Piroplasms in brown hyaenas (*Parahyaena brunnea*) and spotted hyaenas (*Crocuta crocuta*) in Namibia and South Africa are closely related to *Babesia lengau*

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Abstract The objective of our study was identification and molecular characterisation of piroplasms and rickettsias occurring in brown (*Parahyaena brunnea*) and spotted hyaenas (*Crocuta crocuta*) from various localities in Namibia and South Africa. Whole blood (n=59) and skin (n=3) specimens from brown (n=15) and spotted hyaenas (n=47) were screened for the presence of *Babesia*, *Theileria*, *Ehrlichia* and *Anaplasma* species using the Reverse Line Blot (RLB) hybridization technique. PCR products of 52/62 (83.9%) of the specimens hybridized only with the *Theileria/Babesia* genus-specific probes 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 DNA could be detected. Parasite 18S rRNA gene of brown (n=3) and spotted hyaena (n=6) specimens was subsequently amplified, cloned and the recombinants sequenced. Homologous sequence searches of databases indicated that the obtained sequences were most closely related to *B. lengau*, originally described from cheetahs (*Acinonyx jubatus*). 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 six distinct groups, of which groups I to V represented novel *B. lengau* genotypes and/or gene variants. We suggest that these genotypes cannot be classified as new *Babesia* species, but rather as variants of *B. lengau.* This is the first report of occurrence of piroplasms in brown hyaenas.

Keywords: *Babesia lengau*, *Crocuta*, Hyaena, Namibia, *Parahyaena*, South Africa

Acknowledgements

This research was supported financially by the National Research Foundation (NRF), South Africa (grant no 95836 to BLP and grant no 76529 to MCO). Specimens were obtained from (i) the South African National Parks (SANParks) Biobank, Skukuza, and (ii) the NRF Biobank at the National Zoological Gardens, Pretoria.

Conflict of interest The authors declare that they have no conflict of interest.

Introduction

There is mounting evidence that piroplasms, e.g. *Babesia* spp., commonly occur in wildlife hosts that do not exhibit any clinical signs of infection (Penzhorn 2006), but there is a dearth of information on occurrence of *Babesia* spp. in hyaenas. The first evidence stems from a spotted hyaena (*Crocuta crocuta*) from Virunga National Park (previously Albert National Park) in the Democratic Republic of the Congo that was found to be infected with a small piroplasm, which was assigned the name *Nuttalia alberti* (Van Den Berghe 1937, 1942) (Figure 1). This species was later referred to as *Babesia alberti* (Peirce et al. 2001). Based on molecular characterisation, a piroplasm close to *Babesia lengau* was recently reported from 6/19 (32%) spotted hyaenas from the Liuwa Plain National Park and South Luangwa National Park, Zambia (Williams et al. 2013). As yet, there are no published reports on piroplasms from brown hyaenas (*Parahyaena brunnea*), a species endemic to southern Africa.

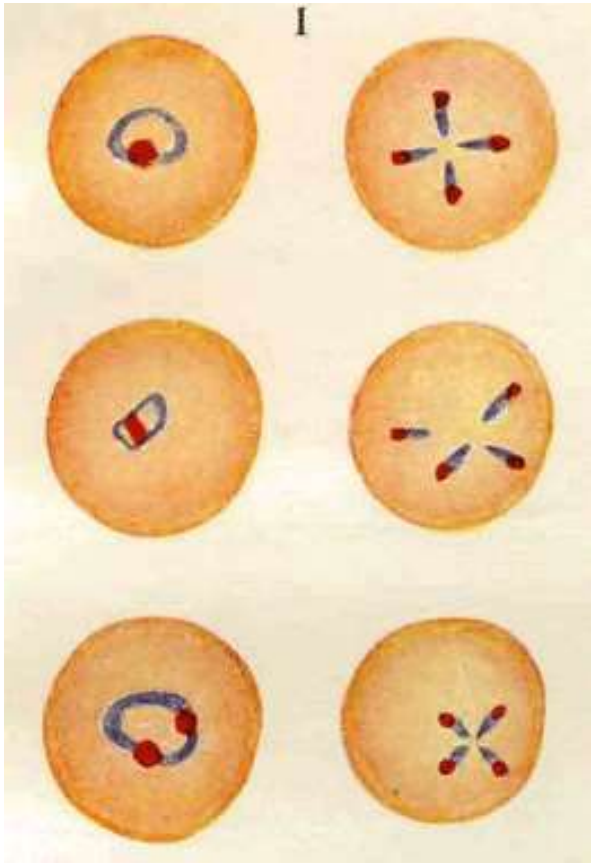

Figure 1: Original illustrations of "*Nuttalia alberti*". ©RBINS: Plate 1, L. Van Den Berghe (1942). Enquête parasitologique (1-Parasites du sang des vertébrés). 15 p., 2 pls, Exploration of National Park Albert and Kagera National Park: Mission L. Van Den Berghe (1936) used with permission from the Royal Belgian Institute of Natural Sciences.

