Babesia bicornis, _Theileria bicornis_ and _Theileria equi_ in metapopulations of two black rhinoceros (_Diceros bicornis_) subspecies in South Africa and their potential impact on conservation

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Highlights

- Populations in mesic habitats are more likely to be infected with piroplasms.
- Black rhinoceroses can be subclinically infected with *Theileria equi.*
- *Theileria bicornis* haplotypes H1, H3 and H4 were identified, H3 being the most common.
- Risk of infection must be assessed prior to rhinoceros reintroduction.
- Prophylactic action should be taken to prevent potentially fatal babesiosis.

Abstract

The two black rhinoceros subspecies (*Diceros bicornis bicornis* and *D. b. minor*) in South African conservation areas are managed as separate metapopulations. Since infection with *Babesia bicornis* can be fatal in black rhinoceroses, occurrence of this and other piroplasms in the two metapopulations was determined to assess possible risk. Blood specimens were collected from 156 black rhinoceroses: 80 from *D. b. bicornis* and 76 from *D. b. minor*. DNA was extracted; the V4 hypervariable region of the parasite 18S rRNA gene was amplified and subjected to the Reverse Line Blot (RLB) hybridization assay. There was a significant difference in occurrence of piroplasms: 18/80 (23%) in *D. b. bicornis* and 39/76 (51%) in *D. b. minor*. *Theileria bicornis* occurred in significantly more of the *D. b. minor* population (36/76; 47%) than the *D. b. bicornis* population (1/80; 1%); with *B. bicornis* the difference was not significant: *D. b. bicornis* 5/80 (6%) and *D. b. minor* 9/76 (11%). Three individuals were infected with *Theileria equi*. Results were confirmed using molecular characterization of the near full-length parasite 18S rRNA gene of 13 selected specimens. We identified four (Tb1, Tb2, Tb3 and Tb4) 18S rDNA sequence types for *T. bicornis*, two for *B. bicornis* (Bb1 and Bb2) and one for *T. equi* (Teq1). We furthermore identified *T. bicornis* haplotypes H1, H3 and H4 in 10 rhinoceroses; H3 was the most common haplotype identified. Rhinoceroses inhabiting

more arid areas are apparently free of *T. bicornis* and *B. bicornis*, probably due to the absence or scarcity of vectors. When individuals are relocated for metapopulation management purposes, appropriate prophylactic action should be taken to minimise the risk of babesiosis, which could be fatal.

Keywords: Black rhinoceros; *Babesia bicornis*; *Theileria bicornis*; *Theileria equi*; Metapopulation management; reintroduction

1. Introduction

Black rhinoceroses (*Diceros bicornis*) were historically widespread throughout sub-Saharan Africa, with the exception of the equatorial rain forests (Emslie and Adcock, 2013). Hunting and poaching, in combination with man-made alteration of the landscape leading to the fragmentation and isolation of a once continuous wildlife ecosystem, has resulted in black rhinoceroses existing in only a few isolated pockets within their former range (Friedmann and Daly, 2004). To keep isolated and small populations genetically viable, metapopulation management is required to avoid further loss of genetic variability as a result of inbreeding (Brooks, 1989). This management includes the translocation of rhinoceroses from areas of high density to low-density areas, or areas that are suitable for reintroducing new populations. It is a dynamic process that integrates the management of various subpopulations, population genetic management and habitat management, with the ultimate aim of reintroducing black rhinoceroses to areas where they have been extirpated. Appropriate translocations can be beneficial but can also lead to problems such as the transfer of pathogens (Ewen et al., 2012).

The spread of diseases to new environments, via translocations, may have important effects on wildlife, agriculture or public health, and may affect the success of the translocation effort itself (Leighton, 2002). All translocations involve moving an organism from one environment to another and hence will necessarily affect both source and release locations. Some effects may be negative to the species being translocated or to the wider ecosystem, and it is the responsibility of managers to identify and reduce or prevent negative aspects of these conservation initiatives. One important risk involved with moving organisms is that they will carry pathogens (Ewen et al., 2012). Any animal translocation contains two main types of risks or health concerns: (a) the introduction of the disease or infectious agent by the translocated animals to the destination ecosystem may result in ecological or economic harm, and/or (b) health hazards present at the destination ecosystem may adversely affect the translocated animals.

