Identification and molecular characterization of *Babesia* species in brown (*Parahyaena brunnea*), striped (*Hyaena hyaena*) and spotted hyaenas (*Crocuta crocuta*)

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DECLARATION

I hereby declare that this dissertation, which I hereby submit for the Master of Science degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, to be my own work and has not been previously submitted by me for degree purposes at another tertiary institution.

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REJ Burroughs

December 2014

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SUMMARY

Haemoparasites have been a source of economic and research interest for animal managers for some time. Of these, the role played by the Apicomplexan *Babesia* and *Theileria* species in both domesticated animals and wildlife has been of major importance, as has been the role that tick vectors play in their life cycle and the routes of transmission to domesticated animals. *Babesia* and *Theileria* species differ in morphology, life cycle, epidemiology and the clinical signs produced in host species. Both genera are obligate intracellular parasites. A major difference between the two genera is the fact that *Theileria* species undergo schizogony and transstadial transmission in the tick vector, while *Babesia* species do not undergo schizogony and have a transovarial mode of transmission in the tick vector. Investigating the role that

Babesias in general play in domestic animals has largely focussed on the species that infect livestock, dogs, horses and, to a lesser extent, cats. Until the advent of molecular techniques, Babesias were identified on the basis of direct light microscopic morphology, serological tests and life cycle, with associated symptomatology and pathology caused in different species.

The role that wild animals play in a variety of diseases that affect domesticated animals has long been implied, but is often unsubstantiated. In some instances, these parasites have recently been identified as of major potential significance, particularly in asymptomatic carriers of the infection. The relationships between the *Babesia* species infecting hyaenas and those of other carnivores need to be further explored. Fluctuations of mammal populations occur in small managed reserves, but more importantly, in larger ecosystems. The role that disease, and particularly babesiosis, may play in changing interactions and population numbers between these predators needs to be investigated further. This can only be done if the parasites that play a role in disease can be accurately identified. Molecular tools are particularly suited to opening up of potential avenues for investigating potential disease aspects of predator and scavenger populations, and the role that this may have in disease ecology implications for these animals, and possibly for related and other wild carnivores.

A total of 55 blood and skin samples collected from different hyaenas were obtained from the biobanks of the SANParks and the National Zoological Gardens of SA (NZG). Twelve of the samples were from brown hyaenas (*Parahyaena brunnea*), 39 were from spotted hyaenas (*Crocuta crocuta*) and four were from striped hyaenas (*Hyaena hyaena*) These were screened for the presence of *Babesia* and *Theileria* species using the Reverse Line Blot (RLB) hybridization technique. Samples were simultaneously screened for the presence of *Ehrlichia* and *Anaplasma* spp. The RLB results revealed that the PCR products of 74.5% of the samples hybridized only with the *Theileria/Babesia* genus-specific probe, 80% with the *Babesia*-1, 74.5% with the *Babesia*-2 genus-specific probe and not with any of the species-

specific probes, suggesting the presence of a novel species or variant of a species. No *Ehrlichia* and/or *Anaplasma* species could be detected. The parasite 18S rRNA gene of five spotted hyaena samples was subsequently amplified, cloned and the recombinants sequenced. Homologous sequence searches of databases that were performed using the BLASTn package indicated that the obtained sequences were most closely related (98 – 99% identity) to *B. lengau*, previously identified in cheetah in South Africa. The observed sequence similarities were subsequently confirmed by phylogenetic analyses which showed that the obtained hyaena sequences formed a monophyletic group with *B. lengau, B. conradae* and sequences previously isolated from humans and wildlife in the western USA. Within the *B. lengau* clade, the obtained sequences and the published *B. lengau* sequences grouped into four distinct groups, of which groups I, II and III each represented a novel *B. lengau* genotype. It remains difficult to establish how much 18S rRNA gene sequence variation must exist for the source organism to be considered a different species or to be considered merely a variant and/or genotype of a species. Based on this, as well as the fact that we do not have any data on the morphology of the parasites, their possible vectors or their role in clinical disease, we suggest that these genotypes cannot be classified as new *Babesia* species, but rather as variants of *B. lengau.*

The study confirms that hyaena are susceptible to infection by a *Babesia* sp similar to *B. lengau*, but also demonstrates that they are not clinically affected by the infection. Their role as carriers of this organism and their ability to carry this infection over to other species still needs elucidation.

CHAPTER 1

INTRODUCTION

Haemoparasites have been a source of economic and research interest for animal managers for some time. Of these, the role played by the Apicomplexan *Babesia* and *Theileria* species in both domesticated animals and wildlife has been of major importance, as has been the role that tick vectors play in their life cycle and the routes of transmission to domesticated animals. *Babesia* and *Theileria* species differ in morphology, life cycle, epidemiology and the clinical signs produced in host species (Uilenberg, 2006). Both genera are obligate intracellular parasites. A major difference between the two genera is the fact that *Theileria* species undergo schizogony and transstadial transmission in the tick vector (Mehlhorn, Schein & Ahmed, 1994), while *Babesia* species do not undergo schizogony and have a transovarial mode of transmission in the tick vector (Uilenberg, 2006). The role of *Babesia* in animals in general has been reviewed by Uilenberg (2006), and more recently by Schnittger, Rodriguez, Florin-Christensen & Morrison (2012). Investigating the role that Babesias in general play in domestic animals has largely focussed on the species that infect livestock, dogs, horses and, to a lesser extent, cats. Until the advent of molecular techniques, Babesias were identified on the basis of direct light microscopic morphology, serological tests and life cycle, with associated symptomatology and pathology caused in different species (Uilenberg, 2006).

The role that wild animals play in a variety of diseases that affect domesticated animals has long been implied, but is often unsubstantiated. In some instances, these parasites have recently been identified as of major potential significance. The role that *Theileria parva* plays in carrier African buffaloes (*Syncerus caffer*) for instance is a case in point. Buffaloes are

asymptomatic carriers of the infection, which is highly fatal for domestic cattle in Southern Africa. Equally, forms of *Theileria*, *Anaplasma*, and *Babesia* have been described that are largely asymptomatic in their wildlife hosts, but under certain conditions, can prove fatal. This is particularly true in newly introduced animals, or animals that have been subject to some sort of stress factor that suppresses immunity to that particular parasite (Penzhorn, 2006). This may be as a result of a direct management procedure that the animal is subjected to, but can also be as a result of population-modulating factors such as drought and accompanying malnutrition, or social/behavioural influences.

JUSTIFICATION AND AIM OF THIS STUDY

The relationships between the *Babesia* species infecting hyaenas and those of other carnivores need to be further explored. Fluctuations of mammal populations occur in small managed reserves, but more importantly, in larger ecosystems. Large carnivores such as hyaenas and lions (*Panthera leo*) are continually in a state of flux in relation to numbers of other predators and/or scavengers such as black-backed jackals (*Canis mesomelas*), and to lesser extent with African wild dogs (*Lycaon pictus*). The role that disease, and particularly babesiosis, may play in changing interactions and population numbers between these predators needs to be investigated further. This can only be done if the parasites that play a role in disease can be accurately identified.

Molecular tools are particularly suited to opening up potential avenues for investigating potential disease aspects of predator and scavenger populations, and the role that this may have in disease ecology implications for these animals, and possibly for related and other wild carnivores.

The objective of the study was the identification and molecular characterisation of *Babesia* species that occur in brown (*Parahyaena brunnea*), striped (*Hyaena hyaena*) and spotted (*Crocuta crocuta*) hyaenas.

Specific objectives of the study were:

1. Collection and screening of hyaena blood samples for the presence of *Babesia* and *Theileria* species using the Reverse Line Blot (RLB) hybridization assay. Samples were simultaneously screened for the presence of *Ehrlichia* and *Anaplasma* spp.
2. Molecular characterization (by cloning and sequencing of the 18S rRNA gene) of novel *Babesia* spp. found.
3. Phylogenetic analysis and design of species-specific RLB oligonucleotide probes in the hyper-variable V4 region of the 18S rRNA gene of the novel species found.

CHAPTER 2

LITERATURE REVIEW

Host susceptibility and taxonomy

Babesia species have been described in a number of domestic and wild animal species, including carnivores, ungulates (perissodactyls and artiodactyls), rock hyraxes (Kjemtrup, Thomford, Robinson & Conrad 2000; Nijhof, Penzhorn, Lynen, Mollel, Morkel, Bekker & Jongejan, 2003; Penzhorn, 2006; Bosman, Venter & Penzhorn, 2007; Oosthuizen, Zweygardt, Collins, Troskie & Penzhorn, 2008; Oosthuizen, Allsopp, Troskie, Collins, & Penzhorn, 2009; Schnittger *et al*., 2012), as well as rodents and humans (Persing, Mathiesen, Marshall, Telford, Spielman, Thomford & Conrad, 1992).

The taxonomy of *Babesia* species of some domestic carnivores has undergone significant changes within the last 20 years. The species of significance in dogs were separated into a morphologically large *B. canis* grouping and those associated with a morphologically smaller *B. gibsoni*. The differentiation of *B. canis* into 3 species, *B. rossi*, *B. vogeli*, and *B. canis*, has also allowed a more precise study of the epidemiology of the diseases and the vectors associated with each species of parasite (Matjila, Penzhorn, Bekker, Nijhof & Jongejan, 2004; Uilenberg, 2006). The vector for *B. rossi* has been identified as *Haemaphysalis elliptica* (previously regarded as a synonym of *H. leachi)*, *B. vogeli* as *Rhipicephalus sanguineus* and *B. canis* as *Dermacentor reticularis* (Uilenberg, Franssen, Perie & Spanjer, 1989; Lewis, Penzhorn, Lopez-Rebollar & De Waal, 1996; Matjila, Leisewitz, Jongejan & Penzhorn, 2008a). Other species of *Babesia* have also been identified, e.g. *B. conradae* (Kjemtrup, Wainwright,

Miller, Penzhorn & Carreno, 2006; Kjemtrup & Conrad, 2006), which have specific local epidemiological significance. More and more species of *Babesia* are being identified that are capable of infecting dogs, as described by Birkenhauer, Neel, Ruslander, Levy & Breitschwerdt (2004). The most commonly found *Babesia* in domestic cats is still classified as *B. felis*, but clinical signs in domestic cats have also been attributed to *B. lengau*. (Bosman, Oosthuizen, Pierce, Venter & Penzhorn, 2010; Bosman, Oosthuizen, Venter, Steyl, Gous and Penzhorn, 2013).