Blood smears taken from brown hyaenas at Lüderitz, Namibia, as part of a larger study were submitted to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, for screeing for the presence of parasites. Small piroplasms were observed in the erythrocytes. Since specific identification of piroplasms cannot be based on morphological characteristics, this prompted further investigation. Additional brown hyaena as well as spotted hyaena specimens were obtained from various sources in Namibia and South Africa. The objective of the study was identification and molecular characterisation of some tick-transmitted pathogens occurring in these two hosts, i.e. piroplasms (*Babesia* and *Theileria* spp.) as well as rickettsias (*Anaplasma* and *Ehrlichia* spp.). Such information not only enhances basic biodiversity data, but could be important in understanding risks facing these hosts. For example, when subclinical carrier hosts experience stressful conditions, clinical signs may manifest, as has been demonstrated with *Babesia bicornis* in black rhinoceroses (*Diceros bicornis*) (Nijhof et al. 2003).

Table 1: Origin of the brown hyaena and spotted hyaena specimens analysed

Country	**Locality**	**Free-ranging or captive**
BROWN HYAENA (n=15)		
Namibia (n=11)	Lüderitz (n=8)	Free-ranging
	Outjo (n=3)	Free-ranging
South Africa (n=4)	National Zoological Gardens, Pretoria (n= 1)	Captive
	Lion & Safari Park, Gauteng (n=1)	Captive
	Pretoria, Gauteng (n=1)	Free-ranging
	Waterberg, Limpopo (n=1)	Free-ranging
SPOTTED HYAENA (n=47)		
Namibia (n=8)	Etosha National Park (n=8)	Free-ranging
South Africa (n=39)	National Zoological Gardens, Pretoria (n=9)	Captive
	Kruger National Park (n=30)	Free-ranging

Materials and methods

Sample collection and DNA extraction

Specimens from 62 animals were analysed: 15 brown hyaenas and 47 spotted hyaenas (Table 1). Most specimens were sourced from the biobanks of South African National Parks (Skukuza), and the National Research Foundation (National Zoological Gardens, Pretoria). Free-ranging animals (n=52) supplied the bulk of the specimens. With the exception of skin specimens from 3 brown hyaenas, all specimens were whole blood collected in EDTA or, in one instance, in heparin.

Genomic DNA was extracted from the blood and skin specimens using the QIAmp DNA. Blood was eluted in 100 µl elution buffer and stored at –20°C.

Reverse Line Blot (RLB) hybridization

The reverse line blot (RLB) hybridisation assay was done according to Gubbels et al. (1999) and Nagore et al. (2004).*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') and biotin labelled reverse primer Ehr-R (5'-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3') (Bekker et al. 2002) were used to amplify the V1 hypervariable region of the 16S rRNA gene for *Ehrlichia* and *Anaplasma*. 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 was applied for amplification under stringent conditions according to the methods described by Nijhof et al. (2005). Known *Babesia, Theileria, Ehrlichia* and *Anaplasma* and genus- and species-specific oligonucleotide probes were used at predetermined concentrations.

18S rRNA amplification, cloning, sequencing and phylogenetic analysis

The full-length 18S rRNA gene of six spotted hyaena and three brown hyaena specimens that tested positive for piroplasms 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 1700 bp fragment of the 18S rRNA gene (Oosthuizen et al. 2008). PCR was performed using the High Fidelity PCR Master Mix (Roche Diagnostics, Mannheim, Germany) and according to the manufacturer's instructions. Six separate reactions were prepared per sample.

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. The pure DNA was eluted in 20 µl elution buffer.

Using the pGEM-T Easy Vector system (Promega, Madison, WI, USA), 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). Colonies were picked and placed into Falcon tubes containing imMedia Amp Liquid broth (Invitrogen, USA); 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. 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').

The obtained sequences were assembed and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Staden et al. 2000). Homologous sequence searches of databases were performed using the BLASTn package (Altschul et al. 1990), and a multiple sequence alignment was performed using ClustalX (version 1.81 for Windows) which included all related available genera from GenBank (Thompson et al. 1997). The alignment was truncated to the size of the smallest sequence (1334 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 MEGA7 (Kumar et al. 2016) using both neighbour-joining (Saitou and 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 MEGA7 (Kumar et al. 2016).