Wildlife can be carriers of *Babesia* species, which under stressful conditions, such as translocations or drought, can become pathogenic to the host (Penzhorn, 2006). In the 1960s, in East Africa, mortalities of black rhinoceroses after physical capture were attributed to babesiosis (McCullough and Achard, 1969; Mugera and Wandera, 1967). Subsequently, deaths of two black rhinoceroses in Ngorongoro Conservation Area (Tanzania) and two in South Africa [Hluhluwe Game Reserve and Addo Elephant National Park (NP)] were positively linked to babesiosis (Nijhof et al., 2003). In all four cases the animals died soon after capture or during periods of environmental stress, e.g. drought. Based on molecular characterization, a novel species designated *Babesia bicornis* was found in three of these animals, as well as in 5 of 11 apparently healthy

black rhinoceroses in the Great Fish River Nature Reserve (NR) (Nijhof et al., 2003). After the two black rhinoceroses had died of babesiosis, the other rhinoceroses in the Ngorongoro Conservation Area were treated prophylactically with diminazene aceturate administered by darting, to apparent good effect (Fyumagwa et al., 2004).

Theileria bicornis, which has not been incriminated in causing mortality, was also described from black rhinoceroses (Nijhof et al., 2003). It was subsequently reported from white rhinoceroses (*Ceratotherium simum*) in Kruger NP (Govender at al., 2011) and in both white and black rhinoceroses in Kenya (Otiende et al., 2015). Three *T. bicornis* 18S rRNA haplotypes (396 bp) were reported from Kenya: haplotypes H1 and H3 occurring in black rhinoceroses and H2 in white rhinoceroses (Otiende et al., 2015). These haplotypes were shown to be widespread among the major rhinoceros subpopulations in Kenya, although geographical location could not be correlated with a specific haplotype. The authors emphasized the important ecological and conservation implications of their findings, especially for population management programmes and as a means of avoiding the transport of infected animals into non-affected areas.

Theileria bicornis was also detected in Australia in captive white and black rhinoceroses translocated from Africa (Yam et al., 2018). Haplotype H2 was detected in two white rhinoceroses, while a new haplotype (H4) was described in a black rhinoceros. Six captive-bred black rhinoceroses tested *T. bicornis*-negative, indicating that *T. bicornis* was introduced to Australia with wild-caught rhinoceroses and that transmission amongst the Australian captive population did not occur (Yam et al., 2018).

The aim of this study was to determine the prevalence of piroplasms, especially *B. bicornis* and *T. bicornis,* and tick-transmitted rickettsias in various South African *D. b. bicornis* and *D. b. minor* populations living under different ecological conditions. The long-term aim was a better understanding of potential disease risks involved in translocating black rhinoceroses for metapopulation management, in order to anticipate and mitigate the risk of piroplasm-associated disease during stressful and unfavourable conditions.

2. Materials and Methods

2.1. Specimens

Blood specimens in EDTA collected from immobilized black rhinoceroses during routine management operations by South African National Parks (SANParks) veterinary teams are stored in biobanks at Skukuza, Kruger NP, and Kimberley, Northern Cape Province. Biobank specimens from 156 individual black rhinoceroses, collected between 2002 and 2014, were available: 80 from *D. b. bicornis* populations and 76 from *D. b. minor* populations (Fig. 1).