A number of *Babesia* species have been described in wild carnivores (Penzhorn, 2006): *B. cynicti* in yellow mongoose (*Cynictis penicillata*) (Penzhorn & Chaparro, 1994), and *B. canis rossi* in black-backed jackal (*Canis mesomelas*), side-striped jackal (*Canis adustus*), and African wild dogs (*Lycaon pictus*) (Penzhorn, 2006; Matjila, Leisewitz, Jongejan, Bertschinger & Penzhorn, 2008b). *Babesia lotori* has been described in raccoons (*Procyon lotor*) (Anderson, Magnarelli & Sulzer, 1981), and new *Babesia* species in racoons have been found in Japan (Jinnai, Kawabuchi-Kurata, Tsuji, Nagajima, Fujisawa, Nagata, Koide, Matoba, Asakawa, Takahashi & Ishihara, 2009). The role that *Babesia* plays in wild carnivores in Southern Africa has been reviewed by Bosman *et al*. (2007). *Babesia felis* has been described in African wild cat (*Felis sylvestris*), an unidentified *Babesia* in cheetah (Averbeck, Bjork, Packer & Herbst, 1990) which was later described as *Babesia lengau* (Bosman *et al*., 2010), *Babesia leo* in lions (*Panthera leo*) (Lopez-Rebollar, Penzhorn, De Waal, & Lewis, 1999; Penzhorn, Kjemtrup, López-Rebollar & Conrad, 2001; Bosman *et al*., 2007) and *Babesia pantherae* in leopards (*Panthera pardus*) in Kenya (Dennig & Brocklesby, 1972). Subsequent work by Githaka, Konnai, Kariuki, Kandama, Murata & Ohashi (2012) in Kenya confirmed the existence of a unique *Babesia* sequence in leopards, but it could not be correlated back to the original parasite described by Dennig and Brocklesby (1972).

Experimental infection of coyotes (*Canis latrans*) with *B. gibsoni* highlights the potential role that wild species may play in infecting domestic animals (Evers, Kocan, Reichard & Meinkoth, 2003). Some of these were identified on the basis of serological tests such as the fluorescent antibody tests, which provide an indication of past infection only (Gubbels, De Vos, Van Der Weide, Viseras, Schouls, De Vries, & Jongejan, 1999). Many of these have been described by older morphological means only, and the similarities and differences between the parasites have not been adequately explored.

The incidence of *Babesia* in hyaenas is largely unknown; similarly, as for many carnivores, the method of transmission between individuals is also not known (Penzhorn *et al.,* 2001). A piroplasm reported from a spotted hyaena (*Crocuta crocuta*) was initially assigned the name *Nuttalia alberti* (Van Den Berghe 1937), but was later referred to as *Babesia alberti* (Peirce, Anderson & Penzhorn, 2001; Penzhorn, 2006). Species classification status accredited to this parasite needs to be verified, however. *Babesia lengau* has recently been described in spotted hyaena in Zambia (Williams, Berentsen, Shock, Teixera, Dunbar, Becker & Yabsley, 2013). Blood samples obtained by the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria indicated the presence of a *Babesia* species, which needed to be elucidated.

Transmission and Life cycle

Babesia spp. are transmitted by ticks of the family Ixodidae, and show typical life cycle characteristics of the Apicomplexa, with both a gametocyte stage present in the vertebrate host, and a sporozooite stage in the invertebrate host (Chauvin, Moeau, Bonnet, Plantard & Malandrin, 2009). This is presented schematically in Figure 1.

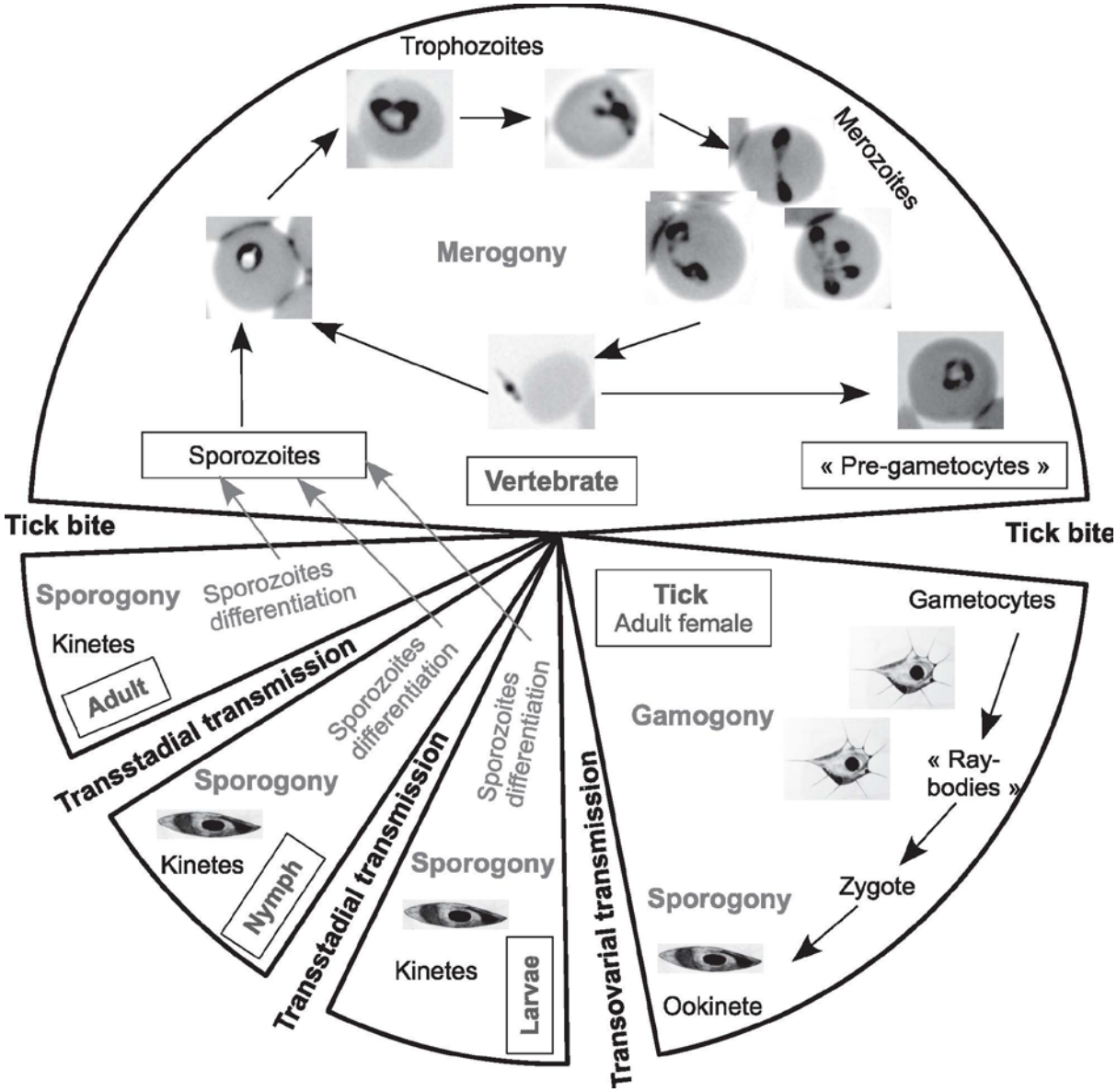

Figure 1: Schematic representation of the life cycle of *Babesia* spp. (Chauvin *et al.,* 2009)

There are indications that other genera of ticks may be involved in the transmission of *Babesia* spp., particularly the argasid or soft ticks. Under experimental conditions, *Ornithodoros moubata* could transmit *B. gibsoni* to dogs (Battsetseg, Matsuo, Xuan, Boldbataar, Chee & Umemiya, 2007), and there are suggestions that potentially pathogenic *Babesia* spp. can infect bats and birds using argasid ticks as a vector (Schnittger *et al.,* 2012).

In the vertebrate host, sporozoites are transmitted from the salivary glands of the ticks, and enter the red blood cells directly without undergoing any further development, as is the case in *Theileria* spp, and are then called trophozoites (Uilenberg, 2006). This trophozoite proliferates by merogony (binary fission) to form 2 or more merozoites in the cell. This process may eventually result in lysis and death of the host cell. These merozoites then invade other red blood cells, and repeat the process. A pre-gametocyte stage is also produced, which is then the infective stage for ticks if they attach on an infected host (Uilenberg, 2006; Chauvin *et al*., 2009).

In the tick, the pre-gametocyte stage consumed during the blood meal by the adult female tick transforms into gametocytes in the lumen of the gut. These gametocytes fuse to form zygotes, which penetrate the cells lining the gut of the tick. This process is referred to as gamogony (Chauvin *et al*., 2009). In the cells lining the gut, the cells change to a motile ookinete stage during which meiosis occurs (sporogony). Penetration of various organs in the tick now occurs (ovaries, salivary glands), and the gamonts change to sporozoites, which is the infective form for vertebrates (Uilenberg, 2006; Chauvin *et al*., 2009).

The role of hyaena as hosts

Hyaenas have a zoological classification distinct from other members of the Order Carnivora, namely within the Family Hyaenidae. Their closest relative is the aardwolf (*Proteles cristatus*: Family Protelidae), in which a species of *Babesia* has been described (Peirce *et al*., 2001), which to date has not been further characterised.

The social organization and ecological niche that brown (*Parahyaena brunnea*) and spotted hyaenas (*Crocuta crocuta*) fill are slightly different. Spotted hyaenas often form packs for both social and foraging purposes, and can be predators rather than scavengers exclusively

(Skinner & van Aarde, 1981; Mills, 1984). Brown hyaenas are solitary foragers, but can be encountered in small groups (Mills, 1984). In interactions between the 2 species, spotted hyaena are clearly dominant (Mills,1984). Striped hyaena (*Hyaena hyaena*) are generally solitary and much smaller than spotted hyaena, but occupy much the same ecological niche where their distributions overlap (Kruuk, 1976). Striped hyaena are scavengers, but will predate on smaller animals such as hares and smaller antelope, and will consume insects, fruits, birds and reptiles (Kruuk, 1976).

The role of disease in scavengers is poorly documented in relation to that described in other carnivores, and their role in disease ecology in general is not well understood. A noted feature of scavengers thriving in ecosystems where they consume carcasses infected with various pathogens is that they often suffer no apparent ill-effects. This is the case in the Kruger National Park of South Africa, where the incidence of tuberculosis is high, with no reports of infection in hyaena.

It is clear that hyaenas under free-living conditions do succumb to infectious diseases such as canine distemper (Alexander, Kat, Frank, Holekamp, Smale, House & Appel, 1995; Haas, Hofer, East, Wohlein, Liess & Barret, 1996), *Streptococcus* infection (Höner, Wachter, Speck, Wibbelt, Ludwig, Fyumaga, Wohlsein, Lieckfeldt, Hofer & East, 2006) and hepatozoonosis (East, Wibbelt, Liekfeldt, Goller, Wilhem, Schares, Thierer & Hofer, 2008). In none of the reports is there mention of concomitant *Babesia* infection.

The role of parasites in ecosystems in general has also been the subject of debate and has led to the construction of models proposing reasons for this (Linnel & Strand, 2000; Seilacher, Reif & Wenk, 2007). The dynamics of parasites within monoxenic and heteroxenic hosts have specific evolutionary advantages, and are essential to the well-being of all ecosystems over evolutionary time (Seilacher *et al.,* 2007). These interactions can work at a number of levels –

molecular, physiological, genetic and ecological (Seilacher *et al.,* 2007). The roles of host specificity and long-term persistence of *Babesia* parasites in hosts have been described by Chauvin *et al.* (2009). Besides ecological mechanisms that may exist, molecular responses and adaptations of parasites, ticks and hosts are of major importance (Chauvin *et al.,* 2009). Although tolerance and resistance can be expected to be balanced over time, this is subject to significant fluctuations to accommodate many factors, including genetic variation and co-evolution (Råberg, Sim & Read, 2007).

