetuated by the erroneous designation of *A. centrale* as a separate species by Ristic (1968) without a formal species description. Initially, Theiler (1911) had described it as *Anaplasma marginale* variety *centrale.* Moreover, the lack of molecularly characterized *A. centrale* strains with corresponding genome sequences has contributed to this uncertainty. In this study, we have shown that *A. centrale* has a gene, *msp1aS*, that is a homolog of *msp1α* of *A. marginale*. The gene, *msp1aS*, encodes Msp1aS which has similar structural features to Msp1a of *A. marginale*. The *msp1aS* gene contains repeats at the 5' end that vary both in sequence and in number. In this study, we showed that the gene is useful as a genetic marker for genotyping *A. centrale* strains and is a valuable tool in determination of genetic diversity of *A. centrale* strains. Our results revealed that there is high genetic diversity of *A. centrale* strains in South Africa. Unfortunately, our results were limited to geographic areas within South Africa; it will be interesting to learn about *A. centrale* genetic diversity on a global scale. We can only speculate at this time that Msp1aS is a good phylogeographic marker, due to the high degree of sequence variation, as has been shown for Msp1a (de la Fuente et al., 2005). *Anaplasma marginale* Msp1a is involved in adhesion to bovine erythrocytes and tick cells (Kocan et al., 2010), and it is tempting to speculate that Msp1aS may function in the same manner, but no functional assays have been conducted to determine the function of Msp1aS. Another possibility is that Msp1aS could be involved in tick transmission of *A. centrale*, however, this would need to be addressed in future experiments.

For many years, *A. centrale* was thought to lack a homolog of *A. marginale* Msp1a, however, the genome sequence revealed a divergent gene residing in a syntenic position to *A. marginale msp1α.* The genome sequence highlighted key dissimilarities such as that detected for *msp1aS* and *msp1α* and indicated divergence between *A. marginale* and *A. centrale*. In order to further explore the divergence of the two organisms a comparative phylogenetic analysis was performed for sequences of three conservative genes (16S rRNA, *groEL* and *msp4*) of *A. marginale* and *A. centrale* obtained from cattle, buffalo, and black wildebeest DNA samples originating from different geographical areas in South Africa. Phylogenetic analysis was employed to resolve the appropriate taxonomic position of *A. centrale*. The phylogenetic classification based on the sequences of three genes of the two organisms, revealed two distinct clades for *A. centrale* and *A. marginale*. These findings suggested that *A. centrale* is a separate species from *A. marginale.* Further comparisons, including the differences in genome architecture, *msp1α*/*msp1aS* gene sequence, and the biology of tick transmissibility, provided

additional evidence for divergence between *A. centrale* and *A. marginale*. We amended the description of *A. centrale* as a separate species based on the above comparisons. With this clarification of the taxonomic standing, the correct description will be adopted in the literature.

It has been experimentally proven through a transmission study that *R. simus* ticks are capable of transmitting *A. centrale* in South Africa (Potgieter & van Rensburg, 1987). The presence of high rates of *A. centrale* infections in unvaccinated cattle and wildlife in this study clearly indicates that there must be a vector responsible for transmitting this pathogen. We attempted three collections of tick samples seeking to identify *R. simus*, however, no *R. simus* ticks were found in uThukela district during these collection periods. This therefore necessitated the investigation of other tick species in the uThukela district, as they could be responsible for transmission of *A. centrale* infections. Amongst the four tick species present in this area, *R. evertsi evertsi* and *R. appendiculatus* were the most prevalent. We examined the *msp1aS* genotypes from cattle and ticks that were positive for *A. centrale* infection. One new tandem repeat was identified. Two *msp1aS* genotypes were found in common in cattle samples and in samples of *R. evertsi evertsi* infesting those cattle. The presence of *A. centrale* infections and common *msp1aS* genotypes suggests a high probability that these ticks may be vectors of *A. centrale*. However, at this stage we can only speculate that these ticks are responsible for transmission of *A. centrale*. In order to confirm a vector, a tick-transmission study is necessary. Tick-transmission studies unambiguously demonstrate: 1) the ability of a tick to acquire *A. centrale* from persistently infected cattle with known level of parasitaemia during tick-feeding; 2) successful invasion of the tick midgut epithelium and replication of *A. centrale* in the midgut epithelial cells and invasion of the salivary glands, 3) formation of the dense infective form of the *A. centrale* organism in the salivary glands, and 4) the dose level of parasitaemia that is successfully transmitted by a tick to the cattle during feeding.

All these factors are determinants of vectoral capacity; interestingly the pathogen must also be adaptive to the tick-specific physiological and behavioral pattern and distinctively adaptive to biological processes pertaining during feeding of blood meal to molting of the tick (Liu & Bonnet, 2014). This demonstrates good vector-pathogen adaptability. The transmissibility depends on the level of parasitaemia (Futse et al., 2003).

A study by Futse et al (2003), demonstrated transmission of *A. marginale* (Puerto Rico and St. Maries strains) by *R. microplus* and *D. andersoni*. These ticks were acquisition-fed on

persistently infected cattle to examine their ability to acquire these *A. marginale* strains, and thereafter, these ticks were transmission-fed on naïve cattle to examine their ability to transmit the *A. marginale* strains. Approximately 90% of *R. microplus* and *D. andersoni* ticks were infected with *A. marginale* strains within the salivary gland. These ticks successfully transmitted both the Puerto Rico and St. Maries strains to the naïve cattle and the transmitted strain identity was confirmed based on the *msp1α* genotype. Like our study, this study employed *msp1α* genotyping of infected cattle and infected tick salivary glands to verify strain identity. A major difference being the experimental and the natural conditions under which the two studies were undertaken. In our study, the *A. centrale* strain identity between tick species and the host was not well represented, and the reason may be the natural conditions and low sampling density of the cattle. In contrast, the evidence of infected salivary glands and at least two similar *msp1aS* genotypes that were found to be common in the tick salivary glands and the cattle, suggests that *msp1α* and *msp1aS* genes are powerful genotyping tools that may shed a light in the tick-pathogen-host interaction.

In conclusion, this study contributes to the knowledge of anaplasmosis epidemiology in wildlife species in African countries. The results described here highlight that buffalo, black wildebeest, blue wildebeest, eland and waterbuck may be contributing factors to be considered in the epidemiology and spread of anaplasmosis. The *msp1aS* genotyping tool developed here indicates that wildlife and cattle are infected with *A. centrale* strains that are different from the vaccine strain, suggesting a natural infection cycle. This study contributes greatly to the description and the taxonomic status of *A. centrale.*

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Appendices

Appendix 1. Accession numbers for GenBank reference sequences used in Chapter 4.

Accession number	Species	Origin/Strain	Reference	Host
16SrRNA				
AB211163	*A. bovis*	Japan	Kawahara et al., 2006	Deer
HM131218	*A. bovis*	Japan	Sakamoto et al., 2010	Dog
AF283007	*A. capra* (previously *A. centrale* Aomori)	Japan	Inokuma et al., 2001	Cattle
KX417207	*A. capra*	China	Sun et al., 2015	Tick
KX417195	*A. capra*	China	Yang et al., 2016	Sheep
KX417207	*A. capra*	China	Yang et al., 2016	Sheep
KY007144	*A. capra*	China	Zhuang et al., 2017	Tick
AF318944	*A. centrale*	South Africa	Bekker et al., 2002	Vaccine Onderstepoort
AF414868	*A. centrale*	South Africa	Lew et al., 2003	Vaccine Onderstepoort
AF414869	*A. centrale*	South Africa L strain	Lew et al., 2003	Tick
CP001759	*A. centrale*	Israel strain	Herndon et al., 2010	Cattle
EF520688	*A. centrale*	Italy	Ceci at al., 2008	Cattle
EF520690	*A. centrale*	Italy	Ceci at al., 2008	Cattle
KC189841	*A. centrale*	South Africa	Debeila, 2012	Buffalo
KU686784	*A. centrale*	Uganda	Byaruhanga et al., 2016	Cattle
AF414871	*A. marginale*	South Africa	Lew et al., 2003	Black wildebeest
AF414878	*A. marginale*	Zimbabwe	Lew et al., 2003	Not known
CP000030	*A. marginale*	St. Maries	Brayton et al., 2005	Cattle
CP001079	*A. marginale*	Florida	Dark et al., 2008	Cattle
DQ341369	*A. marginale*	China	Du & Zhao., 2006	Buffalo
AF414870	*A. ovis*	South Africa	Lew et al., 2003	Sheep
EF587237	*A. ovis*	China	Mo et al., 2007	Sheep
AB196720	*A. phagocytophilum*	Japan	Kawahara et al ., 2006	Deer
KC470064	*A. phagocytophilum*	China	Zhao et al., 2013	Rat
AY077619	*A. platys*	Japan	Inokuma et al., 2002	Dog
EF139459	*A. platys*	Thailand	Pinyoowong et al., 2008	Dog
U11021	*Rickettsia rickettsii*	USA	Stothard et al., 1994	Tick
GroEL				
AF414866	*A. centrale*	South Africa L strain	Lew et al., 2003	Tick
CP001759	*A. centrale*	Israel strain	Herndon et al., 2010	Cattle
EF520694	*A. centrale*	Italy	Ceci et al., 2008	Cattle
AF414860	*A. marginale*	Australia	Lew et al., 2003	Cattle
CP000030	*A. marginale*	St. Maries	Brayton et al., 2005	Cattle
FJ226455	*A. marginale*	Japan	Ooshiro et al., 2009	Cattle
AF441131	*A. ovis*	South Africa	Lew et al., 2003	Goat
FJ460441	*A. ovis*	Cyprus	Psaroulaki et al., 2009	Goat
AF482760	*A. phagocytophilum*	Germany	Von Loewenich et al., 2003	Horse
KJ677107	*A. phagocytophilum*	South Korea	Kim et al., 2014	Human
AF478129	*A. platys*	DRC	Sanogo et al., 2016	Tick
AY077621	*A. platys*	Japan	Inokuma et al., 2002	Dog
AY050315	*Neorickettsia helminthoeca*	USA	Rikihisa et al., 2016	Dog
Msp4				
KR261641	*A. capra*	China	Sun & Yu, 2016	Tick
KX417357	*A. capra*	China	Yang et al., 2017	Sheep
CP001759	*A. centrale*	Israel strain	Herndon et al., 2010	Cattle
AY283194	*A. marginale*	Brazil	de la Fuente et al., 2004	Cattle

AY702922	*A. marginale*	Italy	de la Fuente et al., 2005	Tick
CP001079	*A. marginale*	Florida	Dark et al., 2008	Cattle
CP000030	*A. marginale*	St. Maries	Brayton et al., 2005	Cattle
EU436159	*A. marginale*	Italy	Torina et al., 2008	Cattle
HQ456348	*A. ovis*	China	Ma et al., 2011	Sheep
JQ663993	*A. ovis*	Iran	Yasini et al., 2012	Sheep
HQ661163	*A. phagocytophilum*	Slovakia	Derdáková et al., 2011	Sheep
KP861636	*A. phagocytophilum*	Spain	Alberdi et al., 2015	Tick
AF020068	*Wolbachia sp.*	USA	Braig et al., 1998	Fly

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Appendix 2. Accession numbers for the 16S rRNA gene, *groEL* and *msp4* gene sequences generated in the study (Chapter 4).