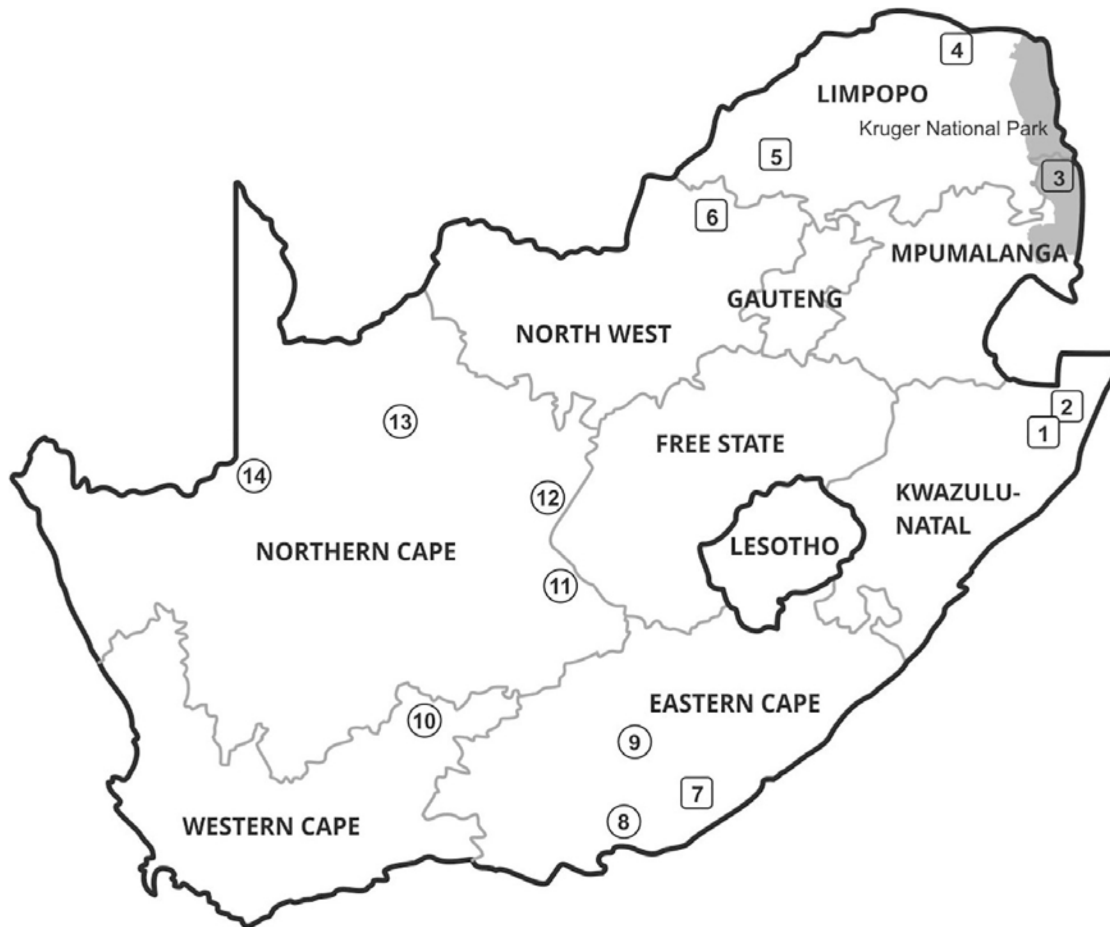

Fig. 1. Locations of South African black rhinoceros (*Diceros bicornis*) populations mentioned in the text. *Diceros b. minor* (squares): (1) Hluhluwe-iMfolozi Park; (2) Mkuzi Game Reserve; (3) Kruger National Park; (4) Maremani Nature Reserve; (5) Marakele National Park; (6) Pilanesberg National Park; (7) Great Fish River Nature Reserve. *Diceros b. bicornis* (circles): (8) Addo Elephant National Park; (9) Mountain Zebra National Park; (10) Karoo National Park; (11) Private conservancy; (12) Vaalbos and Mokala National Parks; (13) Tswalu Private Game Reserve; (14) Augrabies Falls National Park.

2.2. DNA extraction and PCR

DNA was extracted from 200 µl of EDTA-anticoagulated blood using the QIAamp® DNA Blood MiniKit (QIAGEN, Southern Cross Biotechnologies, South Africa), following the manufacturer's instructions. Once extracted, the DNA was subjected to Polymerase Chain Reaction (PCR), as described by Nijhof et al. (2003). The V4

hypervariable area of the parasite 18S rRNA gene was amplified using the *Theileria* and *Babesia* genus-specific forward primer RLB F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and the reverse-biotin-labelled primer RLB R2 (5'-Biotin-CTA AGA ATT TCA CCT CTG ACA GT-3') (Nijhof et al., 2003). *Anaplasma* and *Ehrlichia* genus-specific primers Ehr-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') (Schouls et al., 1999) and Ehr-R (biotin 5'-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 parasite 16S rRNA. The primers used were obtained from Inqaba Biotechnical Industries (Pty) Ltd (South Africa). A positive and negative control was included for each batch of samples processed, during the PCR amplification. The positive control was of known *B. bovis* DNA (Onderstepoort Biological Products, South Africa) and the negative control consisted out of the PCR master mix without template DNA.

The PCR products were analysed using the Reverse Line Blot (RLB) hybridization technique (Gubbels et al., 1999).

2.3. Reverse Line Blot (RLB) hybridisation assay

The PCR products were then hybridized to a Biodyne® C blotting membrane (Separations, South Africa), containing the *Theileria* and *Babesia* genus-specific probe, as well as *B. bicornis*, *T. bicornis* and *T. equi* species-specific probes. A list of all the probes used on the membrane is summarized in Table 1.

Table 1. List of genus and species-specific probes used during the Reverse Line Blot (RLB) hybridization assay. R = A / G, W = A / T are the symbols used to indicate degenerate positions.