The concept of "ecological fitting" provides a model for the long-term evolutionary relationship between host, parasite and transmission in response to inevitable climate change (Polley & Thompson, 2009).

Techniques for identification of *Babesia sp*.

Conventional methods of identification and classification have in the past proved inconsistent. Parasites may not have been present in specific stained blood smears, or the level of parasitaemia, particularly in chronic cases, was inadequate. The advance in molecular diagnostic techniques has allowed precise and consistent identification of specific *Babesia* parasites, and indeed has allowed re-classification of some *Babesia* species. *Theileria equi*, for example, was until recently regarded as *Babesia equi (*Uilenberg, 2006*)* (Laveran, of malaria fame, initially named this organism *Piroplasma equi;* it has been classified in at least 8 other genera). *Babesia* and *Theileria* species have been characterised using the 18S rRNA gene (Gubbels *et al*., 1999; Cacciò, Antunovic, Moretti, Mangili, Marinculic, Baric, Slemenda & Pieniazek, 2002; Schnittger, Yin, Qi, Gubbels, Beyer, Niemann, Jongejan, & Ahmed, 2004), while other members of the group such as *Anaplasma* and *Ehrlichia* are analysed using the 16S rRNA gene (Matjila *et al*., 2008a).

Using Polymerase Chain Reaction (PCR) techniques has been invaluable in amplifying parasitic DNA, but very few techniques have been readily available that identify mixed infections (Nagore, García-Sanmartín, García-Pérez, Juste & Hurtado, 2004). This may be crucial in wild animals, where both mixed species and mixed genus infections may be present. The application of a standard PCR in combination with a reverse-line blot (RLB) hybridisation technique has made this a much simpler process (Gubbels *et al.,* 1999; Schnittger *et al.,* 2004; Bhoora, Franssen, Oosthuizen, Guthrie, Zweygarth, Penzhorn, Jongejan & Collins 2009; Oosthuizen *et al*., 2009). With the addition of a genus-specific *Babesia*/*Theileria* probe, all PCR products are identified – those that are not specific and that bind to the genus-specific probe would indicate that a new species or variant of a species may be present, and can be targeted for further analysis (Nagore *et al.,* 2004; Oosthuizen *et al.,* 2009).

Complete 18S rRNA gene sequence analysis, after cloning into a suitable plasmid vector, then becomes possible. These sequences can be aligned and compared with sequences that are available on known databases to provide identity and phylogeny.

These techniques have been used on haematozoan parasites found in a variety of wildlife species (Nijhof *et al.,* 2003; Oosthuizen *et al*., 2009), and on a number of *Babesia* species associated with carnivores (Penzhorn *et al.,* 2001; Bosman *et al.,* 2007; Matjila *et al.,* 2008b). To date there has not been a study investigating *Babesia* parasites that have been described in hyaena using molecular techniques. On the basis of current knowledge, molecular techniques are thus well suited for the envisaged study.

CHAPTER 3

MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN AND METHODOLOGY

A schematic presentation of the laboratory processes and analysis is given in Figure 2.

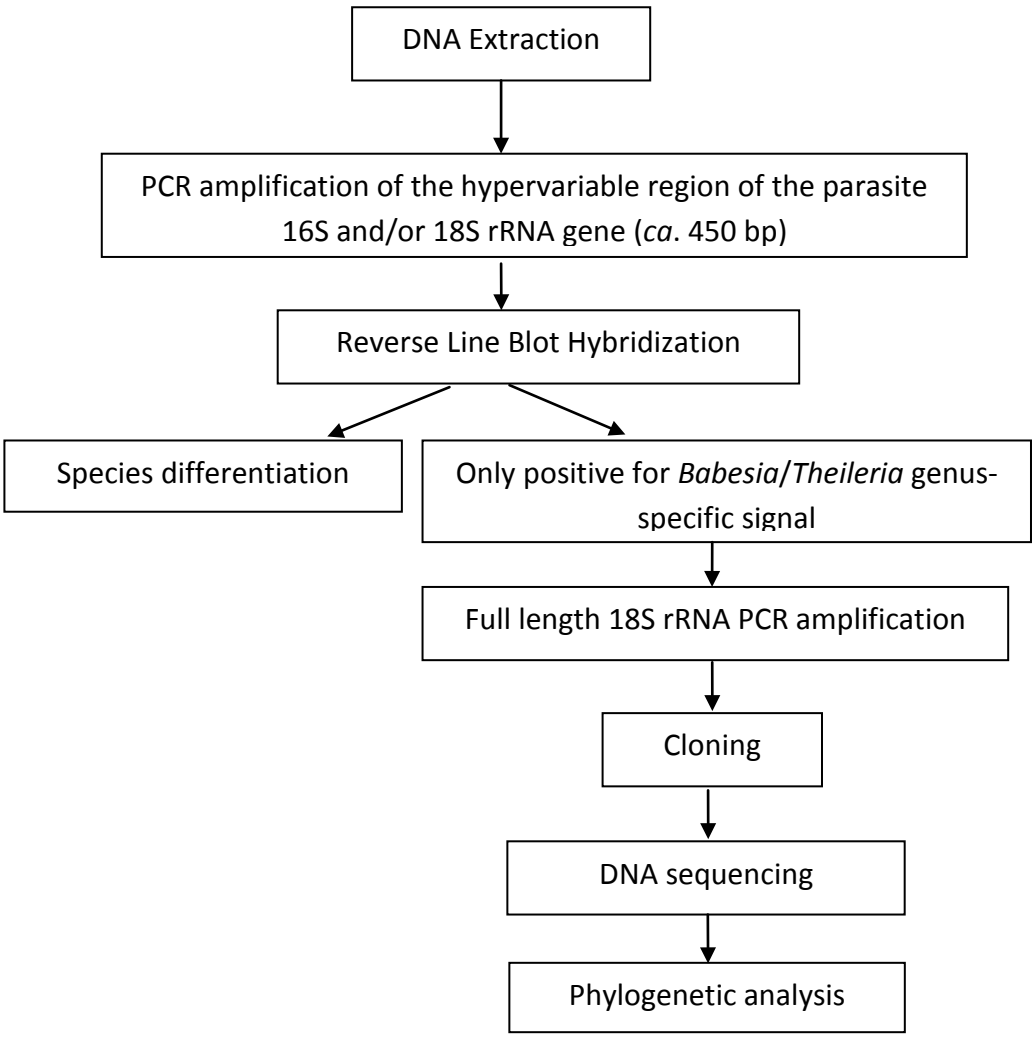

Figure 2: Work-flow diagram of laboratory processes

3.2 SAMPLE COLLECTION

A number of sample sources were utilized; 8 DNA and 2 whole blood samples were available from the Biobank of the Department of Veterinary Tropical Diseases, which had been obtained from a previous study from the Biobank of the National Research Foundation. Further samples were obtained from the Biobank of the National Research Foundation, which consisted of the following: seven spotted hyaena whole blood samples collected in EDTA, one heparin whole blood sample from a spotted hyaena, three skin samples from brown hyaena and four whole blood samples from striped hyaena. An additional 30 samples were obtained from the BioBank of the South African National Parks in Skukuza, which were all spotted hyaena. These are summarized in Table 1.

Table 1: Summary of hyaena samples used in the analysis, indicating date of DNA extraction and origin.

Extraction Number	Sample ID	Species	Sample Type	DNA Extraction date	Origin
National Research Foundation Biobank:					
1	11658	Spotted Hyaena	Blood	31/10/2011	NZG
2	12091	Spotted Hyaena	Blood	31/10/2011	NZG
3	12092	Spotted Hyaena	Blood	31/10/2011	NZG
4	13184	Spotted Hyaena	Blood	31/10/2011	NZG
5	13185	Spotted Hyaena	Blood	31/10/2011	NZG
6	13186	Spotted Hyaena	Blood	31/10/2011	NZG
7	13211	Spotted Hyaena	Blood	31/10/2011	NZG
8	13223	Spotted Hyaena	Blood	31/10/2011	NZG
9	14175	Striped Hyaena	Blood	31/10/2011	JHB Zoo
10	15921	Striped Hyaena	Blood	31/10/2011	NZG
11	15935	Striped Hyaena	Blood	31/10/2011	NZG
12	15942	Striped Hyaena	Blood	31/10/2011	NZG
13	1484	Brown Hyaena	Skin	31/10/2011	Lion Safari Park, RSA
14	13668	Brown Hyaena	Skin	31/10/2011	NZG
15	5198	Brown Hyaena	Skin	31/10/2011	Waterberg, RSA
16	RLB 505/07	Brown Hyaena	Blood	31/10/2011	Pretoria, RSA
17	RLB 462/07	Spotted Hyaena	Blood	31/10/2011	Pretoria, RSA

18	RLB 28/04	Brown Hyaena	DNA	2004	Luderitz, Namibia
19	RLB 29/04	Brown Hyaena	DNA	2004	Luderitz
20	RLB 30/04	Brown Hyaena	DNA	2004	Luderitz
21	RLB 31/04	Brown Hyaena	DNA	2004	Luderitz
22	RLB 32/04	Brown Hyaena	DNA	2004	Luderitz
23	RLB 33/04	Brown Hyaena	DNA	2004	Luderitz
24	RLB 34/04	Brown Hyaena	DNA	2004	Luderitz
25	RLB 206/04	Brown Hyaena	DNA	2004	Namibia
SANParks Biobank:					
26	929/12	Spotted Hyaena	Blood	22/11/2012	KNP
27	930/12	Spotted Hyaena	Blood	22/11/2012	KNP
28	931/12	Spotted Hyaena	Blood	22/11/2012	KNP
29	932/12	Spotted Hyaena	Blood	22/11/2012	KNP
30	933/12	Spotted Hyaena	Blood	22/11/2012	KNP
31	934/12	Spotted Hyaena	Blood	22/11/2012	KNP
32	935/12	Spotted Hyaena	Blood	22/11/2012	KNP
33	936/12	Spotted Hyaena	Blood	22/11/2012	KNP
34	937/12	Spotted Hyaena	Blood	22/11/2012	KNP
35	938/12	Spotted Hyaena	Blood	22/11/2012	KNP
36	939/12	Spotted Hyaena	Blood	22/11/2012	KNP
37	940/12	Spotted Hyaena	Blood	22/11/2012	KNP
38	941/12	Spotted Hyaena	Blood	22/11/2012	KNP
39	942/12	Spotted Hyaena	Blood	22/11/2012	KNP
40	943/12	Spotted Hyaena	Blood	22/11/2012	KNP
41	944/12	Spotted Hyaena	Blood	22/11/2012	KNP
42	945/12	Spotted Hyaena	Blood	22/11/2012	KNP
43	946/12	Spotted Hyaena	Blood	22/11/2012	KNP
44	947/12	Spotted Hyaena	Blood	22/11/2012	KNP
45	948/12	Spotted Hyaena	Blood	22/11/2012	KNP
46	949/12	Spotted Hyaena	Blood	22/11/2012	KNP
47	950/12	Spotted Hyaena	Blood	22/11/2012	KNP
48	951/12	Spotted Hyaena	Blood	22/11/2012	KNP
49	952/12	Spotted Hyaena	Blood	22/11/2012	KNP
50	953/12	Spotted Hyaena	Blood	22/11/2012	KNP
51	954/12	Spotted Hyaena	Blood	22/11/2012	KNP
52	955/12	Spotted Hyaena	Blood	22/11/2012	KNP
53	956/12	Spotted Hyaena	Blood	22/11/2012	KNP
54	957/12	Spotted Hyaena	Blood	22/11/2012	KNP
55	958/12	Spotted Hyaena	Blood	22/11/2012	KNP

Notes:
NZG: National Zoological Gardens, Pretoria
JHB Zoo: Johannesburg Zoo
KNP: Kruger National Park

3.3 LABORATORY TECHNIQUES

3.3.1 DNA Extraction

Genomic DNA was extracted from the whole blood and the skin samples using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions.