Accession number	Sample name	Species	Origin	Host	Genotype
16S rRNA					
KY287616	HiP_4_B	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac1
KY287615	HiP_3_B	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac2
KY287623	KNP_581_B	*A. centrale*	Kruger National Park	Buffalo	Ac2
KY287630	KNP_584_F	*A. centrale*	Kruger National Park	Buffalo	Ac4
KY287618	HiP_6_L	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac4
KY287614	HiP_2_A	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac4
KY287600	AEP_1007_2	*A. centrale*	Addo Elephant National Park	Buffalo	Ac4
KY287622	HiP_6_a	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac4
KY287605	AEP_1013_G	*A. centrale*	Addo Elephant National Park	Buffalo	Ac4
KY287624	KNP_581_C	*A. centrale*	Kruger National Park	Buffalo	Ac4
KY287627	KNP_584_A2	*A. centrale*	Kruger National Park	Buffalo	Ac4
KY287611	CNP_978_B	*A. centrale*	Camdeboo National Park	Buffalo	Ac4
KY287612	CNP_985_B	*A. centrale*	Camdeboo National Park	Buffalo	Ac4
KY287607	Berg_27_d	*A. centrale*	Bergville Farm 2	Cattle	Ac4
KY287636	MNP_999_2	*A. centrale*	Mokala National Park	Buffalo	Ac4
KY287619	HiP_6_L1	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac4
KY287613	CNP_976_A	*A. centrale*	Camdeboo National Park	Buffalo	Ac4
KY287606	Berg_27_a	*A. centrale*	Bergville Farm 2	Cattle	Ac3
KY287632	MNP_1000_4	*A. centrale*	Mokala National Park	Buffalo	Ac3
KY287626	KNP_584_A	*A. centrale*	Kruger National Park	Buffalo	Ac3
KY287621	HiP_6_Z	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac3
KY287620	HiP_6_p	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac3
KY287604	AEP_1013_F	*A. centrale*	Addo Elephant National Park	Buffalo	Ac3
KY287631	MNP_1000_1	*A. centrale*	Mokala National Park	Buffalo	Ac3
KY287671	HiP_6_aJan	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am3
KY287625	KNP_581_H	*A. marginale*	Kruger National Park	Buffalo	Am3
KY287629	KNP_584_C2	*A. marginale*	Kruger National Park	Buffalo	Am3
KY287628	KNP_584_C	*A. marginale*	Kruger National Park	Buffalo	Am3
KY287610	CNP_976_3	*A. marginale*	Camdeboo National Park	Buffalo	Am3
KY287609	CNP_976_1	*A. marginale*	Camdeboo National Park	Buffalo	Am3
KY287603	AEP_1013_A	*A. marginale*	Addo Elephant National Park	Buffalo	Am3
KY287602	AEP_1007_8	*A. marginale*	Addo Elephant National Park	Buffalo	Am3
KY287608	Berg_27_E	*A. marginale*	Bergville Farm 2	Cattle	Am3
KY287634	MNP_1021_1	*A. marginale*	Mokala National Park	Buffalo	Am3
KY287638	MNP_999_A	*A. marginale*	Mokala National Park	Buffalo	Am3
KY287639	MNP_999_D	*A. marginale*	Mokala National Park	Buffalo	Am3
KY287635	MNP_1021_2	*A. marginale*	Mokala National Park	Buffalo	Am3
KY287633	MNP_1000_A	*A. marginale*	Mokala National Park	Buffalo	Am3
KY287637	MNP_999_5_1	*A. marginale*	Mokala National Park	Buffalo	Am1
KY287601	AEP_1007_5	*A. marginale*	Addo Elephant National Park	Buffalo	Am2
GroEL					
KY305539	HiP_6_A	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac1
KY305547	HiP_3_A	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac1
KY305540	AEP_1013_C	*A. centrale*	Addo Elephant National Park	Buffalo	Ac1
KY305541	CNP_976_1	*A. centrale*	Camdeboo National Park	Buffalo	Ac1
KY305542	CNP_985_A2	*A. centrale*	Camdeboo National Park	Buffalo	Ac1
KY305543	AEP_1013_D	*A. centrale*	Addo Elephant National Park	Buffalo	Ac1
KY305544	AEP_1003_B	*A. centrale*	Addo Elephant National Park	Buffalo	Ac1
KY305553	AEP_1007_1	*A. centrale*	Addo Elephant National Park	Buffalo	Ac1
KY305556	AEP_1003_E	*A. centrale*	Addo Elephant National Park	Buffalo	Ac1

KY305545	CNP_978_C	*A. centrale*	Camdeboo National Park	Buffalo	Ac1
KY305546	CNP_978_A	*A. centrale*	Camdeboo National Park	Buffalo	Ac1
KY305550	CNP_978_B	*A. centrale*	Camdeboo National Park	Buffalo	Ac1
KY305548	CNP_985_A	*A. centrale*	Camdeboo National Park	Buffalo	Ac1
KY305549	CNP_985_C	*A. centrale*	Camdeboo National Park	Buffalo	Ac1
KY305551	KNP_584_A	*A. centrale*	Kruger National Park	Buffalo	Ac1
KY305557	KNP_581_B	*A. centrale*	Kruger National Park	Buffalo	Ac1
KY305560	KNP_581_A	*A. centrale*	Kruger National Park	Buffalo	Ac1
KY305552	MNP_1000_1	*A. centrale*	Mokala National Park	Buffalo	Ac1
KY305555	MNP_999_3	*A. centrale*	Mokala National Park	Buffalo	Ac1
KY305554	Berg_27_C	*A. centrale*	Bergville Farm 2	Cattle	Ac1
KY305559	Berg_27_A	*A. centrale*	Bergville Farm 2	Cattle	Ac1
KY305558	Berg_27_B	*A. centrale*	Bergville Farm 2	Cattle	Ac1
KY305561	CNP_976_2	*A. marginale*	Camdeboo National Park	Buffalo	Am1
KY305562	AEP_1007_3	*A. marginale*	Addo Elephant National Park	Buffalo	Am1
KY305563	MNP_1021_3	*A. marginale*	Mokala National Park	Buffalo	Am2
KY305564	MNP_1021_2	*A. marginale*	Mokala National Park	Buffalo	Am2
KY305565	MNP_1000_4	*A. marginale*	Mokala National Park	Buffalo	Am2
KY305567	MNP_1000_5	*A. marginale*	Mokala National Park	Buffalo	Am2
KY305568	KNP_582_A	*A. marginale*	Kruger National Park	Buffalo	Am2
KY305569	Berg_27_F	*A. marginale*	Bergville Farm 2	Cattle	Am2
KY305570	Berg_27_Z	*A. marginale*	Bergville Farm 2	Cattle	Am2
KY305571	KNP_582_A1	*A. marginale*	Kruger National Park	Buffalo	Am2
KY305572	HiP_6_D	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am2
KY305573	HiP_7_C	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am2
KY305574	MNP_958_C	*A. marginale*	Mokala National Park	Buffalo	Am2
KY305575	MNP_999_1	*A. marginale*	Mokala National Park	Buffalo	Am2
KY305576	MNP_999_4	*A. marginale*	Mokala National Park	Buffalo	Am2
KY305566	MNP_1000_2	*A. marginale*	Mokala National Park	Buffalo	Am2
KY305577	HiP_2_A	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am2
KY305578	KNP_584_2A2	*A. marginale*	Kruger National Park	Buffalo	Am2
KY305579	HiP_3_B	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am2
KY305580	AEP_1002_E	*A. marginale*	Addo Elephant National Park	Buffalo	Am2
KY305581	AEP_1002_A	*A. marginale*	Addo Elephant National Park	Buffalo	Am2
KY305582	HiP_4_A	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am2
KY305583	HiP_2_D	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am2
Msp4					
KY305601	CNP_976AC2	*A. centrale*	Camdeboo National Park	Buffalo	Ac4
KY305603	CNP_979AC2	*A. centrale*	Camdeboo National Park	Buffalo	Ac4
KY305602	CNP_978AC2	*A. centrale*	Camdeboo National Park	Buffalo	Ac4
KY305604	Berg_25AC2	*A. centrale*	Bergville Farm 2	Cattle	Ac4
KY305605	Berg_19AC2	*A. centrale*	Bergville Farm 1	Cattle	Ac4
KY305606	Berg_27AC2	*A. centrale*	Bergville Farm 1	Cattle	Ac4
KY305607	HiP_3AC	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac4
KY305608	HiP_7AC	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac4
KY305609	MNP_1000AC	*A. centrale*	Mokala National Park	Buffalo	Ac4
KY305610	HiP_4AC	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac4
KY305611	HiP_2AC	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac4
KY305612	CNP_976AC	*A. centrale*	Camdeboo National Park	Buffalo	Ac4
KY305613	AEP_1007AC	*A. centrale*	Addo Elephant National Park	Buffalo	Ac4
KY305614	HiP_6AC	*A. centrale*	Hluhluwe iMfolozi Park	Buffalo	Ac4
KY305615	CNP_978AC	*A. centrale*	Camdeboo National Park	Buffalo	Ac4
KY305616	KNP_581AC	*A. centrale*	Kruger National Park	Buffalo	Ac1
KY305617	Berg_27AC	*A. centrale*	Bergville Farm 2	Cattle	Ac3
KY305618	Berg_25AC	*A. centrale*	Bergville Farm 2	Cattle	Ac3
KY305619	AEP_999AC	*A. centrale*	Addo Elephant National Park	Buffalo	Ac3
KY305620	KNP_584AC	*A. centrale*	Kruger National Park	Buffalo	Ac3
KY305612	Berg_19AC	*A. centrale*	Bergville Farm 1	Cattle	Ac2

KY305590	KNP_581_AM	*A. marginale*	Kruger National Park	Buffalo	Am3
KY305584	HiP_17AM	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am3
KY305585	HiP_7AM	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am3
KY305595	HiP_6AM	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am3
KY305586	Berg_27AM	*A. marginale*	Bergville Farm 2	Cattle	Am3
KY305587	KNP_584AM	*A. marginale*	Kruger National Park	Buffalo	Am3
KY305588	MNP_999AM	*A. marginale*	Mokala National Park	Buffalo	Am3
KY305589	HiP_2AM	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am3
KY305591	HiP_10AM	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am3
KY305592	MNP_1000AM	*A. marginale*	Mokala National Park	Buffalo	Am3
KY305593	MNP_1021AM	*A. marginale*	Mokala National Park	Buffalo	Am3
KY305596	AEP_1007AM	*A. marginale*	Addo Elephant Park	Buffalo	Am3
KY305594	HiP_13AM	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am3
KY305597	HiP_6REPAM	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am3
KY305597	KNP_582AM	*A. marginale*	Kruger National Park	Buffalo	Am2
KY305599	HiP_5AM	*A. marginale*	Hluhluwe iMfolozi Park	Buffalo	Am2
KY305600	CNP_976AM	*A. marginale*	Camdeboo National Park	Buffalo	Am1

Appendix 3. Estimates of evolutionary divergence between 16S rRNA gene sequences (Chapter 4). The number of nucleotide differences between selected sequences is shown. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1105 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*A. marginale_StMaries_CP000030*																							
2	*A. marginale_Florida_NR074556*	1.0																						
3	*A. marginale_China_DQ341369*	0.0	1.0																					
4	*A. marginale_SA_BW_AF414871*	1.0	2.0	1.0																				
5	*A. marginale_Zimbabwe_AF414878*	1.0	2.0	1.0	2.0																			
6	**Am2**	**2.0**	**3.0**	**2.0**	**3.0**	**3.0**																		
7	**Am3**	**0.0**	**1.0**	**0.0**	**1.0**	**1.0**	**2.0**																	
8	**Am1**	**1.0**	**2.0**	**1.0**	**2.0**	**2.0**	**1.0**	**1.0**																
9	*A. centrale_Israel_CP001759*	4.0	5.0	4.0	5.0	5.0	2.0	4.0	3.0															
10	*A. centrale_SA_KC189841*	6.0	7.0	6.0	7.0	7.0	4.0	6.0	5.0	2.0														
11	**Ac4**	**4.0**	**5.0**	**4.0**	**5.0**	**5.0**	**2.0**	**4.0**	**3.0**	**0.0**	**2.0**													
12	**Ac3**	**3.0**	**4.0**	**3.0**	**4.0**	**4.0**	**3.0**	**3.0**	**4.0**	**1.0**	**3.0**	**1.0**												
13	**Ac1**	**4.0**	**5.0**	**4.0**	**5.0**	**5.0**	**4.0**	**4.0**	**5.0**	**2.0**	**4.0**	**2.0**	**1.0**											
14	**Ac2**	**5.0**	**6.0**	**5.0**	**6.0**	**6.0**	**3.0**	**5.0**	**4.0**	**1.0**	**3.0**	**1.0**	**2.0**	**3.0**										
15	*A. ovis_China_EF587237*	6.0	7.0	6.0	7.0	7.0	4.0	6.0	5.0	6.0	8.0	6.0	7.0	8.0	7.0									
16	*A. ovis_SA_AF414870*	4.0	5.0	4.0	5.0	5.0	4.0	4.0	5.0	6.0	8.0	6.0	5.0	6.0	7.0	2.0								
17	*A. phagocytophilum_China_KC470064*	27.0	28.0	27.0	28.0	28.0	27.0	27.0	28.0	29.0	31.0	29.0	28.0	29.0	30.0	31.0	29.0							
18	*A. phagocytophilum_Japan_AB196720*	37.0	38.0	37.0	38.0	38.0	35.0	37.0	36.0	37.0	39.0	37.0	38.0	39.0	38.0	37.0	39.0	16.0						
19	*A. bovis_Japan_AB211163*	41.0	42.0	41.0	42.0	42.0	39.0	41.0	40.0	39.0	41.0	39.0	40.0	41.0	40.0	40.0	42.0	28.0	31.0					
20	*A. bovis_Japan_HM131218*	43.0	44.0	43.0	44.0	44.0	41.0	43.0	42.0	41.0	43.0	41.0	42.0	43.0	42.0	42.0	44.0	30.0	33.0	5.0				
21	*A. platys_Japan_AY077619*	30.0	31.0	30.0	31.0	31.0	30.0	30.0	31.0	32.0	34.0	32.0	31.0	32.0	33.0	33.0	31.0	13.0	22.0	32.0	34.0			
22	*A. platys_Thailand_EF139459*	30.0	31.0	30.0	31.0	31.0	30.0	30.0	31.0	32.0	34.0	32.0	31.0	32.0	33.0	33.0	31.0	13.0	22.0	32.0	34.0	0.0		
23	*R. rickettsii _U11021*	168.0	169.0	168.0	169.0	169.0	170.0	168.0	169.0	172.0	174.0	172.0	171.0	172.0	173.0	171.0	170.0	171.0	168.0	179.0	181.0	176.0	176.0	