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

Results

The results of the RLB hybridization are shown in Table 2. PCR products of 52/62 specimens (83.9%) hybridized with the *Theileria/Babesia* genus-specific probes only, and not with any of the species-specific probes. The remaining 10/62 specimens (16.1%) tested negative (or below detection limit of the test). Specimens from all 38 free-ranging spotted hyaenas were positive, while specimens from 4/9 captive animals were positive. All specimens tested negative (or below detection limit of the test) for *Ehrlichia* and/or *Anaplasma* species.

Table 2: Occurrence of haemoparasite infection in brown and spotted hyaena specimens as determined by Reverse Line Blot hybridisation

	Brown hyaena (n=15) (%)	**Spotted hyaena (n=47) (%)**	**Total (n=62) (%)**
Theileria/*Babesia* genus-specific	8 (53.3%)	42 (89.4%)	50 (80.6%)
Babesia genus-specific 1	10 (66.7%)	42 (89.4%)	52 (83.9%)
Babesia genus-specific 2	8 (53.3%)	42 (89.4%)	50 (80.6%)
Ehrlichia/*Anaplasma* genus-specific	0	0	0
Negative / below detection limit	5 (33.3%)	5 (10.6%)	10 (16.1%)

Six of the spotted hyaena specimens (free-ranging; Kruger National Park) and three of the brown hyaena specimens (free-ranging; Outjo and Lüderitz, Namibia) were selected for molecular

characterization by cloning and sequencing of the 18S rRNA gene. The amplification of the near full-length (~1700 bp) 18S rRNA gene from all nine specimens was successful (data not shown). The PCR products were cloned and a total of 23 recombinants were sequenced. Sequences were assembled, edited and aligned with sequences of related genera from Genbank

BLASTn homology searches indicated that the obtained recombinant sequences 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) detected in two domestic cats in South Africa suffering from severe cerebral and haemolytic babesiosis, respectively (Bosman et al. 2013). The obtained sequences further showed 97–98% sequence identity with *B. conradae* (AF158702 and AF231350), isolated from a Californian dog (Kjemtrup and Conrad 2006); and 97% sequence identity with *B. duncani* (HQ289870 and HQ285838), isolated from humans in the USA (Conrad et al. 2006).

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 (1276 bp). Five of the obtained sequences were identical (933-2, 929-1, 949-6, 275-2, 32-4); the remainder differed from each other by one to 17 nucleotides. Comparisons of the novel sequences to published *B. lengau, B. conradae* and *B. duncani* 18S rRNA sequences over a region of 1276 bp indicated that sequence 933-8 difered by only one nucleotide from the published *B. lengau* sequences, while 939-3 differed by three nucleotides. The rest of the novel sequences differed by 6 to 20 nucleotides from the published *B. lengau* sequences.

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 2. 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 2).

Within the *B. lengau* clade, the obtained sequences and the published *B. lengau* sequences grouped into six distinct groups (Figure 2), of which groups I, II, III, IV and V each represented a novel