The whole blood (200 µl) was mixed with 20 µl of protein kinase and incubated at 46°C for 3 hours. A lysis buffer was applied to the solution, which was then centrifuged for 3 seconds at 8000 rpm, and incubated for 20 minutes. Two centrifugation steps using washing buffers were then done prior to the addition of the elution buffer.

In the case of the skin samples, approximately 25 mg of tissue was mixed with 30 µl of protein kinase, and incubated at 46°C for 3 hours. The procedure used was then identical to that used for the whole blood.

DNA obtained from both the whole blood and the skin samples was eluted in 100 µl elution buffer and stored at –20 °C pending further analysis.

3.3.2 Polymerase Chain reaction

Theileria and *Babesia* genus-specific primers RLB F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and biotin-labelled RLB R2 (5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3') (Nijhof *et al*. 2005) were used to amplify the V4 hypervariable area of the parasite 18S rRNA gene. For the simultaneous detection of *Ehrlichia* and *Anaplasma* spp, primers Ehr-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') (Schouls *et al*., 1999) and biotin labelled reverse primer Ehr-R (5'-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3')

(Bekker, de Vos, Taoufik, Sparagano & Jongejan, 2002) were used to amplify the V1 hypervariable region of the 16S rRNA gene for *Ehrlichia* and *Anaplasma*. The PCR reaction mixture consisted of 12.5 µl of Platinum Quantitative PCR SuperMix-UDG (which contained of 3 mM MgCl2, 200 µM each of dGTP, dATP and dCTP, 400 µM dUTP, 0.75 U Platinum® Taq DNA polymerase and 0.5 U uracil deoxy-glycosylase) (Invitrogen, The Scientific Group, South Africa), 0.5 µM of each primer, 2.5 µl of DNA to achieve a total volume of 25 µl. Positive and negative controls were included in each batch of samples; the positive control was *Babesia bovis* DNA extracted from the *B. bovis* vaccine (Onderstepoort Biological Products, South Africa), and the negative control was water.

A touchdown PCR thermocycler program (Table 2) was applied for amplification under stringent conditions according to the methods desrcibed by Nijhof *et al.* (2005), after which 5 µl of the PCR product was examined on a 2% agarose gel that had been stained with ethidium-bromide and visualized under an ultra-violet transilluminator.

Table 2: Thermocycler program for *Ehrlichia/Anaplasma* and *Babesia/Theileria* touchdown PCR (adapted from DVTD laboratory manuals)

No of cycles	Duration	Temperature in °C	
1	3 min	37°C	Activation of UDG
1	10 min	94°C	Inactivation of UDG & activation of *Taq*
2	20 min 30 sec 30 sec	94°C 67°C 72°C	Denaturation Annealing Elongation
2	20 min 30 sec 30 sec	94°C 65°C 72°C	Denaturation Annealing Elongation
2	20 sec 30 sec 30 sec	94°C 61°C 72°C	Denaturation Annealing Elongation
2	20 sec 30 sec 30 sec	94°C 61°C 72°C	Denaturation Annealing Elongation

2	20 sec 30 sec 30 sec	94°C 59°C 72°C	Denaturation Annealing Elongation
40	20 sec 30 sec 30 sec	94°C 57°C 72°C	Denaturation Annealing Elongation
2	7 min	72°C	Final extension

3.3.3 Reverse Line Blot Hybrisiation Assay

The reverse line blot (RLB) hybridisation assay was followed according to the methods described by Gubbels *et al.* (1999) and Nagore *et al.* (2004). Known *Babesia, Theileria, Anaplasma* and *Ehrlichia* genus- and species-specific oligonucleotide probes were used at predetermined concentrations and their positions noted on the membrane. An in-house membrane was prepared containing the relevant genus- and species-specific probes as listed in Table 2. The preparation, hybridization, subsequent stripping and storage of the membrane were carried out as described by Gubbels *et al*. (1999). Briefly, the PCR products were denatured at 99.9°C for 10 minutes, and applied perpendicular to the probes on the membrane in a miniblotter. The positive control was loaded into lane 2. Cross flow into empty slots was prevented by filling these with 2X SSPE/0.1% SDS. Hybridization of the PCR products was carried out at 42.5°C for 60 minutes, after which the samples were removed by aspiration and the membrane washed twice in 2X SSPE/0.5% SDS at 50°C for 10 minutes with gentle shaking to remove non-specific PCR products that may have hybridized onto the membrane.

Table 3: List of genus- and species-specific primers used during the PCR and RLB hybridization. The degenerate position R denotes either A or G, W denotes either A or T.

Oligonucleotide probe	**Sequence (5' – 3')**	**Reference**
Ehrlichia/Anaplasma genus-specific	GGG GGA AAG ATT TAT CGC TA	Bekker, *et al.*, 2002
Anaplasma bovis	GTA GCT TGC TAT GAG AAC A	Bekker, *et al.*, 2002
Anaplasma centrale	TCG AAC GGA CCA TAC GC	RLB manual, Isogen
Anaplasma (Ehrlichia) sp. *Omatjenne*	CGG ATT TTT ATC ATA GCT TGC	Bekker, *et al.*, 2002
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	Bekker, *et al.*, 2002
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG	Bekker, *et al.*, 2002
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	Bekker, *et al.*, 2002,
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT	RLB manual, Isogen
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	RLB manual, Isogen
Theileria/Babesia genus-specific	TAA TGG TTA ATA GGA RCR GTT G	Gubbels, *et al.,* 1999
Theileria genus-specific1	ATT AGA GTG TTT CAA GCA GAC	Nijhof * (unpublished)
Babesia genus-specific 1	ATT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
Babesia genus-specific 2	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	Nijhof, *et al.,* 2003
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels, *et al.,* 1999
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels, *et al.,* 1999
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	Butler, *et al.,* 2008
Babesia canis	TGC GTT GAC GGT TTG AC	Matjila, *et al.,* 2004
Babesia rossi	CGG TTT GTT GCC TTT GTG	Matjila, *et al.,* 2004
Babesia vogeli	AGC GTG TTC GAG TTT GCC	Matjila, *et al.,* 2004
Babesia divergens	ACT RAT GTC GAG ATT GCA C	Nijhof, *et al,* 2003
Babesia felis	TTA TGC TTT TCCGAC TGG C	Bosman, *et al.,* 2007
Babesia gibsoni	TAC TTG CCT TGT CTG G TTT	Nijhof, *et al.* 2003
Babesia leo	ATC TTG TTG CTT GCA GCT T	Bosman, *et al.,* 2007
Babesia major	TCC GAC TTT GGT TGG TGT	Georges, *et al.,* 2001
Babesia microti	GRC TTG GCA TCW TCT GGA	Nijhof, *et al.,* 2003
Babesia occultans	CCT CTT TTG GCC CAT CTC GTC	He, *et al.,* 2012
Babesia sp. (sable)	GCT GCA TTG CCT TTT CTC C	Oosthuizen, *et al.,* 2008
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG	Matjila *et al.*, 2008
Theileria annulata	CCT CTG GGG TCT GTG CA	Georges, *et al.,* 2001
Theileria bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof, *et al.,* 2003
Theileria buffeli	GGC TTATTT CGG WTT GAT TTT	Gubbels, *et al.,* 1999
Theileria equi	TTC GTT GAC TGC GYT TGG	Butler, *et al.,* 2008
Theileria lestoquardi	CTT GTG TCC CTC CGG G	Schnittger, *et al.,* 2004
Theileria mutans	CTT GCG TCT CCG AAT GTT	Gubbels, *et al.,* 1999
Theileria ovis	TTG CTT TTG CTC CTT TAC GAC	Altay *et al.,* 2007
Theileria parva	GGA CGG AGT TCG CTT TG	Nijhof, *et al.,* 2003
Theileria separata	GGT CGT GGT TTT CCT CGT	Schnittger, *et al.,* 2004
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T	Oura, *et al.,* 2004
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG	Nijhof, *et al.,* 2005
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C	Nijhof, *et al.,* 2005
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels, *et al.,* 1999
Theileria velifera	CCT ATT CTC CTT TAC GAG T	Gubbels, *et al.,* 1999

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The membrane was then incubated with 10 ml 2X SSPE/0.5 SDS and 2.5 µl streptavidin-POD (peroxidase-labeled) conjugate (Roche Diagnostics, South Africa) at 42°C for 30 minutes, washed, spread with a chemiluminescence solution (ECL1 + ECL2) (DNA Thunder, Perkin Elmer, Separation Scientific, South Africa) and exposed to an X-ray film (X-OMAT Blue XB-1, Kodak, Separation Scientific, South Africa). After development of the X-ray film, the biotin-streptavidin-peroxidase complex that was formed produced dark spots where hybridization occurred and these were used to identify the specific species. Samples that were positive for genus-specific probes only, and not for the species-specific probes, were further characterized.

Stripping of the membrane was performed by washing the membrane twice in 1% SDS preheated to 80°C for 30 minutes under gentle shaking. Thereafter the membrane was washed in 20mM EDTA for 15 minutes at room temperature, also under gentle shaking. The membrane was stored in a plastic bag at 4°C in a solution of 20mM EDTA at pH 8.

3.5 Full-length 18S rRNA Amplification

The full-length 18S rRNA gene of five spotted hyaena samples was amplified using primers, Nbab_1F [5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3'] and Nbab_1R [5'-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC-3'] that were designed for the amplification of the 1 700 bp fragment of the 18S rRNA gene (Oosthuizen *et al*., 2008; Bhoora *et al*., 2009). PCR were performed using the High Fidelity PCR Master Mix (Roche Diagnostics, Mannheim, Germany) and according to the manufacturer's instructions. This Master Mix consisted of 1.5 mM $MgCl_2$, 200 µM dNTPs, High Fidelity Enzyme blend (concentration unknown) and nuclease-free water. Approximately 75 ng (2.5 µl) of the extracted DNA was mixed with 22.5 µl of this Master Mix, which then included 20 pmol/µl of each primer to achieve a total volume of 25 µl.

Six separate reactions were prepared per sample. To enable verification of which of the selected samples were successfully amplified, visualization on an agarose gel was performed. Five µl of the PCR product were loaded onto an agarose gel again together with 2 µl of a loading dye, and run for 40 minutes at 120 volts, using a Labnet Gl XL Enduro machine. Use was made of a GeneRuler 100 bp DNA ladder (Fermentas, Invitrogen, USA) as a marker.