Appendix 4. Estimates of evolutionary divergence between GroEL amino acid sequences (Chapter 4). The number of amino acid differences between selected sequences is shown. The analysis involved 16 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 488 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1	*A. marginale_StMaries_CP000030*																
2	*A. marginale_Japan_FJ226455*	0.0															
3	*A. marginale_Australia_AF414860*	0.0	0.0														
4	**Am1**	**0.0**	**0.0**	**0.0**													
5	**Am2**	**3.0**	**3.0**	**3.0**	**3.0**												
6	*A. centrale_Israel_CP001759*	4.0	4.0	4.0	4.0	7.0											
7	*A. centrale_SA_AF414866*	5.0	5.0	5.0	5.0	8.0	1.0										
8	*A. centrale_Italy_EF520694*	6.0	6.0	6.0	6.0	9.0	2.0	1.0									
9	**Ac1**	**4.0**	**4.0**	**4.0**	**4.0**	**7.0**	**0.0**	**1.0**	**2.0**								
10	*A. ovis_SA_AF441131*	9.0	9.0	9.0	9.0	8.0	9.0	10.0	11.0	9.0							
11	*A. ovis_Cyrus_FJ460441*	47.0	47.0	47.0	47.0	46.0	46.0	47.0	48.0	46.0	41.0						
12	*A. phagocytophilum_Germany_AF482760*	38.0	38.0	38.0	38.0	37.0	39.0	38.0	39.0	39.0	37.0	70.0					
13	*A. phagocytophilum_SKorea_KJ677107*	40.0	40.0	40.0	40.0	39.0	41.0	40.0	41.0	41.0	39.0	72.0	2.0				
14	*A. platys_Congo_AF478129*	38.0	38.0	38.0	38.0	39.0	38.0	37.0	38.0	38.0	38.0	72.0	23.0	25.0			
15	*A. platys_Japan_AY077621*	38.0	38.0	38.0	38.0	39.0	38.0	37.0	38.0	38.0	38.0	72.0	23.0	25.0	0.0		
16	*Neoric helminthoeca_AAL12494*	227.0	227.0	227.0	227.0	226.0	225.0	225.0	226.0	225.0	226.0	246.0	227.0	229.0	228.0	228.0	

Appendix 5. Estimates of evolutionary divergence between Msp4 amino acid sequences (Chapter 4).The number of amino acid differences between selected sequences is shown. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 198 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2015).

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	*A. marginale_StMaries_CP000030*																	
2	*A. marginale_Florida_CP001079*	1.0																
3	*A. marginale_Italy_AY702922*	1.0	0.0															
4	*A. marginale_Brazil_AY283194*	2.0	1.0	1.0														
5	**Am3**	**2.0**	**1.0**	**1.0**	**2.0**													
6	**Am2**	**2.0**	**2.0**	**2.0**	**3.0**	**1.0**												
7	**Am1**	**1.0**	**0.0**	**0.0**	**1.0**	**1.0**	**2.0**											
8	*A. centrale_Israel_CP001759*	22.0	21.0	21.0	21.0	22.0	23.0	21.0										
9	**Ac4**	**21.0**	**20.0**	**20.0**	**20.0**	**21.0**	**22.0**	**20.0**	**1.0**									
10	**Ac1**	**20.0**	**19.0**	**19.0**	**19.0**	**20.0**	**21.0**	**19.0**	**2.0**	**1.0**								
11	**Ac2**	**18.0**	**17.0**	**17.0**	**17.0**	**18.0**	**19.0**	**17.0**	**4.0**	**3.0**	**2.0**							
12	**Ac3**	**20.0**	**19.0**	**19.0**	**19.0**	**20.0**	**21.0**	**19.0**	**4.0**	**3.0**	**2.0**	**4.0**						
13	*A. ovis_Iran_JQ663993*	7.0	7.0	7.0	8.0	8.0	7.0	7.0	20.0	19.0	18.0	18.0	18.0					
14	*A. ovis_China_HQ456348*	7.0	7.0	7.0	8.0	8.0	7.0	7.0	20.0	19.0	18.0	18.0	18.0	0.0				
15	*A. phagocytophilum_Europe_HQ661163*	77.0	78.0	78.0	78.0	78.0	78.0	78.0	77.0	76.0	75.0	74.0	75.0	77.0	77.0			
16	*A. phagocytophilum_Spain_KP861636*	77.0	78.0	78.0	78.0	78.0	78.0	78.0	76.0	75.0	74.0	74.0	74.0	77.0	77.0	5.0		
17	*Wolbachia.sp_AF020068*	149.0	149.0	149.0	148.0	149.0	148.0	149.0	145.0	145.0	145.0	145.0	145.0	148.0	148.0	140.0	141.0	

Appendix 6. Pictures of tick species collected from uThukela dip tanks showing dorsal and ventral view (Chapter 5).

A. *Rhiphicephalus microplus,* dorsal (left) and ventral (right) view of a male tick.

B. *Rhiphicephalus microplus* dorsal (left) and ventral (right) view of a female tick.

C. *Rhiphicephalus appendiculatus* dorsal (left) and ventral (right) view of a male tick.

D. *Rhiphicephalus appendiculatus* dorsal (left) and ventral (right) view of a female tick.

E. *Rhiphicephalus evertsi evertsi* dorsal (left) and ventral (right) view of a male tick.

F. *Rhiphicephalus evertsi evertsi* dorsal (left) and ventral (right) view of a female tick.

Appendix 7. Approval for the PhD study 'Detection, Differentiation and phylogenetic relationship between *A. marginale* and *A. marginale* subspecies *centrale*. issued by the Research Ethics Committee, Faculty of Humanities, University of Pretoria, South Africa.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Detection, differentiation and phylogenetic relationship between *Anaplasma marginale* and *A. marginale ss centrale***
PROJECT NUMBER	**V085-14**
RESEARCHER/PRINCIPAL INVESTIGATOR	**A Khumalo**

STUDENT NUMBER (where applicable)	**140 867 44**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Biobank samples**	
NUMBER OF ANIMALS	**To be reported**	
Approval period to use animals for research/testing purposes		**October 2014-October 2015**
SUPERVISOR	**Prof. M Oosthuizen**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	27 October 2014
CHAIRMAN: UP Animal Ethics Committee	Signature	

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension of Application

PROJECT TITLE	**Detection, differentiation and phylogenetic relationship between *Anaplasma marginale* and *A. marginale ss centrale***
PROJECT NUMBER	**V085-14**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Z Khumalo**

STUDENT NUMBER (where applicable)	**UP_140 867 44**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Biobank samples**	
NUMBER OF ANIMALS	**To be reported**	
Approval period to use animals for research/testing purposes		**October 2016-October 2017**
SUPERVISOR	**Prof. M Oosthuizen**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 20 October 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Prevalence of anaplasmosis in cattle in the Bergville and Ladysmith areas, KwaZulu Natal**
PROJECT NUMBER	**V088-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Z Khumalo**

STUDENT NUMBER (where applicable)	**UP_14086744**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	***Cattle***	
NUMBER OF ANIMALS	**150**	
Approval period to use animals for research/testing purposes		**July 2016-July 2017**
SUPERVISOR	**Prof. M Oosthuizen**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 25 July 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

Appendix 8. Permission to do research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) for the research project.

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agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1

Prof Marinda Oosthuizen
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

Dear Prof Oosthuizen

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 13 June 2016, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. No blood samples or ticks from buffalo may be collected for this study. Furthermore no buffalo blood samples that were already collected or ticks that were already collected from buffalo may be utilised for this study;
4. Only the blood and ticks currently stored at DVTD, that were collected from cattle in the the Bergville and Ladysmith areas under the supervision of the control animal health technician prior to having obtained Section 20 approval may be utilised for this study;
5. No new blood samples or ticks may be collected for this study;
6. Oricol must be used as accredited waste management company;
7. The study may not be started until an amended ethics approval was obtained for amendment of the protocol to cattle instead of buffalo.

Title of research/study: Prevalence of Anaplasmosis in Cattle in the Bergville and Ladysmith Areas, KwaZulu-Natal
Researcher (s): Prof Marinda Oosthuizen
Institution: Department of Veterinary Tropical Diseases, UP.
Your Ref./ Project Number:
Our ref Number: 12/11/1/1

Kind regards,

pp

DR. MPHO MAJA

DIRECTOR OF ANIMAL HEALTH

Date: 2016 -06- 27

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1

Prof Marinda Oosthuizen
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

Dear Prof Oosthuizen

RE: Dispensation on Section 20 Approval in Terms of the Animal Diseases Act, 1984 (Act No 35 of 1984) for: Prevalence of Anaplasmosis in Cattle in the Bergville and Ladysmith Areas, KwaZulu-Natal

Your email dated 13 June 2016 refers.

A dispensation is hereby granted on point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Extracted DNA may be stored at the Biobank of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria;
ii) Stored extracted DNA may only be used for further research after having obtained new Section 20 approval;
iii) Stored extracted DNA may not be outsourced.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH

Date: 2016 -06- 27

Appendix 9. Wildlife samples were obtained from the South African National Parks SANParks Biobank under reference number LARBJ1118 Conservation Genetics and from the Wildlife Biological Resource Center (WBRC) and Biobank SA under the auspices of the National Zoological Gardens (NZG) of South Africa and Johannesburg Zoo.