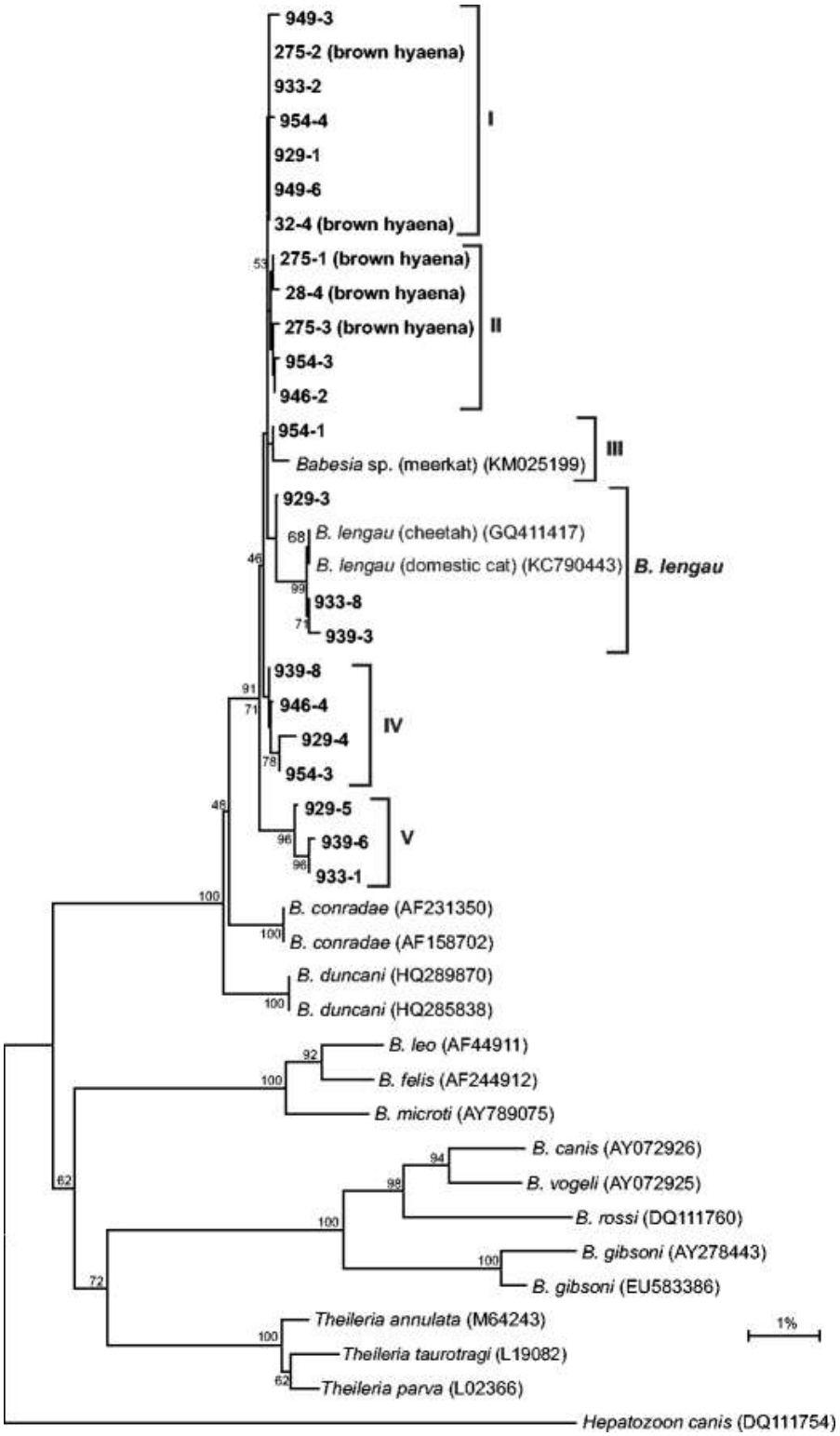

Figure 2: Neighbour-joining tree, with the Kimura two-parameter distance calculation showing the phylogenetic relationship of the obtained hyaena sequences to related species based on the near full-length 18S rRNA gene sequences. Sequences obtained from brown hyaena are indicated in parenthesis; the remainder were obtained from spotted hyaena. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. *Hepatozoon canis* (DQ111754) was used as outgroup.

Table 3: 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)	*B. lengau* genotype
SPOTTED HYAENA:					
929/12	KNP*	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific *Babesia* 2 genus specific	929-1	1334	I
			929-3	1334	*B. lengau*
			929-4	1334	IV
			929-5	1335	V
933/12	KNP	*Theileria/Babesia genus-spesific* *Babesia 1 genus specific* *Babesia 2 genus specific*	933-1	1335	V
			933-2	1334	I
			933-8	1334	*B. lengau*
939/12	KNP	*Theileria/Babesia genus-spesific* *Babesia 1 genus specific* *Babesia 2 genus specific*	939-3	1334	*B. lengau*
			939-6	1335	V
			939-8	1334	VI
946/12	KNP	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific *Babesia* 2 genus specific	946-2	1334	II
			946-4	1334	IV
949/12	KNP	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific *Babesia* 2 genus specific	949-3	1334	I
			949-6	1334	I
954/12	KNP	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific *Babesia* 2 genus specific	954-1	1334	III
			954-2	1334	IV
			954-3	1334	II
			954-4	1334	I
BROWN HYAENA:					
28/04	Lüderitz, Namibia	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific *Babesia* 2 genus specific	28-4	1334	II
32/04	Lüderitz, Namibia	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific	32-4	1334	I
275/15	Outjo, Namibia	*Theileria/Babesia* genus-spesific *Babesia* 1 genus specific *Babesia* 2 genus specific	275-1	1334	II
			275-2	1334	I
			275-3	1334	II