Amplicons of all six reactions per sample were pooled to avoid Taq polymerase induced errors and cleaned-up using the QIAquick PCR Purification Kit (Qiagen, Southern Cross Biotechnologies), according to the manufacturer's instructions. Purification of the products was done to remove all primers, nucleotides, enzymes and other impurities that might interfere with subsequent analysis. The pure DNA was eluted in 20 µl elution buffer.

3.6 Cloning of the Full Length 18S rRNA Gene

Using the pGEM-T Easy Vector system (Promega, Madison, WI, USA), which included the T4 DNA ligase, the purified PCR fragment was ligated into the pGEM-T Easy vector and transformed into competent *E. coli* JM109 cells (JM109 High Efficiency Competent Cells, Promega, USA), which was plated out using standard techniques onto two imMedia AMP Blue culture plates (Introgen, USA) and incubated overnight at 37°C.

White colonies were picked and placed into Falcon tubes containing imMedia Amp Liquid incubator broth (Invitrogen, USA); blue colonies were discarded. Following an incubation step, isolation of the recombinant plasmids was done by means of the High Pure Plasmid Isolation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. This involved the use of stages of washing with suspension buffers (in the presence of RNase), lysis and binding buffers, and then eluted into 50 µl, and stored at -20°C.

3.7 DNA Sequencing and Phylogenetic analysis

Sequencing was performed using the ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), 350 ng plasmid DNA and 3.2 pmol of primer. The near full-length 18S rRNA gene sequences were sequenced using the RLB F2 and RLB R2 primers, as well as the vector primers SP6 (5'-TTA TAC GAC TCA CTA TAG GG-3') and T7 (5'-TAT TTA GGT GAC ACT ATA-3'). Sequencing was performed using an ABI3100 genetic analyser by Inqaba Biotech Systems located in Pretoria, South Africa.

The obtained sequences were assembed and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Staden, Beal, & Bonfield, 2000). Homologous sequence searches of databases were performed using the BLASTn package (Altschul, Gish, Miller, Meyer & Lipman, 1990), and a multiple sequence alignment was performed using ClustalX (version 1.81 for Windows) which included all related available genera from GenBank (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997). The alignment was truncated to the size of the smallest sequence (1321 bp) using BioEdit v7 (Hall, 1999). Similarity matrices were constructed from the aligned sequence data by single distance, using the two-parameter model of Kimura (1980). The Jukes and Cantor (1969) correction model was applied for multiple base changes. Phylogenetic trees were constructed using MEGA4 (Tamura, Dudley, Nei & Kumar, 2007) using both neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony. Bootstrapping was applied using 1000 replicates/tree for the distance method and 100 replicates/tree for the parsimony method (Felsenstein, 1985). All consensus trees generated were edited using MEGA4 (Tamura *et al.,* 2007). The GenBank accession numbers used to construct the phylogenetic tree is shown in Table 4.

The 18S rRNA gene sequences of the sequences identified in this study have been submitted to GenBank.

Table 4: 18S rRNA gene sequences used in the phylogenetic analyses.

Accession Number	Taxonomic Classification	Location	Source
AY072926	*B. canis*	Croatia	Dog
DQ111760	*B. rossi*	Sudan	Dog
AF158702	*B. conradae*	USA	Dog
AF231350	*B. conradae*	USA	Dog
HQ289870	*B. duncani*	USA	Human
HQ285838	*B. duncani*	USA	Human
AF244912	*B. felis*	South Africa	Lion
AY278443	*B. gibsoni*	Spain	Dog
EU583386	*B. gibsoni*	USA	Dog
GQ411417	*B. lengau*	South Africa	Cheetah
KC790443	*B. lengau*	South Africa	Domestic cat
AF244911	*B. leo*	South Africa	Lion
AY789075	*B. microti*	Poland	*Microtus agrestis*
AY072925	*B. vogeli*	Italy	Dog
DQ111754	*H. canis*	Sudan	Dog
M64243	*T. annulata*	NA	NA
L02366	*T. parva*	NA	NA
L19082	*T. taurotragi*	South Africa	NA

Note: NA = Not available or unknown.

CHAPTER 4

RESULTS

4.1 Reverse Line Blot Hybridisation

The RLB results revealed that the PCR products of 74.5 % of the samples (n = 41) hybridized only with the *Theileria/Babesia* genus-specific probes and not with any of the species-specific probes (Table 5 and Figure 3). The remaining 20 % (n = 11) of samples were negative (or below detection limit of the test) for any of the *Theileria* and/or *Babesia* species. All samples tested negative (or below detection limit of the test) for *Ehrlichia* and/or *Anaplasma* species. A representative RLB result is shown in Figure 4.

Table 5: Occurrence of haemoparasite species infections in hyaena samples as determined by RLB hybridization.

	Brown hyaena (n = 12) (%)	**Spotted hyaena (n = 39) (%)**	**Striped hyaena (n = 4) (%)**	**TOTAL (n = 55) (%)**
Theileria/Babesia genus-specific only	7 (58.3%)	34 (87.1%)	0 (0)	**41 (74.5%)**
Babesia genus-specific 1	8 (66.6%)	34 (87.1%)	2 (50%)	**44 (80%)**
Babesia genus-specific 2	6 (50.1%)	34 (87.1%)	1 (25%)	**41 (74.5%)**
Negative/below detection limit	4 (33.3%)	5 (12.8%)	2 (50%)	**11 (20%)**
Ehrlichia/Anaplasma genus-specific only	0 (0)	0 (0)	0 (0)	**0 (0)**

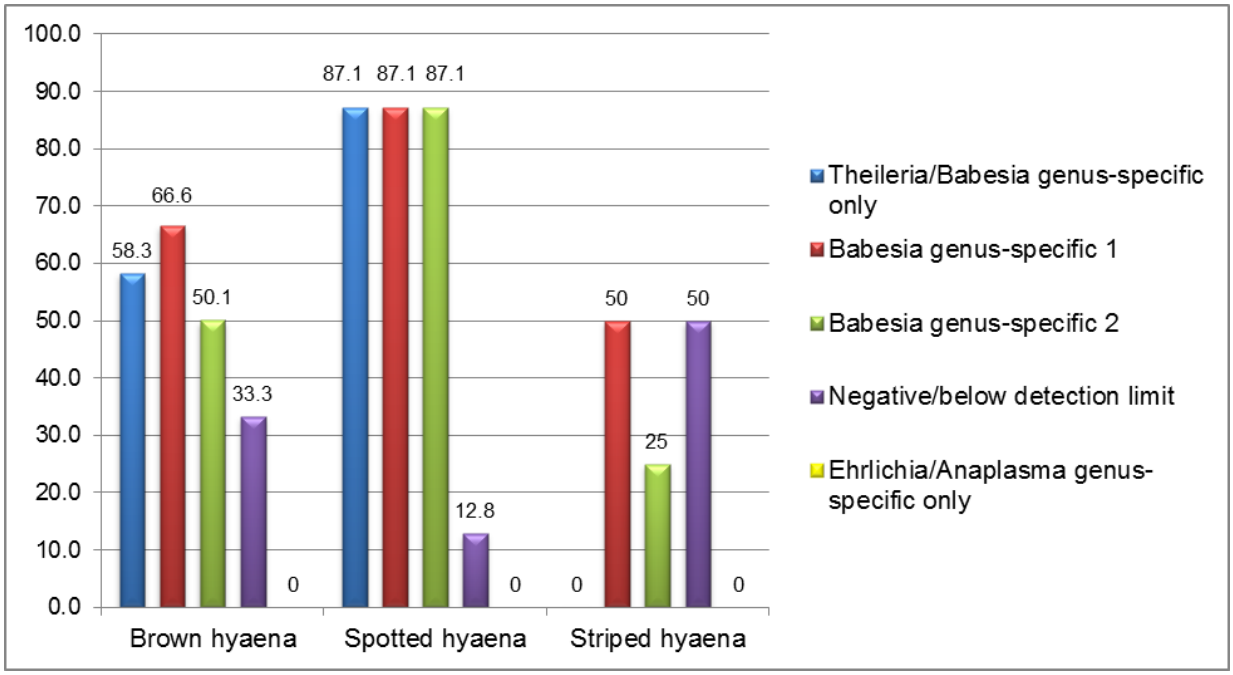

Figure 3: Occurrence of haemoparasite species infections (%) in hyaena samples as determined by RLB hybridization.

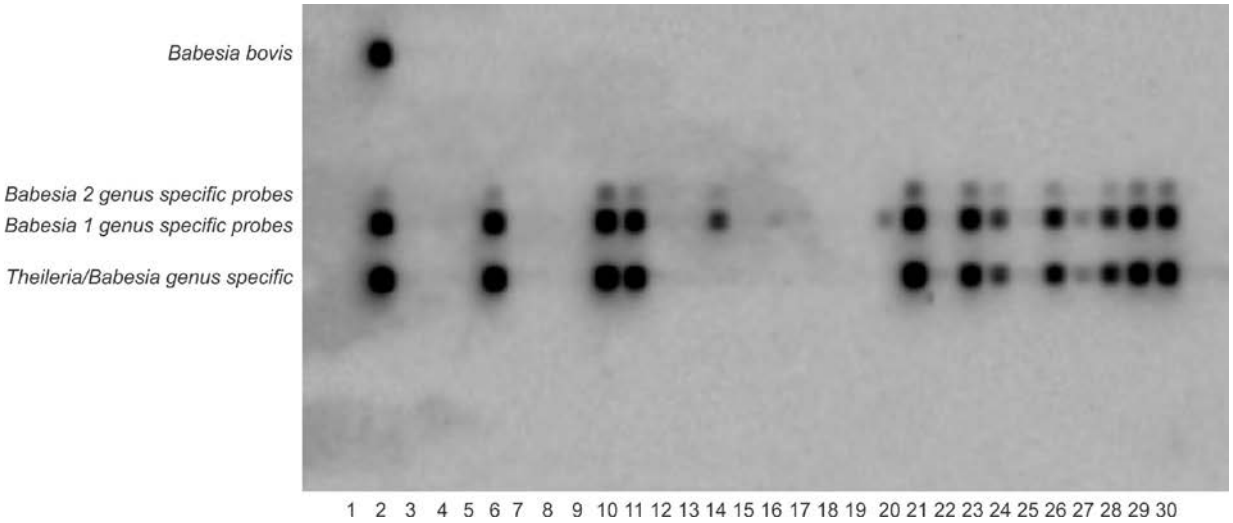

Figure 4: Representative reverse line blot (RLB) hybridization assay results for the simultaneous detection of *Theileria, Babesia, Ehrlichia*, and *Anaplasma* species in the hyaena samples.

4.2 Cloning, sequencing and phylogenic analysis

Five of the spotted hyaena samples were selected for molecular characterization by cloning and sequencing of the 18S rRNA gene. The amplification of the full-length (~1 700 bp) 18S rRNA gene from all five samples was successful (data not shown). The PCR products were cloned and two recombinants per sample were sequenced (Table 6). Sequences were assembled, edited and aligned with sequences of related genera from Genbank (Table 7).

Table 6: Origin and results of the samples selected for 18S rRNA characterization by cloning and sequencing.