Reference: LARBJ1118 Conservation Genetics

South African
NATIONAL PARKS

MATERIAL TRANSFER AGREEMENT

BETWEEN

SOUTH AFRICAN NATIONAL PARKS
herein represented by **__Dr. Peter Buss__**
in his capacity as **__Senior GM: Veterinary Wildlife Services__**
(hereinafter referred to as "SANParks")

AND

[NAME OF INSTITUTION]
herein represented by **__Dr Marinda Oosthuizen__**
in her capacity as **__Researcher__**
(hereinafter referred to as "Recipient")

WHEREAS the Recipient has requested the Material from SANParks;

AND WHEREAS SANParks accepted the Recipients' request for the Material subject to the terms and conditions as stipulated hereunder:

National Research Foundation
NZG National Zoological Gardens of South Africa

PO Box 754
Pretoria, 0001
South Africa
Tel: 012 328 3265
Fax: 012 323 4540
Int. Code: +27
info@nzg.ac.za

www.nzg.ac.za

NZG/ P13/05

14 June 2013

Prof Marinda C Oosthuizen
University of Pretoria
Private Bag X20
Hatfield
0028

Dear Prof Oosthuizen

APPROVAL OF RESEARCH PROPOSAL

This letter serves to inform you that your research proposal P13/05 "Can eland, waterbuck and impala, act as reservoir host for anaplasmosis, theileriosis and heartwater? And more specifically, what is the role of the African buffalo as reservoir hosts of *Anaplasma marginale* and *A. centrale*?" has been approved by the NZG Research Ethics and Scientific Committee (RESC) with the following provisos:

1. Inform the RESC of completion or termination (with reason) of the research at the NZG.
2. Submission of an annual progress report on request. Failure to submit a progress report may result in approval to be withdrawn.
3. Submission of a written request for an extension or for any changes within the research project
4. Acknowledgement of the NZG in all research outputs emanating from this research project (please include PDF documents of all publications)
5. Submission of a final report on completion of the study.
6. Inform the Occupational Health and Safety Practitioner of the NZG of the project.

Thank you for making use of the NZG as a research platform.

Yours sincerely

Prof Antoinette Kotze
Chair: NZG Research Ethics & Scientific Committee

RESEARCH OUTPUTS

CONFERENCES

YEAR 2014

KHUMALO, Z.T.H., COLLINS, N.E., CHAISI, M.E., BRAYTON, K.A., OOSTHUIZEN, M.C. 2014. Molecular detection and characterization of *Anaplasma* species in African buffalo (*Syncerus caffer*) in the Kruger National Park and Hluhluwe-iMfolozi Park, South Africa. Joint 8th International Ticks and Tick-borne Pathogens (TTP-8) and 12th Biennial Society for Tropical Veterinary Medicine (STVM) Conference, Cape Town South Africa, 24-29 August 2014. (POSTER).

KHUMALO, Z.T.H., COLLINS, N.E., SIBEKO, K.P., CHAISI, M.E., BRAYTON, K.A., OOSTHUIZEN, M.C. 2014. Molecular detection and characterization of *Anaplasma* species in African buffalo (*Syncerus caffer*) in the Kruger National Park and Hluhluwe-iMfolozi Park, South Africa. International Congress on Parasites of Wildlife, Skukuza, Kruger National Park, 14 – 17 September 2014. (ORAL).

YEAR 2015

KHUMALO Z,T.H., COLLINS, N.E., OOSTHUIZEN, M.C. 2015 Bovine Anaplasmosis in South Africa: Natural History, Biology and Phylogeny. Wildlife Symposium, Faculty of Veterinary Science, University of Pretoria, Onderstepoort. South Africa. October 2015 (ORAL).

KHUMALO, Z.T.H., COLLINS, N.E., CHAISI, M.E., BRAYTON, K.A., OOSTHUIZEN, M.C. 2015. Molecular detection and characterisation of *Anaplasma* species in African Buffalo (*Syncerus caffer*) and black wildebeest (*Connochaetes gnou*) populations in South Africa. 25th International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP), 16-20 August 2015, Liverpool, United Kingdom. (POSTER) **(Zama Khumalo = Winner of WAAVP-AF travel award)**

KHUMALO, Z.T.H., COLLINS, N.E., CHAISI, M.E., BRAYTON, K.A, OOSTHUIZEN M.C. 2015. Molecular detection and characterization of *Anaplasma* species in African buffalo (*Syncerus caffer*) and black wildebeest (*Connochaetes gnou*) populations in South Africa. ESCCAR International Congress on *Rickettsia* and other Intracellular Bacteria held in Lausanne, Switzerland from 13 - 16 June 2015. (POSTER).

YEAR 2016

KHUMALO, Z.T.H., CATANESE, H.N., LIESCHING, N., PAIDASHE, H., COLLINS, N.E., CHAISI, M.E., GEBREMEDHIN, A.E., OOSTHUIZEN, M.C., BRAYTON, K.A. 2016. *Anaplasma marginale* subspecies *centrale* strains using *msp1aS* genotype reveals a wildlife reservoir. 45th conference of the Parasitological Society of Southern Africa, Cape Town, South Africa, 28- 31 August 2016. (ORAL).

KHUMALO, Z.T.H., CATANESE, H.N., LIESCHING, N., PAIDASHE, H., COLLINS, N.E., CHAISI, M.E., GEBREMEDHIN, A.E., OOSTHUIZEN, M.C., BRAYTON, K.A. 2016. *Anaplasma marginale* subspecies *centrale* strains using *msp1aS* genotype reveals a wildlife reservoir. Faculty Day, 25 August 2016 (POSTER).

BRAYTON, K.A., KHUMALO, Z.T.H., CATANESE, H.N., LIESCHING, N., PAIDASHE, H., COLLINS, N.E., CHAISI, M.E., GEBREMEDHIN, A.E., OOSTHUIZEN, M.C. 2016. Msp1aS genotyping of *Anaplasma centrale* indicates a wildlife reservoir. 1st Joint conference of the Association of Institutions for Tropical Veterinary Medicine (AITVM) and the Society of Tropical Veterinary Medicine (STVM), Berlin, Germany, 4-8 September 2016 (ORAL).

YEAR 2017

KHUMALO, Z.T.H, BRAYTON, K.A., COLLINS, N.E., CHAISI, M.E., Quan, M., OOSTHUIZEN, M.C. 2017. Confirmation of *Anaplasma marginale* variety *centrale* (Theiler 1911) as a separate species, *Anaplasma centrale* (non Theiler 1911) sp. nov., comb. nov (Ristic & Kreier 1984). 9th Tick and Tick Borne Pathogen Conference (TTP9) & 1st Asia Pacific Ricketssia Conference (APR1), Cairns, Australia, 27 August–1 September 2017 (ORAL).

MANUSCRIPTS

Published

KHUMALO, Z.T.H., CATANESE, H.N., LIESCHING, N., HOVE, P., COLLINS, N.E., CHAISI, M.E., GEBREMEDHIN, A.H., OOSTHUIZEN, M.C., BRAYTON, K.A. 2016. Characterization of *Anaplasma marginale* subsp. *centrale* strains by use of *msp1aS* genotyping reveals a wildlife reservoir. Journal of Clinical Microbiology 54, 2503-2512.

Submitted

KHUMALO, Z.T.H., BRAYTON, K.A., COLLINS, N.E., CHAISI, M.E., QUAN M., OOSTHUIZEN, M.C. Evidence confirming the phylogenetic position of *Anaplasma centrale* (ex Theiler1911) Ristic & Kreier 1984. Submitted to International Journal of Systematic and Evolutionary Microbiology (October 2017).

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology

Characterization of *Anaplasma marginale* subsp. *centrale* Strains by Use of *msp1aS* Genotyping Reveals a Wildlife Reservoir

Zamantungwa T. H. Khumalo,[a] Helen N. Catanese,[b] Nicole Liesching,[a] Paidashe Hove,[a,c] Nicola E. Collins,[a] Mamohale E. Chaisi,[a] Assefaw H. Gebremedhin,[b] Marinda C. Oosthuizen,[a] Kelly A. Brayton[a,d]

Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa[a]; School of Electrical Engineering and Computer Science, Washington State University, Pullman, Washington, USA[b]; Biotechnology Platform, Agricultural Research Council, Onderstepoort, Pretoria, South Africa[c]; Program in Vector Borne Diseases, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA[d]

Bovine anaplasmosis caused by the intraerythrocytic rickettsial pathogen *Anaplasma marginale* is endemic in South Africa. *Anaplasma marginale* subspecies *centrale* also infects cattle; however, it causes a milder form of anaplasmosis and is used as a live vaccine against *A. marginale*. There has been less interest in the epidemiology of *A. marginale* subsp. *centrale*, and, as a result, there are few reports detecting natural infections of this organism. When detected in cattle, it is often assumed that it is due to vaccination, and in most cases, it is reported as coinfection with *A. marginale* without characterization of the strain. A total of 380 blood samples from wild ruminant species and cattle collected from biobanks, national parks, and other regions of South Africa were used in duplex real-time PCR assays to simultaneously detect *A. marginale* and *A. marginale* subsp. *centrale*. PCR results indicated high occurrence of *A. marginale* subsp. *centrale* infections, ranging from 25 to 100% in national parks. Samples positive for *A. marginale* subsp. *centrale* were further characterized using the *msp1aS* gene, a homolog of *msp1*α of *A. marginale*, which contains repeats at the 5′ ends that are useful for genotyping strains. A total of 47 Msp1aS repeats were identified, which corresponded to 32 *A. marginale* subsp. *centrale* genotypes detected in cattle, buffalo, and wildebeest. RepeatAnalyzer was used to examine strain diversity. Our results demonstrate a diversity of *A. marginale* subsp. *centrale* strains from cattle and wildlife hosts from South Africa and indicate the utility of *msp1aS* as a genotypic marker for *A. marginale* subsp. *centrale* strain diversity.

Bovine anaplasmosis (gallsickness) is a tick-borne disease caused by the intraerythrocytic rickettsial pathogen *Anaplasma marginale* (1). *A. marginale* is globally prevalent and results in anemia, with mortality rates of up to 30% (2). *Anaplasma marginale* subspecies *centrale* is a less virulent subspecies detected by Sir Arnold Theiler, who recognized its potential as a vaccine against anaplasmosis; 100 years later this live vaccine is still in use in South Africa, Israel, South America, and Australia (3, 4). The strain that is used as a vaccine originated from Theiler's original isolation and was exported at various times to other countries where it has been propagated in the laboratory; the strain known as the "Israel strain" or the "vaccine strain" was sent to Israel in the 1950s and was used to generate the complete genome sequence for *A. marginale* subsp. *centrale* in 2010 (5). *A. marginale* subsp. *centrale* does not provide complete protection against *A. marginale* infection but does protect against severe anaplasmosis (6, 7).

A. marginale infects a wide range of ruminants including buffalo (*Bubalus bubalis* and *Syncerus caffer*), wildebeest (*Connochaetes gnou* and *Connochaetes taurinus*), American bison (*Bison bison*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus hemionus*), black-tailed deer (*Odocoileus hemionus columbianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*) (8–11). Cattle are naturally susceptible to *A. marginale* (4). There has not been much interest in the epidemiology of *A. marginale* subsp. *centrale*, with few reports detecting natural infections of this organism; most often, when detected in cattle it is assumed that it is due to vaccination and is reported as coinfection with *A. marginale* without characterization of the strain (12). Reported *A. marginale* subsp. *centrale* single infections were detected by the reverse line blot (RLB) hybridization assay in Italy without characterization of the strain. More recently, the first known case of bovine anaplasmosis caused by *A. marginale* subsp. *centrale* in Europe was reported (13). While this study described genetic heterogeneity of *A. marginale* subsp. *centrale* strains from different geographic areas in Italy, it is not clear how these are related to the vaccine strain.

For *A. marginale*, the Msp1a protein/gene (*msp1*α) has been used as a genotypic marker to differentiate strains (14). Msp1a is encoded by the single-copy gene, *msp1*α and differs among strains due to variable sequence and numbers of an 84/87-bp repeat sequence (28 or 29 amino acids) located near the amino terminus of the protein (14). A number of studies have examined Msp1a repeats in the United States, South America, Australia, the Philippines, Europe, Israel, China, and Mexico, resulting in identification of more than 200 repeats (14–16). In South Africa, two studies have been conducted to genetically characterize strains using *msp1*α (17, 18), revealing that the repeat structure is common between South African, American, and European strains of *A. marginale*; in fact, some of the repeat sequences that were detected were identical to ones that were detected in the United

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Editor: B. W. Fenwick, University of Tennessee
Address correspondence to Kelly A. Brayton, kbrayton@vetmed.wsu.edu.

States. Not surprisingly, there were also new repeat sequences detected that are, thus far, unique to South Africa.