*Kruger National Park, South Africa

B. lengau genotype and/or gene variant. Sequences 929-3, 933-8 and 939-3 (spotted hyaena, KNP) clustered with the published *B. lengau* cheetah and domestic cat sequences. Five of the seven sequences in Genotype I were identical and obtained from three spotted hyaenas (5 clones) and two brown hyaenas (2 clones). Sequences obtained from brown hyaenas (28-4, 32-4, 275-1 and 275-2) grouped within Genotype I and II sequences. One sequence obtained from a spotted hyaena (954-1) grouped in Genotype III with a *Babesia* sp. sequence recently reported from meerkats (*Suricata suricatta*) in South Africa (Leclaire et al., 2015), differing by three nucleotides.

Six of the selected nine hyaenas contained multiple genotypes; the frequency of genotypes was found to be one (34%), two (22%), three (22%) and four (22%) genotypes per sample (Table 3). Samples 28/04, 32/04 and 949/12 each had one genotype per sample, while samples 929/12 and 954/12 had four genotypes per sample.

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). Three of the obtained spotted hyaena sequences in our study (929-3, 946-4 and 949-6), represented in Genotype I, showed 100% identity to this Zambian sequence over an 811 bp region. The remaining sequences showed 98–99% identity to this Zambian *B. lengau*-like 18S rRNA gene sequence.

Discussion

According to the 2015 World Conservation Union (IUCN) Red List of Threatened Species (http://www.iucnredlist.org) brown hyaenas are regarded as “Near Threatened” (Wiesel, 2015). In contrast, spotted hyaenas are listed as “Least Concern” (Bohm & Höner, 2015). This species remains widespread in Africa, and the total population well exceeds 10 000 mature individuals. There is a continuing decline in populations outside protected areas (and even within some protected areas), however, due to persecution and habitat loss (Höner et al. 2008). 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). Haemoparasite infections have been reported in many wildlife species (Kjemtrup et al. 2000; Penzhorn 2006; Bosman et al. 2007; Oosthuizen et al. 2009; Schnittger et al. 2012), but clinical manifestation of such infections in these species is not common. Certain circumstances, such as stress associated with

capture and translocation, habitat degradation, climate change and immunosuppression can result in clinical signs (Penzhorn 2006).

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 (Allsopp and 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 and Allsopp 1999). Furthermore, it remains unknown whether the *Babesia lengau*-like piroplasms detected in our study are the same species as *B. alberti* described from a single spotted hyaena (Van den Berghe 1937). This will probably remain unresolved, since the original blood smears could not be traced for DNA extraction and molecular characterisation.

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 et al. 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). Unfortunately, only partial sequences of the recently described *B. lengau*-like sequence obtained from Zambian hyaenas (Williams *et al.,* 2014) were available for comparison with the sequences identified in this study, which highlights the importance of obtaining full-length gene sequences for phylogenetic analysis. The host range of *B. lengau* now includes four species of African carnivores and possibly domestic cats and sheep. It does not seem to be host specific, and it is feasible that the current host range for this species may be extended. The absence of known or clinical signs in hyaenas 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).

The potential vector for the transmission of *B. lengau* in hyaenas is unknown. Several species of ticks have been recorded from both spotted and brown hyaenas (Horak et al. 2000). Large numbers of immature stages of *Amblyomma hebraeum* have been recorded in the Kruger National Park, as well as adults of *R. simus* and *Haemaphysalis zumpti* and individuals of *R. maculatus*, *R. appendiculatus*, *H. leachi* (presumably *H. elliptica*; Apanaskevich et al. 2007) and *Ixodes* sp. (Horak et al. 2000). Adult ticks of *H. leachi* (presumably *H. elliptica*), *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 hyaenas in general.

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. 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; Alexander et al. 2010; Williams et al. 2014) perhaps points to a immune system that is highly developed, allowing these predators to function optimally within ecosystems.

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 absence of *Anaplasma* and/or *Ehrlichia* spp in all specimens may be significant. Blood specimens collected from 19 spotted hyaenas in two Zambian national parks were also negative for these genera (Williams et al. 2014).

The study confirms that hyaenas are susceptible to infection by a *Babesia* sp similar to *B. lengau*, but also strongly suggests that they are probably not clinically affected to any appreciable degree. Their role as carriers of this organism and their ability to serve as sources of infection to vectors still needs elucidation.

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This study (V058/11) was approved by the Research Committee of the Faculty of Veterinary Science and the Animal Use and Care Committee of the University of Pretoria.

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