Sample no	Place of Origin	RLB results	Clone no	Sequence length (bp)	Phylogenetic classification
929/12	KNP	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific *Babesia* 2 genus specific	929-4	1334	*B. lengau*
			929-5	1335	*B. lengau*
933/12	KNP	*Theileria/Babesia genus-spesific* *Babesia 1 genus specific* *Babesia 2 genus specific*	933-2	1334	*B. lengau*
			933-8	1335	*B. lengau*
939/12	KNP	*Theileria/Babesia genus-spesific* *Babesia 1 genus specific* *Babesia 2 genus specific*	939-6	1335	*B. lengau*
			939-8	1334	*B. lengau*
949/12	KNP	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific *Babesia* 2 genus specific	949-3	1334	*B. lengau*
			949-6	1334	*B. lengau*
954/12	KNP	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific *Babesia* 2 genus specific	954-3	1334	*B. lengau*
			954-4	1334	*B. lengau*

BLASTn homology searches indicated that the obtained sequences of the 10 clones were most similar (98 – 99% identity) to published *B. lengau* 18S rRNA gene sequences (accession numbers GQ411405 to GQ411417) previously identified from cheetah in South Africa (Bosman *et al*., 2010) and *B. lengau* (KC790443) recently detected in two domestic cat cases

of severe cerebral and haemolytic babesiosis in South Africa (Bosman *et al*., 2013) (Table 7). The obtained sequences further showed 97 – 98% sequence identity with *B. conradae* (AF158702 and AF231350), isolated from a Californian dog (Kjemtrup & Conrad, 2006); and 96 – 97% sequence identity with *B. duncani* (HQ289870 and HQ285838), isolated from humans in the USA (Conrad, Kjemtrup, Carreno, Thoford, Wainwright, Eberhard, Quick, Telford & Herwaldt, 2006) (Table 7).

Table 7: Highest percentage identity BLASTn hits of obtained *B. lengau* 18S rRNA genotypes.

Phylogenetic classification	**Sequence (length: bp)**	**GenBank match (Accession number)**			
		B. lengau (cheetah) (GQ411405 to GQ411417)	*B. lengau* (domestic cat) (KC790443)	*B. conradae* (AF158702 and AF231350)	*B. duncani* (HQ289870 andHQ285838)
B. lengau-like	933-8 (1335)	99%	99%	98%	97%
Genotype I	929-4 (1334)	99%	99%	98%	97%
	954-3 (1334)	99%	99%	98%	97%
	939-8 (1334)	99%	99%	98%	97%
Genotype II	929-5 (1335)	98%	98%	98%	96%
	939-6 (1335)	98%	98%	97%	96%
Genotype III	933-2 (1334)	99%	99%	98%	97%
	949-3 (1334)	99%	99%	98%	97%
	949-6 (1334)	99%	99%	98%	97%
	954-4 (1334)	99%	99%	98%	97%

A partial (~ 833 bp) *B. lengau*-like 18SrRNA gene sequence obtained from a spotted hyaena in Zambia (accession number KF270672) was recenty deposited in Genbank (Williams *et al*., 2014). Four of the obtained hyaena sequences in our study (933-2, 939-8, 949-6 and 954-4) showed 100% identity to this Zambian sequence over a 818 bp region. The remaining six obtained sequences showed 98 – 99% identity to this Zambian *B. lengau*-like 18S rRNA gene sequence.

A comparison of estimated evolutionary divergence between the observed gene sequences and those of closely related *Babesia* 18S rRNA gene sequences was subsequently compared by determining the number of base differences per near full-length 18S rRNA gene sequence (1321 bp) (Table 6). None of the obtained sequences were identical; it differed from each other by three to 27 nucleotides. Comparisons of the novel sequences to published *B. lengau, B. conradae* and *B. duncani* 18S rRNA sequences over a region of 1321 bp (Table 8) indicated that sequence 933-8 difered by only two nucleotides from the published *B. lengau* sequences. The rest of the novel sequences differed by 10 to 26 nucleotides from the published *B. lengau* sequences.

Table 8: Estimates of evolutionary divergence between sequences, showing the number of base differences per sequence from analysis between sequences. All results are based on the pairwise analysis of 16 sequences. Analyses were conducted in MEGA4 (Tamura, *et al*., 2007)]. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1321 positions in the final dataset.

	1	2	3	4	5	6	7	8	9	10	11	12	13	15	16
(1) 949-3															
(2) 954-4	3														
(3) 933-2	5	4													
(4) 949-6	4	3	5												
(5) *B. lengau* (cheetah) (GQ411417)	11	10	12	11											
(6) *B. lengau* (domestic cat) (KC790443)	11	10	12	11	0										
(7) 933-8	12	11	13	10	2	2									
(8) 939-8	5	4	6	5	12	12	13								
(9) 929-4	10	9	11	10	15	15	16	7							
(10) 954-3	8	7	9	8	13	13	14	5	4						
(11) 929-5	15	14	16	15	22	22	23	16	21	19					
(12) 939-6	19	18	20	19	26	26	27	16	19	19	4				
(13) *B. conradae* (AF231350)	21	20	22	19	27	27	26	21	25	24	28	31			
(14) *B. conradae* (AF158702)	21	20	22	19	27	27	26	21	25	24	28	31	0		
(15) *B. duncani* (HQ289870)	31	30	32	31	31	31	33	30	32	32	39	41	31	31	
(16) *B. duncan*i (HQ285838)	31	30	32	31	31	31	33	30	32	32	39	41	31	31	0

The observed sequence similarities were confirmed by phylogenetic analyses. Neighbour-joining and maximum parsimony techniques were used to reveal the relationships between the

obtained sequences and related *Babesia* species previously deposited in GenBank. A representative tree obtained by the neighbour-joining method is shown in Figure 5. All the obtained sequences formed a monophyletic group with the published *B. lengau* which in turn formed a monophyletic group with *B. conradae* and *B. duncani*. This "Western clade of *Babesia* species" was distinct from the *Babesia* spp. sensu stricto (represented by *B. canis* and *B. gibsoni*), *B. microti* and the *Theileria* spp. (Figure 5).

Within the *B. lengau* clade, the obtained sequences and the published *B. lengau* sequences grouped into four distinct groups (Figure 5), of which groups I, II and III each represented a novel *B. lengau* genotype. Sequence 933-8 clustered with the published *B. lengau* cheetah and domestic cat sequences. Genotype I consisted of 929-4, 939-8 and 954-3; Genotype II of 929-5 and 939-6, while Genotype III consisted of the remainder of the sequences (933-2, 949-3, 949-6 and 954-4).

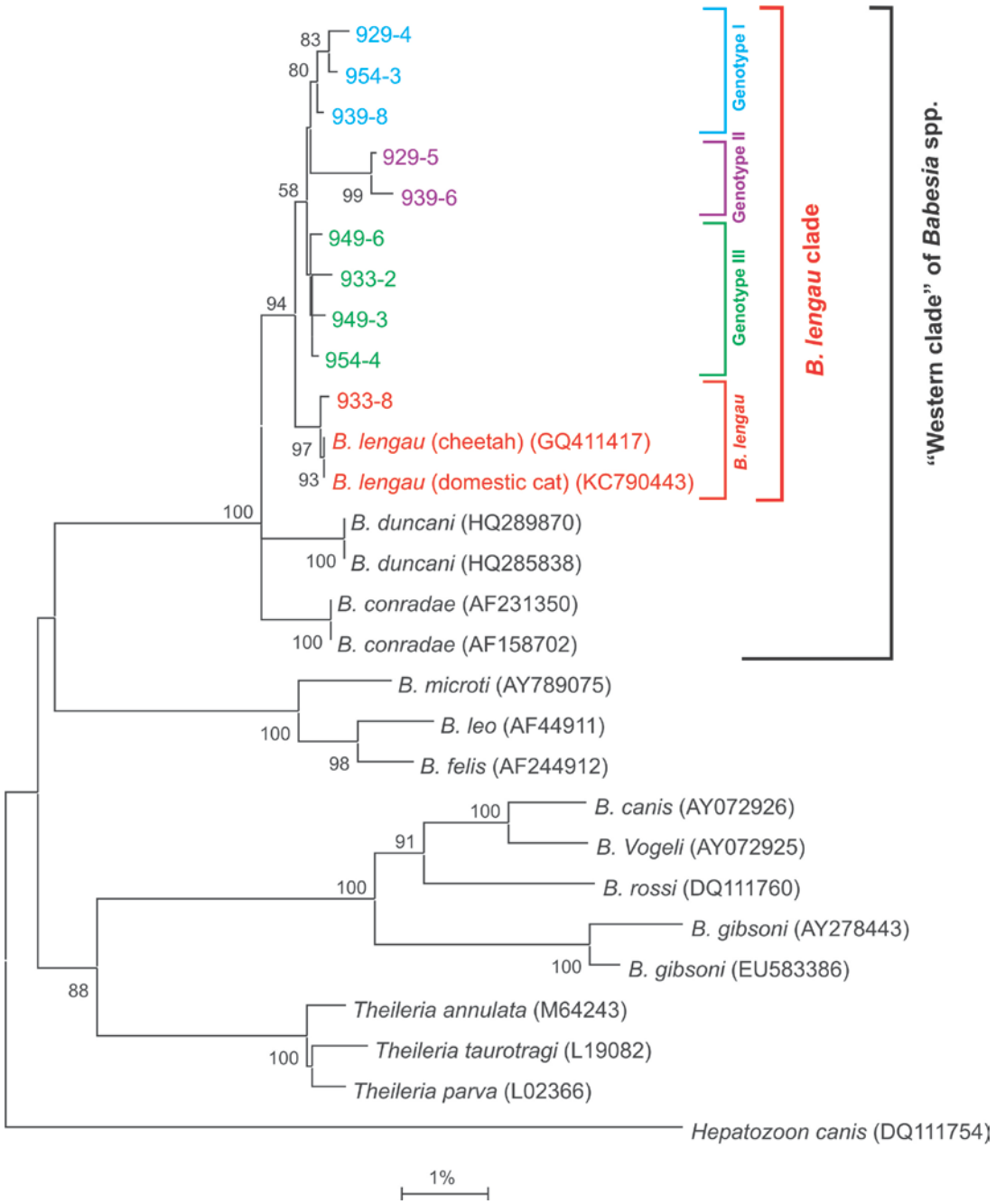

Figure 5: Neighbour-joining tree, with the Kimura two-parameter distance (Kimura, 1980) calculation showing the phylogenetic relationship of the obtained hyaena sequences to related species based on the near full-length 18S rRNA gene sequences (1321 bp). Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. *Hepatozoon canis* (DQ111754) was used as the outgroup. The GenBank accession numbers are indicated in parentheses.

When comparing the obtained hyaena sequences in our study with *B. conradae, B. duncani* and the newly described *B. lengau*-like sequence obtained from a Zambian hyaena over a 818 bp region (Table 9), four of the obtained sequences (949-6, 933-2, 954-4 and 939-8) were identical to that of the *B. lengau*-like sequence. Sequence 949-3 differed by one nucleotide and 939-6 by two nucleotides from the Zambian *B. lengau*-like sequence. The rest of the obtained sequences differed by two to 17 nucleotides from the Zambian sequence.