A. marginale subsp. *centrale* was thought not to have a homolog of *msp1α*; however, complete genome sequencing of the Israel vaccine strain revealed that there is a gene that resides in a position syntenic to *A. marginale msp1α* (5). This gene was named *msp1aS* (S for syntenic; a gene flanked by the same set of genes in two genomes) and has 31 to 36% amino acid sequence identity depending on the *A. marginale* strain compared. Importantly, there are structural similarities, including repeats near the amino terminus and two sets of transmembrane domains near the carboxy terminus that indicate that these proteins are likely homologs (Fig. 1). The repeats in *A. marginale* subsp. *centrale* strain Israel Msp1aS are longer (47 amino acids in length) than the *A. marginale* Msp1a repeats, and there is no sequence identity between the repeats in the two organisms. The vaccine strain has four repeats with an *msp1aS* genotype of Ac1 Ac1 Ac1 Ac2.

In the present study, we have used a duplex quantitative real-time PCR (qPCR) assay to screen for the presence of *A. marginale* subsp. *centrale* and *A. marginale* in vaccinated and unvaccinated cattle and wildlife, indicating that these infections are common and often occur as mixed infections. Samples that tested positive using this screen were then further analyzed for the *msp1aS* genotype, demonstrating that the vaccine strain genotype is prevalent in cattle herds that practice vaccination, while other more divergent genotypes are present in wildlife species.

MATERIALS AND METHODS

Blood collection and DNA extraction. A total of 380 blood samples from wild ruminant species including African buffalo (*Syncerus caffer*, $n = 97$); waterbuck (*Kobus ellipsiprymnus*, $n = 14$); eland (*Taurotragus oryx*, $n = 23$); black wildebeest (*Connochaetes gnou*, $n = 54$); and blue wildebeest (*Connochaetes taurinus*, $n = 23$), together with 86 cattle samples, were obtained from the Wildlife Biological Resource Center (WBRC) and Biobank South Africa (SA) under the auspices of the National Zoological Gardens of South Africa (NZG) and from the South African National Parks (SANParks) Biobank. The remaining buffalo blood samples ($n = 41$) were made available to us by Dave Cooper from Hluhluwe-iMfolozi Park. Additionally, 42 blood samples from vaccinated cattle were obtained from two commercial farms in Bergville, KwaZulu-Natal, South Africa (Table 1). Standard techniques were followed in collecting blood samples for laboratory examination. Genomic DNA was extracted using the QIAmp DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA was eluted in 100 μl of elution buffer and stored at −20°C.

The study was approved by the Animal Ethics Committee of the University of Pretoria, South Africa (V085-14), and permission to use wildlife samples was given by SANParks Biobank under reference number LARBJ1118 Conservation Genetics, by the WBRC, and by Biobank SA under the auspices of the NZG of South Africa and the Johannesburg Zoo with project number NZG/P13/05. Collection of cattle samples was approved by the Department of Agriculture, Forestry and Fisheries under section 20 of the Animal Diseases Act of 1984 with reference 12/11/1/1.

Duplex real-time PCR assay. Quantitative real-time PCR (qPCR) for simultaneous detection and quantification of *A. marginale* and *A. marginale* subsp. *centrale* DNA was performed as described previously (19) with some modifications for use on a LightCycler real-time machine (28) (Roche Diagnostics, Mannheim, Germany). The qPCR was performed in a final reaction volume of 20 μl, containing 2 μl of DNA template (100 to 200 ng of DNA), 12.5 μl of FastStart DNA Master hybridization mix (Roche Diagnostics, Mannheim, Germany), 600 nmol/liter of *A. marginale*-specific primers AM-For (5′ TTG GCA AGG CAG CAG CTT 3′) and AM-Rev (5′-TTC CGC GAG CAT GTG CAT-3′), 900 nmol/liter of *A. marginale* subsp. *centrale*-specific primers AC-For (5′-CTA TAC ACG CTT GCA TCT C-3′) and AC-Rev (5′-CGC TTT ATG ATG TTG ATG C-3′), and 200 nmol/liter of probes AM-Pb (5′-6FAM-TCG GTC TTA ACA TCT CCA GGC TTT CAT-BHQ1-3′) and AC-Pb (5′-LC610-ATC ATC ATT CTT CCC CTT TAC CTC GT-BHQ2-3′). Thermal cycling conditions were as follows: UDG activation at 40°C for 10 min, preincubation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 1 min and annealing-extension at 60°C for 1 min, and a final cooling step at 40°C for 30 s. The results were analyzed using LightCycler software ver-

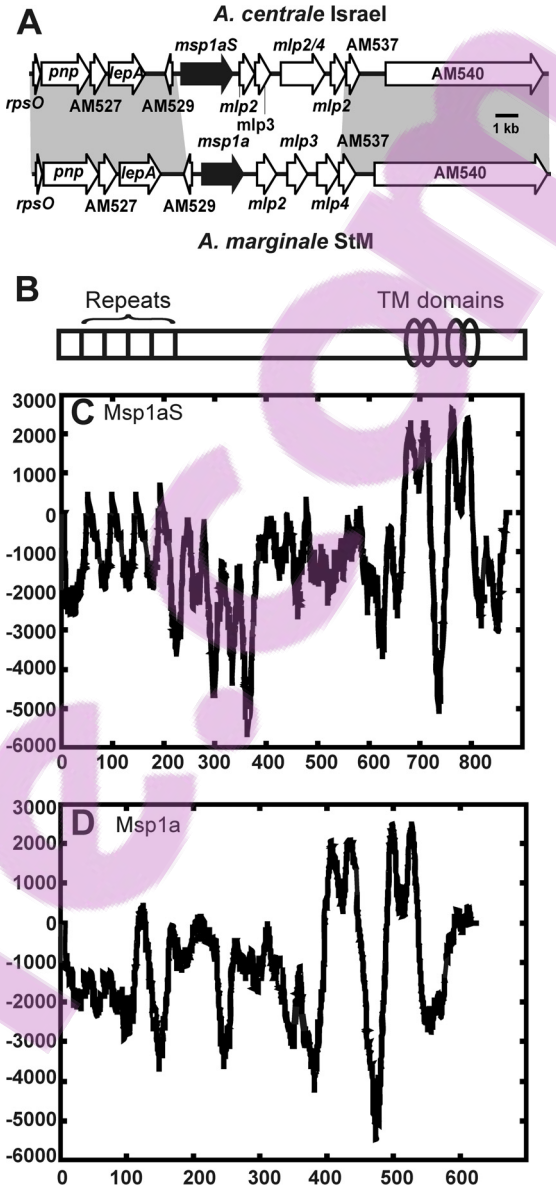

FIG 1 Schematic representation and TMpred plots of Msp1aS. (A) Genomic positioning of Msp1aS of *A. marginale* subsp. *centrale*, also showing that it is syntenic to Msp1a of *A. marginale* St. Maries strain (StM), which suggests that these proteins are homologs. (B) While there is little sequence conservation, these proteins have similar structures: both have a set of repeats near the amino terminus and two sets of transmembrane (TM) domains toward the carboxy terminus. (C and D) TMpred plots show the transmembrane prediction profile for both molecules (Msp1aS from the fully sequenced Israel strain of *A. marginale* subsp. *centrale* and Msp1a from the fully sequenced St. Maries strain of *A. marginale*). Values greater than 500 (*y* axis) indicate transmembrane domains. The repeats of Msp1aS are almost twice as long as those of Msp1a.

TABLE 1 Host samples used in this study

Sample no.	Species	No. of samples	Sample type	Collection site	Origin[a]	Province
565–614	Buffalo	50	EDTA-blood	SANParks[b]	KNP	Mpumalanga
974–987	Buffalo	14	EDTA-blood	SANParks	CNP	Eastern Cape
1002–1016	Buffalo	15	EDTA-blood	SANParks	AEP	Eastern Cape
988–995 and 66/13	Buffalo	9	EDTA-blood	SANParks	GNP	Northern Cape
998–1001 and 1017–1021	Buffalo	9	EDTA-blood	SANParks	MNP	Northern Cape
1–41	Buffalo	41	EDTA-blood	HiP	HiP	KwaZulu-Natal
924–937 and 947–955	Black wildebeest	23	EDTA-blood	SANParks	MTNZNP	Eastern Cape
938–939	Black wildebeest	2	EDTA-blood	SANParks	TMNP	Western Cape
942, 944–953 and 955–972	Black wildebeest	29	EDTA-blood	SANParks	MNP	Northern Cape
1036–1056	Blue wildebeest	21	EDTA-blood	SANParks	MNP	Northern Cape
1057–1058	Blue wildebeest	2	EDTA-blood	SANParks	WCNP	Western Cape
1022–1031	Eland	10	EDTA-blood	SANParks	MNP	Northern Cape
1032–1035	Eland	4	EDTA-blood	SANParks	AEP	Eastern Cape
459–467	Eland	9	FTA filter paper	WBRC, NZG[c]	NZG	Gauteng
1059–1062	Waterbuck	4	EDTA-blood	SANParks	MNP	Northern Cape
468–470	Waterbuck	3	FTA filter paper	WBRC, SA, NZG	Rietvlei NR, JHB Zoological Gardens, Mohale Gate (Gauteng area)	Gauteng
543, 549	Waterbuck	2	EDTA-blood	WBRC, SA, NZG	KNP	Mpumalanga
544–548	Waterbuck	5	EDTA-blood	WBRC, SA, NZG	MaNP	Limpopo
WC103–WC128	Cattle	26	EDTA-blood	NZG collection	WC F3[d]	Western Cape
KZN129–KZN158	Cattle	30	EDTA-blood	NZG collection	KZN F4	KwaZulu-Natal
FS1–FS30	Cattle	30	EDTA-blood	NZG collection	FS F5	Free State
Berg 1–Berg 21	Cattle	21	EDTA-blood	Bergville farm	Bergville F1	KwaZulu-Natal
Berg 22–Berg 42	Cattle	21	EDTA-blood	Bergville farm	Bergville F2	KwaZulu-Natal

[a] Origin, the park/farm from where the sample originates: Kruger National Park (KNP), Cambedoo National Park (CNP), Graspan National Park (GNP), Mokala National Park (MNP) Addo Elephant Park (AEP), Hluhluwe-iMfolozi Park (HiP), Mountain Zebra National Park (MTNZNP), Table Mountain National Park (TMNP), West Coast National Park (WCNP), Marakele National Park (MaNP).
[b] SANParks, South African National Parks.
[c] WBRC, Wildlife Biological Research Center; NZG, National Zoological Gardens, South Africa.
[d] F, farm.

sion 4.0 (Roche Diagnostics). The software indicates a positive result by a C_q value (quantification cycle, synonymous with the C_p, crossing point, value given by the LightCycler instrument), at which fluorescence from amplification exceeds the background fluorescence, and a score of 1 to 5. Negative samples have a score of −1 to −5 and no C_p values. A lower C_q correlates with a higher starting concentration of target DNA in a sample, which then indicates a positive infection. FAM fluorescence (530 nm) was generated in *A. marginale*-positive samples and LC-610 (610 nm) signals were generated in *A. marginale* subsp. *centrale*-positive samples. DNA extracted from the *A. marginale* subsp. *centrale* vaccine strain (Onderstepoort Biological Products [OBP], Pretoria, South Africa) was used as a positive control, and samples C14, C57, or F48 (originating from cattle in the Mnisi Community area, Mpumalanga, South Africa) were used as positive controls for *A. marginale*. The presence of *A. marginale* in these samples was confirmed by sequencing of the *msp1*β genes. A negative and a positive control were included in each set of PCRs that was performed. The analytical specificity of the assay was determined by analyzing DNA from closely related species such as *Anaplasma* sp. Omatjenne and *A. phagocytophilum* (20). The efficiency of the assay was determined from 10-fold serial dilutions of plasmid DNA from clones 9410c (*A. marginale* subsp. *centrale*) and F48a (*A. marginale*).