Table 9: Estimates of evolutionary divergence between sequences, showing the number of base differences per sequence from analysis between sequences. All results are based on the pairwise analysis of 17 sequences. Analyses were conducted in MEGA4 (Tamura, *et al*., 2007)]. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 805 positions in the final dataset.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
(1) 929-5																
(2) 929-6	2															
(3) 929-4	17	17														
(4) 954-3	15	17	4													
(5) *B. lengau* -like	11	13	6	4												
(6) 949-3	12	14	7	5	1											
(7) 949-6	11	13	6	4	0	1										
(8) 933-2	11	13	6	4	0	1	0									
(9) 954-4	11	13	6	4	0	1	0	0								
(10) 939-8	11	13	6	4	0	1	0	0	0							
(11) *B. lengau* (cheetah) GQ411417	20	22	13	11	9	10	9	9	9	9						
(12) *B. lengau* (domestic cat) KC790443	20	22	13	11	9	10	9	9	9	9	0					
(13) 933-8	20	22	13	11	9	10	9	9	9	9	1	1				
(14) B. gibsoni AF231350	23	25	21	20	16	17	16	16	16	16	24	24	24			
(15) *B. conradae* AF158702	23	25	21	20	16	17	16	16	16	16	24	24	24	24		
(16) *B. duncani* HQ289870	33	25	28	28	25	25	25	25	25	25	27	27	28	25	25	
(17) *B. duncan* i HQ285838	33	25	28	28	25	25	25	25	25	25	27	27	28	25	25	0

The observed sequence similarities were subsequently confirmed by phylogenetic analyses. A representative tree obtained by the neighbour-joining method is shown in Figure 6. All the obtained sequences, as well as the Zambian *B. lengau* sequence formed a monophyletic group with *B. lengau.* Within the *B. lengau* clade, the obtained sequences again clustered into the same four groups as was shown for the near full-length 18S rRNA analysis (Figure 6). The only exception was sequence 939-8 which now grouped with Genotype III instead of Genotype I. The Zambian *B. lengau*-like sequence also grouped with Genotype III.

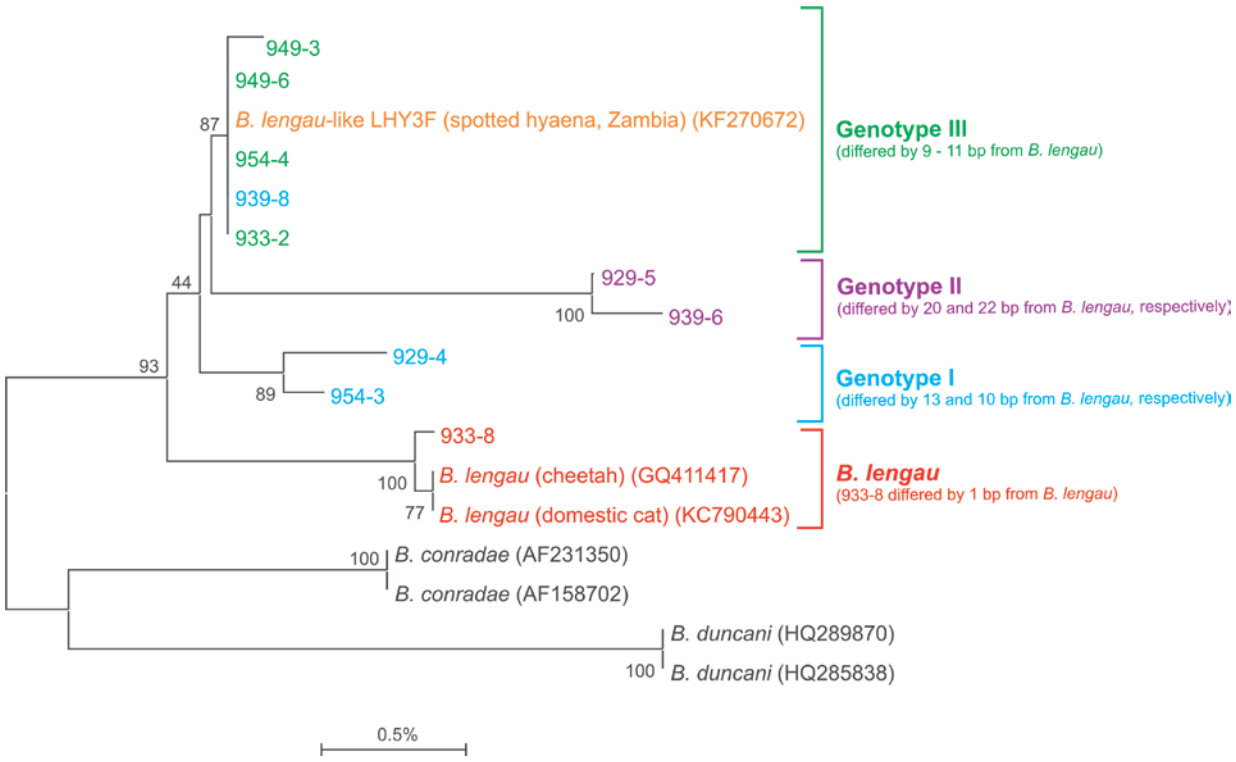

Figure 6: Neighbour-joining tree, with the Kimura two-parameter distance (Kimura, 1980) calculation showing the phylogenetic relationship of the obtained hyaena sequences to the "Western clade" of Babesia species based on the 18S rRNA gene sequences. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. The GenBank accession numbers are indicated in parentheses.

4.4 RLB probe development

In an attempt to design new RLB probes to detect the novel 18S rRNA gene sequences found in this study, the V4 hypervariable region of the 18S rRNA gene of published *B. lengau, B. conradae* and *B. duncani* gene sequences were aligned with the novel hyaena sequences. A 100 bp region of the alignment including the area from which the *B. lengau* RLB oligonucleotide was developed (Bosman *et al*., 2010) is shown in Figure 7. Unfortunately, no

area could be found that displayed enough sequence variation amongst all novel hyaena genotypes obtained, although this could be the result of the low sample size of brown and striped hyaena. One possible sequence identified was 5'-CTG CCA GGA TTT-3', but it was not conserved amongst all the novel sequences (Figure 7). It was, however, shown that should the previously described *B. lengau* RLB probe (Bosman *et al.,* 2010) have been included in this study, the novel *B. lengau* genotypes would most probably not have been detected due to the three nucleotide difference within the probe area. RLB probe sequences for *B. conradae* and *B. duncani* were also suggested (Figure 7). These should be included in future studies to determine specificity.

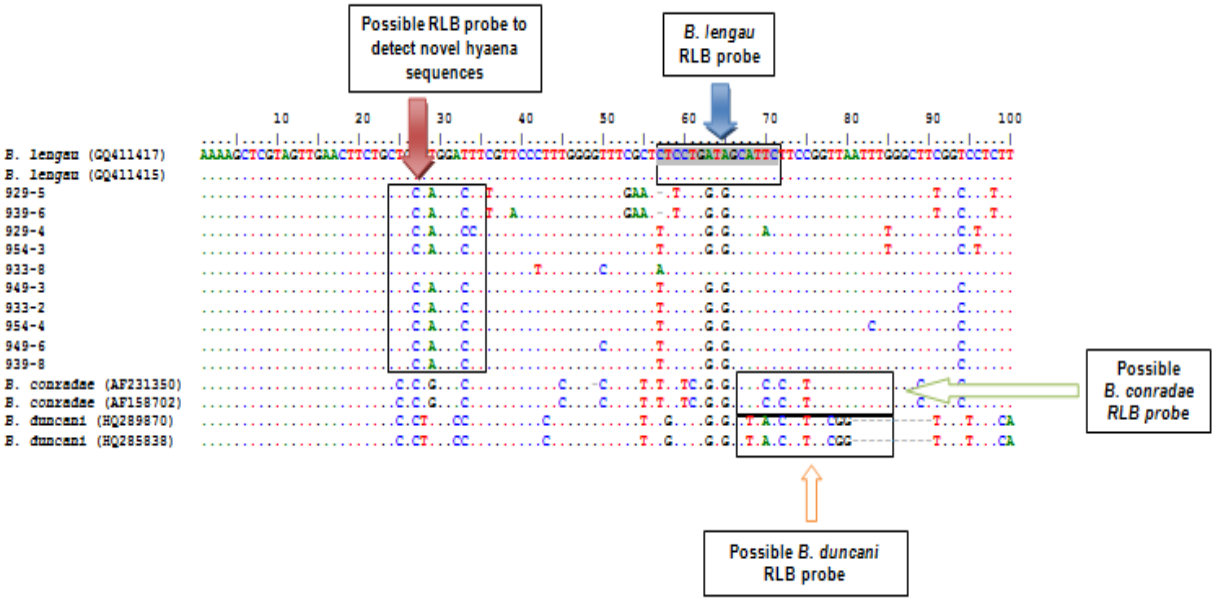

Figure 7: Nucleotide alignment of a 100 bp region each of the V4 hypervariable region of the published 18S rRNA gene of *B. lengau, B. conradae* and *B. duncani* as well as the variants identified in the study.

CHAPTER 5

DISCUSSION

Large predator populations have undergone severe declines in many parts of Africa, primarily due to anthropogenic factors; however, diseases have also caused declines and are of concern for species conservation (Williams *et al.,* 2014). The incidence of haemoparasite infections has been descibed in many wildlife species (Kjemtrup *et al*., 2000; Nijhof *et al*., 2003; Penzhorn, 2006; Bosman *et al*., 2007; Oosthizen *et al*., 2008; Oosthuizen *et al*., 2009; Schnittger *et al*., 2012), but clinical manifestation of such infections in these species is not common. Certain stressful circumstances, such as stress associated with capture and translocation, habitat degradation, climate change and immunosuppression can however result in clinical signs (Penzhorn, 2006; Munson, Terio, Kock, Mlengeya, Roelke, Dubovi, Summers, Sinclair & Packer, 2008; East *et al*., 2008).

Brown (*Parahyaena brunnea*) and striped (*Hyaena hyaena*) hyaena are regarded as “Near Threatened” species according to the 2008 World Conservation Union (IUCN) Red List of Threatened Species (http: //www.iucnredlist.org). The global population size is estimated to be below 10 000 mature individuals per species and experiences ongoing deliberate and incidental persecution such that it may come close to meeting a continuing decline of 10% over the next three generations. This is mainly because of loss of habitat in the wild and conflict with farmers in remaining habitats. They are often shot, poisoned, trapped and hunted with dogs in predator eradication or control programmes, or inadvertently killed in non-selective control programmes. There is also illegal trade in skins and body parts for use in traditional medicine (Arumgam, Wagner & Mills, 2008; Wiesel, Maude, Scott & Mills, 2008).

In contrast, spotted hyaena (*Crocuta crocuta*) are listed as “Least Concern” as the species remains widespread in Africa, and the total world population well exceeds 10 000 mature individuals. There is a continuing decline in populations outside protected areas (and even within some protected areas) due to persecution and habitat loss, although this is not sufficient to warrant listing in a threatened category (Höner, Holekamp & Mills, 2008).

Since little is known about the presence of tick-borne haemoprotozoan parasites in hyaena and their possible role in the decline in hyaena numbers, the aim of this study was to determine the occurrence of these parasites (i.e. *Babesia, Theileria, Anaplasma* and *Ehrlichia* species) in brown, striped and spotted hyaenas using the reverse line blot (RLB) hybridization assay. The *Babesia* species found were subsequently molecularly characterised by using gene sequence and phylogenetic analysis.

The RLB results revealed that the PCR products of 74.5% of the samples hybridized only with the *Theileria/Babesia* genus-specific probe, 80% with the *Babesia*-1, 74.5% with the *Babesia*-2 genus-specific probe and not with any of the species-specific probes, suggesting the presence of a novel species or variant of a species. Spotted hyaena had the highest number of PCR products that hybridized with the respective genus-specific probes (87.1 % for each probe). No *Ehrlichia* and/or *Anaplasma* species could be detected.

The incidence of *Babesia* in hyaenas is largely unknown; similarly, as for many carnivores, the method of transmission between individuals is also not known (Penzhorn *et al.* 2004). Historical reports of *Babesia* from the Family Hyaenidae include *Babesia alberti* (initially named *Nuttalia alberti*) described from the spotted hyaena (Van den Berge, 1937) and an unnamed *Babesia* sp. from the aardwolf (*Proteles cristata*) (Peirce *et al*., 2001). Recently, Williams *et al*. (2014) reported on the presence of a *Babesia* sp., related to *B. lengau,* in six

out of the 19 (32%) spotted hyaena tested originating from the Liuwa Plains National Park and South Luangwa National Park, Zambia.

Five of the spotted hyaena samples were subsequently selected for molecular characterization by cloning and sequencing of the near full-length parasite 18S rRNA gene. BLASTn homology searches indicated that the obtained sequences of the 10 clones were most similar to published *B. lengau* 18S rRNA gene sequences previously identified from cheetah (*Acinonyx jubatus*) (Bosman *et al*., 2010) and *B. lengau* recently detected in two domestic cat cases of severe cerebral and haemolytic babesiosis in South Africa (Bosman *et al.,* 2013). Other closely related species included *B. conradae*, associated with haemolytic anaemia in dogs in the United States (Kjemtrup & Conrad, 2006); and *B. duncani*, isolated from humans in the USA (Conrad *et al*., 2006). The observed sequence similarities were confirmed by phylogenetic analyses which showed that the obtained hyaena sequences formed a monophyletic group with *B. lengau*. Within the *B. lengau* clade, the obtained sequences and the published *B. lengau* sequences grouped into four distinct groups, of which groups I, II and III each represented a novel *B. lengau* genotype. The *B. lengau* clade then formed a monophyletic group with *B. conradae* and *B. duncani*. This "Western clade of *Babesia* species" was shown to be distinct from the *Babesia* spp. sensu stricto (represented by *B. canis* and *B. gibsoni*), *B. microti* and the *Theileria* spp.

Unfortunately, only partial sequences of the recently described *B. lengau*-like sequence obtained from Zambian hyaena (Williams *et al.,* 2014) were available for comparison with the sequences identified in this study. A comparison of estimated evolutionary divergence between the sequences (818 bp) indicated that four of our obtained sequences were identical to that of the Zambian *B. lengau*-like sequence. Phylogenetic analyses confirmed the observed sequence similarities. The Zambian *B. lengau*–like sequence clustered within *B. lengau* Genotype III. The only exception was one sequence (939-8) which now grouped with

Genotype III instead of Genotype I. This highlights the importance of obtaining full-length gene sequences for phylogenetic analysis.

Although variation in the 18S rRNA gene sequence has been widely used to characterize and classify previously unknown *Babesia* and *Theileria* parasites (Gubbels, Hong, van der Weide, Qi, Nijman, Guangyuan & Jongejan, 2000; Nijhof *et al*., 2003; Birkenheuer *et al*., 2004; Schnittger, Yin, Gubbels, Beyer, Niemann, Jongejan & Ahmed, 2003; Nijhof *et al*., 2005; Oosthuizen *et al*., 2008), there is no universally used criterion for classifying organisms to species level based on this variation (Chae, Allsopp, Waghela, Park, Kakuda, Sugimoto, Allsopp, & Wagner, 1999). As a result of the low sample numbers utilized, particularly for brown and striped hyaena, it remains difficult to establish how much 18S rRNA gene sequence variation must exist for the source organism to be considered a different species or to be considered merely a variant and/or genotype of a species (Chae *et al*., 1999; Allsopp & Allsopp, 2006;). Based on this, as well as the fact that we do not have any data on the morphology of the parasites, their possible vectors or their role in clinical disease, we suggest that these genotypes cannot be classified as new *Babesia* species, but rather as variants of *B. lengau.* Futher molecular characterization could be attempted using the second internal transcribed spacer (ITS2) region which displays a higher variability than the 18S rRNA gene, and facilitates distinguishing isolates where results from the 18S rRNA gene is unreliable (Collins & Allsopp, 1999; Schnittger *et al*., 2012). It has previously successfully been used to characterize *B. lengau* in cheetah (Bosman *et al*., 2010). Furthermore, it remains unknown whether the *Babesia* sp. detected in our study is the same as the historically described *B. alberti* from hyaena as blood smears were not available for morphological analysis and genetic characterization of *B. alberti.*

Babesia lengau was first described from clinically healthy cheetahs from Southern Africa (Bosman *et al*., 2010). Subsequently, *B. lengau* was detected from clinically ill domestic sheep

in Greece (Giadinis, Chochlakis, Kritsepi-konstantinou, Makridaki, Tselentis, Kostopoulou, Karatzias & Psaroulaki, 2012) and in two domestic cat cases of severe cerebral and haemolytic babesiosis in South Africa (Bosman *et al.,* 2013). More recently, *B, lengau* was detected in spotted hyaenas and a single lion in Zambia (Williams *et al*., 2014). The host range of *B. lengau* now includes three species of African carnivores (cheetah, hyaena and lion) and possibly domestic cats and sheep. It does not seem to be host specific (as is *B. leo* in lions for example), and it is feasible that the current host range for this species may be extended. The absence of known or clinical signs in hyaena caused by *Babesia* supports the possibility of a subclinical infection situation, which can be determined by a number of factors that relate to parasite, vector and host. In other species, stress or exposure of naïve animals to infection has induced clinical disease (Penzhorn, 2006; Schnittger *et al*., 2012). Clinical signs ascribed as a result of stressful situations have been described in black rhino (*Diceros bicornis)* caused by *Babesia bicornis* (Nijhoff *et al*., 2003) and in lions (*Panthera leo*) by *Babesia leo* (Penzhorn, 2006).

Hyaena as scavengers of carcasses and predators within any ecosystem show little evidence of clinical infection of other diseases, which is an indication of their resilience and ability to adapt to a number of challenges at both an intra-specific and inter-specific level. The prevalence of serologically positive results to a number of infectious diseases such as canine distemper and tuberculosis in the absence of described clinical signs in free-living populations of spotted hyaena (Alexander *et al*., 1995; Höner *et al*., 2006; McNutt, Briggs, Standers, Funston, Hemson, Keet & Van Vuuren, 2010; Williams *et al*., 2014) perhaps points to an immune system that is highly developed, allowing these predators to function optimally within ecosystems. There is no published information about similar studies in brown or striped hyaena.

Exposure to a pathogen does not necessarily imply persistence of that pathogen in the environment. The distribution and density of the host, mortality and population turnover will all play a role (Alexander *et al*., 2010). Wild carnivores often do not occur at sufficient densities to maintain most pathogens (Alexander *et al*., 2010), and hyaena in general are little different. The more the number of susceptible species that occur in a given area, the greater the likelihood of maintaining that pathogen in that area, irrespective of size (Alexander *et al*., 2010). The presence of the *B. lengau* in hyaena, cheetah, lion and other species supports this.

The potential vector for the transmission of *B. lengau* in hyaenas is unknown. No details with regard to the life cycle of *B. lengau* can be inferred, although it is likely to be similar to the typical *Babesia* life cycle as described by Chauvin *et al.* (2009). *B. lengau* associates phylogenetically close to *B. conradae*, of which the known vector is *Rhipicephalus sanguineus (*Kjemtrup & Conrad, 2006; Kjemtrup *et al.*, 2006). Several species of ticks in both spotted and brown hyaena have been recorded (Horak, Braack, Fourie & Walker, 2000), but are often overshadowed by findings in other larger carnivores such as lion. Large numbers of immature stages of *Amblyomma hebraeum* have been recorded in the Kruger National Park, but only adults of *R. simus* and *Haemaphysalis zumpti* in any number, although individuals of *R. maculatus*, *R. appendiculatus*, *H. leachi* and *Ixodes* sp. were recorded also (Horak *et al*., 2000). Adult ticks of *H. leachi*, *R. simus* and *R. nuttali* were recorded in brown hyaena in Gauteng (Horak *et al*., 2000). Of the ticks that infest carnivores, only H. *leachi, H. zumpti*, *R. nuttali* and *R. simus* were considered true parasites of large carnivores by Horak *et al*. (2000), but this needs to be established for hyaena in general.

Of interest is the finding of *B. lengau* DNA in a mouse (*Aethomys* sp.) in association with captive cheetah in South Africa (Golezardy, 2012). These live in underground burrows, and could play a role in maintaining a tick vector that could conceivably transmit the infection to carnivores such as cheetah (Golezardy, 2012). *Haemaphysalis elliptica* and *Rhipicephalus*

simus have been described as being the preferred ticks infesting *Aethomys* sp., and although *B. lengau* DNA was identified from *H. elliptica* in proximity to the infected cheetah, the role that this plays in the transmission of *B. lengau* to cheetah is not clear (Golezardy, 2012; Bosman *et al*., 2012). The potential for having a murine host for a species of *Babesia* that clearly has the potential to infect other species, possibly even hyaena, is interesting as it may have significance in the epidemiology of the disease. The possibility also exists that more than one murine host may be infected and may act as a host for this species, but this needs further investigation.

The geographical distribution of *B. lengau* is unknown, but it can be assumed to be related to distribution range of the various hosts. A species-specific *B. lengau* RLB probe has been developed (Bosman *et al*., 2010); and we have designed RLB probe sequences to detect the *B. lengau* genotypes identified in this study, as well as *B. conradae* and *B. duncani*. These could be used in wider studies to determine which hosts are infected and the geographical distribution of this species. This “landscape genetics” approach may reconstruct parasite movements on a larger scale, but can also have a role in verifying or improving epidemiological approaches to disease surveillance or outbreaks (Archie, Luikart, & Ezenwa, 2009).

Future research that would need to be done on *Babesia* infection in hyaena in South Africa would be to establish distribution ranges for the parasite in spotted, brown and striped hyaena, perhaps linking occurrence of the parasite not only to geographical distribution, but also to different habitat utilization of the various hyaena species. This could include contact between conservation areas and domestic animals, and what dynamics exist at the interface between domestic and wild carnivores What role these parasites play in ecological or population processes as far as hyaena are concerned is unknown, particularly in light of the paucity of

published information confirming clinical infections in hyaena for many known carnivore diseases in Africa.

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