Analysis of the *msp1aS* gene. *A. marginale* subsp. *centrale*-positive samples which had low C_q values as detected by qPCR were selected for analysis of the *msp1aS* gene. Primers MSP1asFZ (5′-CAA GGT CAA GAG TCA GCA TCA TCA GAT G-3′) and MSP1asRZ (5′-CTC CGC GCA CAA TAC TTT CAA CCT CC-3′) were designed based on the *A. marginale* subsp. *centrale* genome sequence (GenBank accession CP001759) to target tandem repeats within the *msp1aS* gene. PCR was performed in a final reaction volume of 25 μl containing Phusion Flash high-fidelity PCR master mix (Thermo Fisher Scientific), 10 pM of each primer, and genomic DNA. Thermal cycling was carried out in a Veriti thermal cycler (Thermo Fisher Scientific) and consisted of an initial denaturation at 98°C for 10 s, followed by 30 cycles of denaturation at 98°C for 1 s, annealing at 67°C for 30 s and extension at 72°C for 15 s, and a final extension at 72°C for 1 min. DNA extracted from the *A. marginale* subsp. *centrale* vaccine obtained from OBP (Pretoria, South Africa) was used as a positive control.

Purified PCR amplicons were cloned into the pJET vector (Thermo Fisher Scientific). Recombinant plasmids were isolated using a High Pure plasmid isolation kit (Roche Diagnostics, Mannheim, Germany) and sequenced using 1 μl of 2 μM M13 primers with an ABI BigDye v3.1 kit on an ABI 3500xL genetic analyzer at Inqaba Biotec (Pretoria, South Africa).

Sequences were assembled, edited, and translated to amino acids using CLC Main Workbench 7.0.3 (Qiagen, Denmark). Tandem repeats were identified using Tandem Repeats Finder (https://tandem.bu.edu/trf/trf.html) (21). The repeats were named A*cn*, to distinguish them from *A. marginale* Msp1a repeats. Truncated repeats were designated with a T at the end of the name. Repeats were curated and analyzed using RepeatAnalyzer (29). Repeat sequences were aligned using the AlignX module of Vector NTI (Invitrogen).

Diversity measures. RepeatAnalyzer calculates four genetic diversity metrics, each of which captures the diversity of repeats in a geographic region in a different way. Broadly, they fall into two groups, those that measure the amount of different repeats and those that measure the distribution of those repeats. Within each of these categories there is a global and a local formulation. The local version of a metric calculates the score independently on each genotype and averages these together to get the final score, while the global version looks at all genotypes together. Specifically, the GDM1-L score can be interpreted as the percentage of unique repeats in each genotype in the region, while the GDM1-G score is the percentage of unique repeats across all genotypes in the region. The

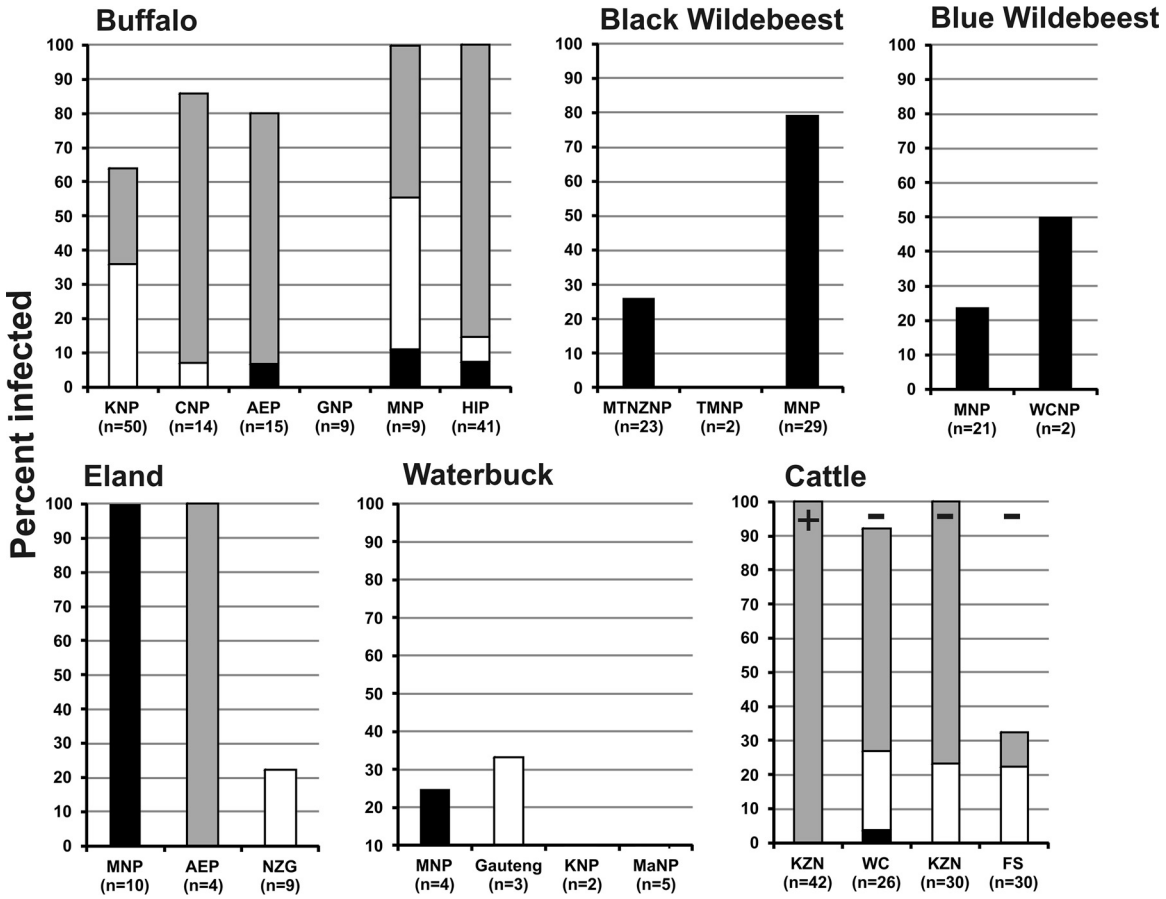

FIG 2 Stacked bar graphs showing occurrence of *Anaplasma* species in wild ruminants and cattle. Buffalo, black and blue wildebeest, eland, waterbuck, and cattle were analyzed by duplex real-time PCR. Animals were sampled from the following national parks and provinces: Kruger National Park (KNP), Cambedoo National Park (CNP), Addo Elephant Park (AEP), Graspan National Park (GNP), Mokala National Park (MNP), Hluhluwe-iMfolozi Park (HIP), Mountain Zebra National Park (MTNZNP), Table Mountain National Park (TMNP), West Coast National Park (WCNP), National Zoological Gardens of South Africa (NZG), Marakele National Park (MaNP), KwaZulu-Natal (KZN), Western Cape (WC), and Free State (FS). Numbers in parentheses indicate the total numbers of animals sampled from that park/province. Samples were collected from vaccinated (+) and unvaccinated (−) cattle. Black indicates animals positive for *A. marginale* subsp. *centrale*, gray indicates animals with mixed infections, and white indicates animals positive for *A. marginale*.

GDM2-L score can be interpreted as the amount of variation (measured as standard deviation) in the number of occurrences of the repeats in a genotype, while the GDM2-G score is the amount of variation in the number of occurrences of all the repeats in all genotypes in the region. A high GDM1 score means that there are more unique repeats, with 0 as the minimum (when all repeats are the same) and 1 being the maximum (when each repeat is unique). A high GDM2 score means that the repeats are distributed more unevenly, with a minimum of 0 (when all repeats occur the same number of times) and values ranging up to but not including 0.5 as the unevenness of repeat distribution increases.

RESULTS

Occurrence of *Anaplasma* species in wild ruminants and cattle in South Africa. Duplex qPCR results indicated that *A. marginale* subsp. *centrale* single infections are common among black wildebeest (Mokala National Park [MNP], 79.3%), blue wildebeest (West Coast National Park [WCNP], 50%), waterbuck (MNP, 25%), and eland (MNP, 100%). Wildebeest did not harbor any *A. marginale* infections. Mixed infections were frequently found in both buffalo and cattle, ranging from 28% to 100% of animals from a given area being positive for both *A. marginale* and *A. marginale* subsp. *centrale* infections. Buffalo samples had high rates of mixed infections and also had lower rates of single infections with *A. marginale* subsp. *centrale* than with *A. marginale*. Interestingly, single infections of both species predominated in sets of animals from specific parks (see eland and waterbuck in Fig. 2), indicating that environment plays a role in exposure to the two pathogens.

Characterization of MSP1aS. Because the sequenced Israel vaccine strain was removed from South Africa more than 60 years ago, we obtained a batch of the vaccine currently produced at OBP in Pretoria, South Africa, and sequenced the *msp1aS* gene. The sequence of the OBP vaccine strain Msp1aS tandem repeat from 2014 was identical to that of the Israel strain (5) with four tandem repeats: Ac1 Ac1 Ac1 Ac2.

Based on the duplex qPCR results, *A. marginale* subsp. *centrale*-positive samples ($n = 25$) were selected for further analysis. *Msp1aS* primers amplified at least one single strong product from all samples tested. Some samples exhibited multiple bands which demonstrated mixed infection (Fig. 3). The *msp1aS* PCR products were cloned and sequenced, and sequence analyses confirmed the presence of tandem repeats similar to those of the vaccine strain (Table 2). The first five columns of Table 2 would combine to

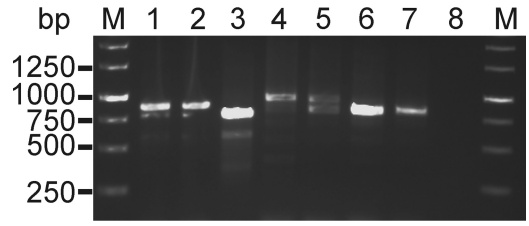

FIG 3 Gel image showing amplicons of *msp1aS*. Lanes 1 and 2, vaccine strain (814 bp); lane 3, animal FS 383 (790 and 637 bp); lane 4, animal Berg10 (922 and 814 bp); lane 5, animal Berg12 (937 and 814 bp); lane 6, animal Berg20 (814 bp); lane 7, animal WC_108 (799 bp); lane 8, negative control. Note that for some samples only a subset of the amplicons were successfully sequenced, while for others, clones with different sequences were obtained from what appeared as a single band. Lanes marked "M" have a 1-kb molecular weight marker.

provide the full strain and sample designation as suggested previously (29), i.e., Ac11 Ac8_ZA, EC_2007_CNP_986; however, we have used shorter names for some of the genotypes for ease of discussion. The strains tested in this study yielded one to five repeat units as predicted from the PCR product sizes; however, there were strains that did not correspond with their PCR products (data not shown). Altogether, 47 different Msp1aS tandem repeats were identified. The repeats ranged from 45 to 51 amino acids with seven truncated repeats ranging from 31 to 33 amino acids (Fig. 4). The most common repeat length was 46 amino acids (Fig. 5A). The Ac1 and Ac2 tandem repeats, contained in the vaccine strain, were detected in cattle, buffalo, and wildebeest.

The vaccine strain was detected in cattle from Bergville which were previously vaccinated with *A. marginale* subsp. *centrale* vaccine. We tested six cattle from Bergville farm 2 which yielded 15 *msp1aS* sequences. The vaccine genotype was detected in five of the six cattle (Table 2). Interestingly, two "vaccine variant" genotypes were detected that were closely related to the vaccine strain genotype and differed by only a single amino acid (VV1 and VV3). Another vaccine variant genotype, VV2 (Ac1 Ac1 Ac1 Ac2 Ac2), that had one additional Ac2 repeat but was otherwise identical to the vaccine strain genotype was noted. Two additional genotypes that were less obviously related to the vaccine strain were detected. Three cattle were tested on Bergville farm 1, resulting in 10 *msp1aS* sequences. Interestingly, the vaccine genotype was only detected in one of these animals despite the fact that these animals were reported as being vaccinated, while two animals contained the related genotype VV3. Seven additional genotypes were detected on farm 1 that were not closely related to the vaccine genotype.

Interestingly, the vaccine genotype and one of the vaccine variant genotypes were also detected in unvaccinated animals, including buffalo (HIP_6, AEP_1003, and KNP_586) and cattle (WC_108). Genotype Ac33 Ac3 Ac6 was detected in a buffalo from Hluhluwe National Park and in a cow from Bergville farm 1. Several truncated repeats were detected (i.e., Ac36T), and although these predominated in the buffalo samples, a genotype containing a truncated repeat was also detected on Bergville farm 1.

RepeatAnalyzer is a program we developed recently to house, curate, and provide metrics for repeat sequences used to characterize bacteria (29). In the present study, we applied it to the analysis of *msp1aS* repeats. The most common genotype structure we detected contained four repeats, with genotypes having from one to five repeats (Fig. 5B). Most repeats occurred only once with two repeats being detected in six different genotypes (Ac1 and Ac6) (Fig. 5C). The Ac1 repeat is not only detected in the vaccine strain but also in several vaccine variant genotypes that were detected on Bergville farm 2. The Ac6 repeat was prevalent in genotypes detected in wildlife and, interestingly, was also detected in genotypes found on Bergville farm 1. In general, we found that the average number of amino acid changes (edit distance) between any two *A. marginale* subsp. *centrale* repeats was high (13.7) and was normally distributed, with 97.8% of data falling within 2 standard deviations. There was a mean of 0.9 and 1.4 repeats at an edit distance of 1 and 2, respectively, from any given repeat. Despite the high level of variation between repeats, we found five repeats within an edit distance of two from Ac1 (Ac2, Ac26, Ac12, Ac20, and Ac48) and seven repeats within two edits of Ac2 (Ac1, Ac14, Ac28, Ac15, Ac16, Ac22, and Ac26).

Diversity analysis and repeat distribution. Using RepeatAnalyzer, we see that South Africa has a large number of unique *A. marginale* subsp. *centrale* repeats (Table 3, GDM1-L), while having an intermediate amount of repeat diversity in general (Table 3, GDM1-G). There is a higher diversity of repeats among the samples isolated from buffalo hosts than among those from cattle hosts, although this would be expected as many of the cattle were vaccinated and would be expected to exhibit the same repeat structure as the vaccine strain. GDM2 measures how uniformly the repeat occurrences in the strains in a region (local) or the region as a whole (global) are distributed. For both GDM2 metrics, the South African values are low, indicating that the repeats are dispersed; i.e., there is not a preponderance of a single repeat type in individual strains or for the country as a whole. The GDM2 values are higher for cattle than for buffalo-derived samples, reflecting more uniformity in the repeats detected in samples from cattle than from buffalo. When examining whether repeats and strains occur in multiple provinces, we have *msp1aS* data from seven of South Africa's nine provinces (Fig. 6). The repeats and strains are mapped according to GPS coordinates, so multiple locations within a province can be visualized and distinguished. Several repeats were detected in multiple locations (Fig. 6A). Repeats Ac1 and Ac2 were found in Mpumalanga, Gauteng, KwaZulu-Natal, Eastern Cape, and Western Cape provinces. The vaccine strain is detected in cattle from KwaZulu-Natal, the Eastern Cape, and the Western Cape (Fig. 6B), which is interesting as we tested vaccinated animals only in KwaZulu-Natal. Gauteng also shows positive for the vaccine strain, but this is due to the purchased vaccine itself.

DISCUSSION

We tested animals from several different parks and farms and showed that *A. marginale* subsp. *centrale* infection is prevalent in black and blue wildebeest, eland, buffalo, waterbuck, and cattle. *A. marginale* subsp. *centrale* has rarely been examined on its own, as typically researchers/ranchers are interested in *A. marginale* infection, and the competitive enzyme-linked immunosorbent assay (cELISA) often used for detection does not discriminate between *A. marginale* and *A. marginale* subsp. *centrale* infection. One study using the cELISA showed high seroprevalence of *Anaplasma* spp. in wildlife from Kenya with eland and blue wildebeest testing at 100% and 96%, respectively. With a reverse line blot assay, it was shown that *Anaplasma* spp. are prevalent in buffalo in northern Botswana with *A. marginale* subsp. *centrale* being the most preva-

TABLE 2 *A. marginale* subsp. *centrale* genotypes detected from South African bovine hosts (cattle, buffalo, and black wildebeest)

Genotype	Country code[a]	Province code[a]	Yr	Animal no.	Sample clone ID	Host species	Origin, park, farm	Vaccine status	Size (bp)	No. of repeats	Short name
Ac1 Ac1 Ac1 Ac2	IL	M	2010	Genome sequence	CP001759	Cattle	Israel 2010	+	814	4	Vaccine
Ac1 Ac1 Ac1 Ac2	ZA	GP	2014		OBP vaccine	Cattle	OBP 2014[b]	+	814	4	Vaccine
SANParks Biobanked samples[c]											
Ac11 Ac8	ZA	EC	2007	CNP_986	G	Buffalo	Cambedoo	−	525	2	
Ac9 Ac8	ZA	EC	2007	CNP_986	C	Buffalo	Cambedoo	−	526	2	
Ac11 Ac11 Ac11 Ac11 Ac8	ZA	EC	2007	CNP_986	C2	Buffalo	Cambedoo	−	940	5	
Ac3 Ac4 Ac5 Ac6	ZA	EC	2007	CNP_987	J2	Buffalo	Cambedoo	−	823	3	
Ac7 Ac8	ZA	EC	2007	CNP_979	D	Buffalo	Cambedoo	−	526	2	
Ac6 Ac35 Ac36T Ac37T Ac6	ZA	NC	2013	MNP_999	L	Buffalo	Mokala	−	889	5	
Ac38 Ac39T Ac34 Ac40T	ZA	NC	2013	MNP_999	N	Buffalo	Mokala	−	759	4	
Ac38 Ac41T Ac42 Ac40T	ZA	NC	2013	MNP_1000	A	Buffalo	Mokala	−	733	4	
Ac6Ac6	ZA	NC	2013	MNP_1000	G	Buffalo	Mokala	−	790	2	
Ac1 Ac1 Ac1 Ac2	ZA	EC	2013	AEP_1003	D	Buffalo	Addo	−	814	4	Vaccine
Ac7 Ac8	ZA	EC	2013	AEP_1006	D	Buffalo	Addo	−	525	2	
Ac38 Ac44T Ac43	ZA	EC	2013	AEP_1006	N	Buffalo	Addo	−	628	3	
Ac31 Ac8	ZA	EC	2013	AEP_1006	S	Buffalo	Addo	−	526	2	
Ac1 Ac1 Ac1 Ac1	ZA	MP	2008	KNP_586	A	Buffalo	Kruger	−	814	4	VV1
Ac26 Ac26 Ac26 Ac2	ZA	NC	2011	MNP_958	F_w	Black wildebeest	Mokala	−	862	4	
Hluhluwe iMfolozi Park											
Ac1 Ac1 Ac1 Ac2	ZA	NL	2008	HiP_6	1	Buffalo	Hluhluwe	−	815	4	Vaccine
Ac30 Ac24 Ac25	ZA	NL	2008	HiP_6	A	Buffalo	Hluhluwe	−	940	3	
Ac29 Ac29 Ac29	ZA	NL	2008	HiP_6	B	Buffalo	Hluhluwe	−	703	3	
Ac33 Ac3 Ac6	ZA	NL	2008	HiP_6	L	Buffalo	Hluhluwe	−	691	3	
NZG Biobanked samples[c]											
Ac20 Ac32 Ac21 Ac10	ZA	WC	2011	WC_107	E	Cattle	WC	−	700	4	
Ac1 Ac1 Ac1 Ac2	ZA	WC	2011	WC _108	A	Cattle	WC	−	799	4	Vaccine
Ac12 Ac12 Ac13 Ac13 Ac14	ZA	NL	2011	KZN_138	B	Cattle	NL	−	919	5	
Ac12 Ac12 Ac13 Ac13Ac14	ZA	NL	2011	KZN_132	A	Cattle	NL	−	941	4	
Ac12 Ac12 Ac13 Ac13 Ac14	ZA	NL	2011	KZN_130	B	Cattle	NL	−	980	5	
Ac15 Ac16 Ac16 Ac16	ZA	FS	2011	FS_56	B	Cattle	FS	−	821	4	
Ac16 Ac16 Ac16	ZA	FS	2011	FS_383	B	Cattle	FS	−	637	3	
Farm 1						Cattle					
Ac33 Ac3 Ac6	ZA	NL	2015	Berg 10	A	Cattle	Bergville	+	691	3	
Ac19 Ac19 Ac3 Ac6	ZA	NL	2015	Berg 10	G	Cattle	Bergville	+	814	4	
Ac17 Ac18 Ac45 Ac46T Ac47	ZA	NL	2015	Berg 10	J	Cattle	Bergville	+	922	5	
Ac1 Ac1 Ac1 Ac22	ZA	NL	2015	Berg 12	B	Cattle	Bergville	+	811	4	VV3
Ac20 Ac21 Ac21 Ac20	ZA	NL	2015	Berg 12	E	Cattle	Bergville	+	937	5	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 12	N	Cattle	Bergville	+	814	4	Vaccine
Ac23 Ac24 Ac25 Ac34	ZA	NL	2015	Berg 19	A	Cattle	Bergville	+	940	5	
Ac26 Ac12 Ac12 Ac27 Ac14	ZA	NL	2015	Berg 19	A_2	Cattle	Bergville	+	946	5	
Ac1 Ac1 Ac1 Ac22	ZA	NL	2015	Berg 19	B	Cattle	Bergville	+	811	4	VV3
Ac19 Ac3 Ac6 Ac6	ZA	NL	2015	Berg 19	I	Cattle	Bergville	+	826	4	
Farm 2											
Ac1 Ac1 Ac1 Ac22	ZA	NL	2015	Berg 25	A	Cattle	Bergville	+	814	4	VV3
Ac1	ZA	NL	2015	Berg 25	E	Cattle	Bergville	+	391	1	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 25	B	Cattle	Bergville	+	814	4	Vaccine
Ac1 Ac1 Ac1 Ac1	ZA	NL	2015	Berg 25	E_2	Cattle	Bergville	+	814	4	VV1
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 25	X	Cattle	Bergville	+	914	5	VV2
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 27	D	Cattle	Bergville	+	814	4	Vaccine
Ac1 Ac1 Ac1 Ac1	ZA	NL	2015	Berg 27	E	Cattle	Bergville	+	956	5	VV1
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 27	B	Cattle	Bergville	+	955	5	VV2
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 17	A	Cattle	Bergville	+	943	5	Vaccine
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 24	A	Cattle	Bergville	+	814	5	Vaccine
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 24	C	Cattle	Bergville	+	955	5	VV2
Ac1 Ac28 Ac2 Ac28	ZA	NL	2015	Berg 24	V	Cattle	Bergville	+	814	4	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 30	G	Cattle	Bergville	+	811	4	Vaccine
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 30	I	Cattle	Bergville	+	954	5	VV2
Ac1 Ac1 Ac1 Ac1	ZA	NL	2015	Berg 20	H3	Cattle	Bergville	+	814	4	VV1

[a] Country and province abbreviations follow ISO 3166-2.
[b] OBP, Onderstepoort Biological Products (Pretoria, South Africa), which produces *A. marginale* subsp. *centrale* vaccine for sale.
[c] SANParks, South African National Parks; NZG, National Zoological Gardens of South Africa Biobanks.

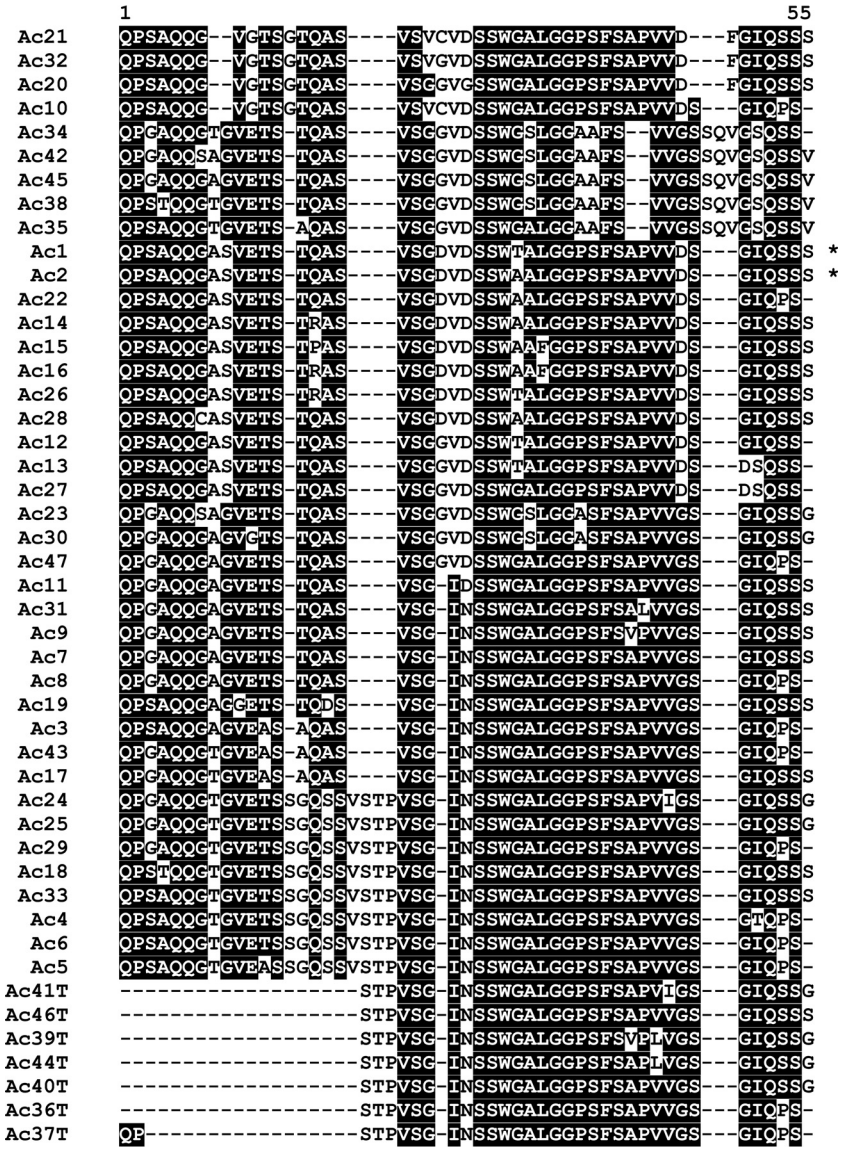

FIG 4 Alignment of *A. marginale* subsp. *centrale* Msp1aS tandem repeats detected from South African cattle, buffalo, and black wildebeest. The 47 repeat types were aligned using the AlignX module of Vector NTI, and groups of identical amino acids are highlighted on a black background. Ac1 and Ac2, the repeats present in the vaccine strain, are indicated with an asterisk.

lent (22). This suggests that wildlife species are reservoirs of *A. marginale* subsp. *centrale*.

We examined positive samples for *msp1aS* genotype, a genotyping scheme that has not previously been employed for *A. marginale* subsp. *centrale*. We identified 47 Msp1aS repeats which corresponded to 32 *A. marginale* subsp. *centrale* genotypes detected in cattle, buffalo, and wildebeest. The most common *A. marginale* subsp. *centrale* genotype among cattle samples was the vaccine genotype. This is not surprising as both farms that we sampled previously vaccinated with *A. marginale* subsp. *centrale* vaccine purchased from OBP. It is worth noting that cattle from farm 1 graze together with goats, sheep, and reedbuck, which might explain the diversity of *A. marginale* subsp. *centrale* strains detected on farm 1. We speculate that there is circulation of *A. marginale* subsp. *centrale* strains among different hosts, which led to the variety of genotypes detected on this farm. Cattle from farm 2 are confined within a grazing area with no interaction with other ruminants. The vaccine genotype was detected in all but one of the animals tested on this farm. In addition to the vaccine genotype, several closely related genotypes were detected, which suggests that the vaccine genotype is changing under selection pressure. This is interesting as we do not see these types of changes in the *msp1α* genotype in *A. marginale*-infected cattle. All repeats detected on farm 2 had an edit distance of two or less from one of the vaccine strain repeats, indicating that these repeats were closely related to the vaccine strain repeats. However, we cannot be sure that the vaccine strain is changing rather than there being an introduction of these new, related genotypes.

The unvaccinated cattle samples from Western Cape and Free State had different *A. marginale* subsp. *centrale* genotypes, while unvaccinated cattle samples from KwaZulu-Natal all had the same *A. marginale* subsp. *centrale* genotype. The vaccine strain was de-

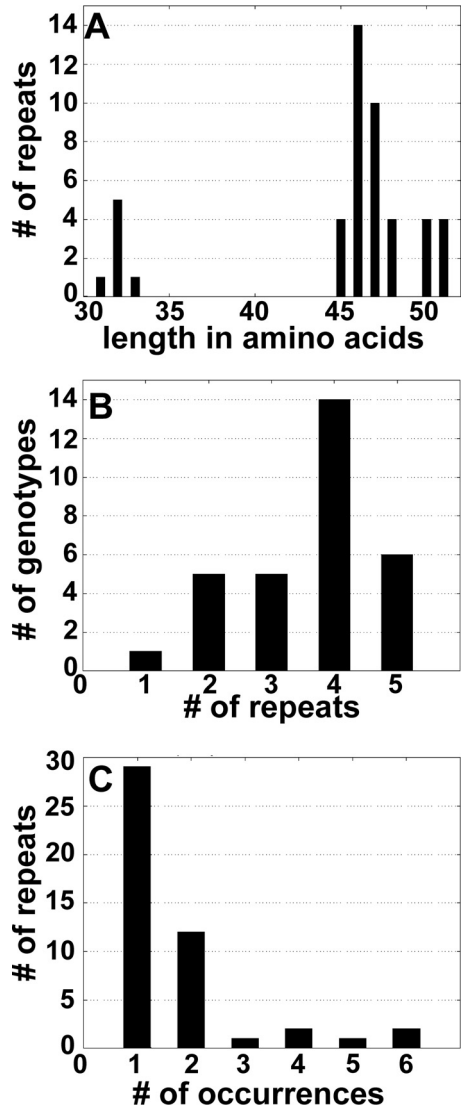

FIG 5 Metrics for *A. marginale* subsp. *centrale* Msp1aS repeats. (A) Number of repeats with a given number of amino acids; i.e., there are four repeats with a length of 45 amino acids. (B) Number of genotypes having a given number of repeats; i.e., 14 genotypes contain four repeats. (C) Number of times a given repeat occurs in our genotype data set; i.e., two repeats occur in six different genotypes.

tected in one of the unvaccinated cattle in the Western Cape. The *A. marginale* subsp. *centrale* genotypes obtained from wild ruminants were diverse, demonstrating geographic segregation of national parks. The repeat Ac8 was common in the *msp1aS* genotypes found in buffalo, even though the buffalo were sourced from parks distributed around South Africa. Ac8 has an edit distance of nine to both repeats Ac1 and Ac2, indicating that it is not closely related to the vaccine strain repeats.

While we have presented diversity metrics broken down by province, we think that the sample size is too small for this to be really meaningful in most cases, i.e., in Mpumalanga and Gauteng, there is an $n = 1$. More importantly, these metrics show us that for South Africa, as a whole, there is a high degree of repeat diversity within genotypes (Table 3, GDM1-L) and a moderate degree of novel genotypes across the country (Table 3, GDM1-G). The low

TABLE 3 Diversity scores for cattle and wildlife hosts by province and host

Location	GDM1-L	GDM1-G	GDM2-L	GDM2-G
All	0.747	0.420	0.065	0.022
Eastern Cape	0.863	0.583	0.069	0.060
Gauteng	0.500	0.500	0.250	0.250
KwaZulu-Natal	0.696	0.419	0.071	0.044
Mpumalanga	0.250	0.250	0	0
Northern Cape	0.760	0.632	0.067	0.050
Free State	0.417	0.286	0.125	0.357
Western Cape	0.750	0.750	0.125	0.093
Buffalo	0.781	0.500	0.051	0.030
Cattle	0.684	0.418	0.081	0.041

GDM2 values indicate that the repeats are dispersed, which is what is expected when the numbers of unique repeats and genotypes are high. This high degree of novel repeats indicates that the repeats have likely been circulating in nature and undergoing selection

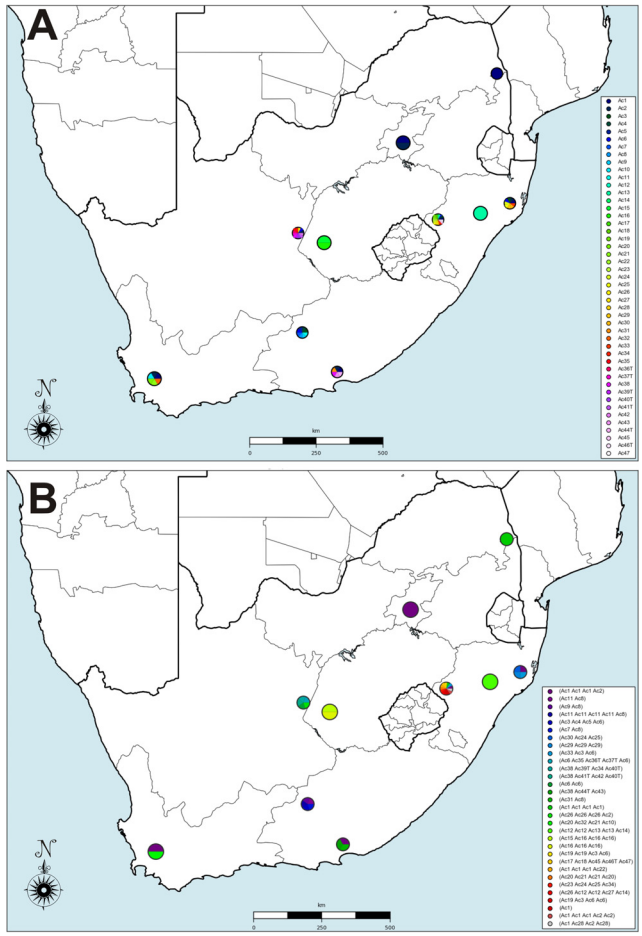

FIG 6 Maps of repeat and strain distribution. (A) Repeats mapped to the provinces of South Africa by GPS coordinates. (B) Strain genotypes mapped to the provinces of South Africa by GPS coordinates. The size of the circle indicates the precision of the location report, with three sizes being possible, corresponding to country, province, and precise GPS location. In these maps, there are no reports that are simply to the country level; i.e., all locations are at the provincial level or more specific. Therefore, there are only two sizes of circles shown. The samples collected from the Free State and Western Cape are marked at the provincial level and, thus, have larger markers.

and change separate from the vaccine strain. As more data are collected, it will be interesting to see if these metrics shift and how these metrics compare with those collected in other countries.

While the *A. marginale* subsp. *centrale* vaccine strain was thought for a long time not to be transmitted by most ticks, it was shown that, in fact, it colonized the tick well but was not secreted into the tick saliva in sufficient quantities for robust transmission (23, 24). Dramatically increasing tick numbers in transmission experiments overcame the transmission barrier (25). Is the reduced ability of the *A. marginale* subsp. *centrale* vaccine strain to be tick transmitted due to long serial needle passage through cattle? Or is there, perhaps, a specific vector-pathogen adaptation? There is a report of apparently efficient tick transmission of *A. marginale* subsp. *centrale* vaccine strain from *Rhipicephalus simus* ticks (26). Although *R. simus* is a proven vector in laboratory conditions, this tick is not found on cattle in large numbers, and the immature stages do not normally infest cattle (27). It would appear that the strains that we have detected circulating in wild animals today are maintained in nature via a natural tick-transmission cycle; however, this remains a speculation at this stage, as we have not tested ticks or performed transmission studies due to the complexities of working with the ecosystem of infections present in South Africa. If, in fact, *A. marginale* subsp. *centrale* is being spread through natural transmission to cattle, it is likely mitigating some of the disease burden of anaplasmosis caused by *A. marginale*.

In conclusion, this paper presents a novel genetic test based on *msp1aS* to discriminate strains of *A. marginale* subsp. *centrale* and shows that the vaccine strain is found widely distributed across South Africa and in animals that do not have a history of vaccination. Further, we present metrics indicating a high degree of Msp1aS repeat diversity in South Africa. Our results indicate the significance of wildlife as reservoir hosts for *A. marginale* subsp. *centrale*.

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