Probe Number	Probe Identification	Probe Sequence (from 5′ – 3′)
1	*Ehrlichia/Anaplasma* genus-specific	GGG GGA AAG ATT TAT CGC TA
2	*A. bovis*	GTA GCT TGC TAT GRG AAC A
3	*A. centrale*	TCG AAC GGA CCA TAC GC
4	*A. marginale*	GAC CGT ATA CGC AGC TTG
5	*A. phagocytophilum*	TTG CTA TAA AGA ATA ATT AGT GG
6	*Anaplasma* sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC
7	*E. canis*	TCT GGC TAT AGG AAA TTG TTA
8	*E. chaffeensis*	ACC TTT TGG TTA TAA ATA ATT GTT
9	*E. ruminantium*	AGT ATC TGT TAG TGG CAG
10	*Theileria/Babesia* genus-specific	TAA TGG TTA ATA GGA RCR GTT G
11	*Babesia* 1 genus-specific	ATT AGA GTG CTC AAA GCA GGC
12	*Babesia* 2 genus-specific	ACT AGA GTG TTT CAA ACA GGC
13	*B. bicornis*	TTG GTA AAT CGC CTT GGT C
14	*B. bigemina*	CGT TTT TTC CCT TTT GTT GG
15	*B. bovis*	CAG GTT TCG CCT GTA TAA TTG AG
16	*B. caballi*	GTG TTT ATC GCA GAC TTT TGT
17	*B. canis*	TGC GTT GAC GGT TTG AC
18	*B. rossi*	CGG TTT GTT GCC TTT GTG
19	*B. vogeli*	AGC GTG TTC GAG TTT GCC
20	*B. divergens*	ACT RAT GTC GAG ATT GCA C
21	*B. felis*	TTA TGC GTT TTC CGA CTG GC
22	*B. gibsoni*	TAC TTG CCT TGT CTG GTT T
23	*B. leo*	TTA TGC TTT TCC GAC TGG C
24	*B. major*	TCC GAC TTT GGT TGG TGT
25	*B. microti*	GRC TTG GCA TCW TCT GGA
26	*B. occultans*	CCT CTT TTG GCC CAT CTC G
27	*Babesia* sp. (sable)	GCG TTG ACT TTG TGT CTT TAG C
28	*Theileria* genus-specific	ATT AGA GTG CTC AAA GCA GGC
29	*T. annae*	CCG AAC GTA ATT TTA TTG ATT G
30	*T. annulata*	CCT CTG GGG TCT GTG CA
31	*T. bicornis*	GCG TTG TGG CTT TTT TCT G
32	*T. buffeli*	GGC TTA TTT CGG WTT GAT TTT
33	*T. equi*	TTC GTT GAC TGC GYT TGG
34	*T. lestoquardi*	CTT GTG TCC CTC CGG G
35	*T. mutans*	CTT GCG TCT CCG AAT GTT
36	*T. ovis*	TGC GCG CGG CCT TTG CGT T
37	*T. parva*	GGA CGG AGT TCG CTT TG
38	*T. separata*	GGT CGT GGT TTT CCT CGT
39	*Theileria* sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
40	*Theileria* sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
41	*Theileria* sp. (sable)	GCT GCA TTG CCT TTT CTC C
42	*T. taurotragi*	TCT TGG CAC GTG GCT TTT
43	*T. velifera*	CCT ATT CTC CTT TAC GAG T

2.4. Cloning, sequencing and phylogenetic analysis

To confirm the RLB results obtained, 13 samples were selected for further molecular analysis. The parasite near-full length 18S rRNA gene was amplified using primers NBab_1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and T/B_Rev (5'-AAT AAT TCA CCG GAT CAC TCG-3') (Oosthuizen et al., 2008; Matjila et al., 2008). The Phusion Flash High-Fidelity (Thermo Scientific) PCR master mix was used to prepare PCRs. Four separate reactions were prepared per sample. Amplicons of all four reactions per sample were pooled to avoid Taq polymerase-induced errors, purified and cloned into the pJET vector (Thermo Fisher Scientific). Three recombinants per sample were sequenced on an ABI 3500XL Genetic Analyzer using vector primers pJET1.2_F and pJET1.2_R at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa).

The obtained gene sequences were assembled, edited and aligned using GAP 4 of the Staden package (Version 1.6.0 for Windows) (Staden et al., 2000). Sequence identities were determined from GenBank using BLASTn (Altschul et al., 1990). Sequences were aligned with appropriate reference sequences from GenBank using ClustalX (Version 1.81 for Windows). The alignment was manually truncated to the size of the smallest sequence using Bioedit (Hall, 1999). The Tamura-Nei (TN93+G+I) substitution model (Tamura and Nei, 1993), determined as the best-fit model using MEGA 7 (Kumar et al., 2016), was used to infer a maximum likelihood phylogenetic tree. This was in combination with the bootstrap method using 1000 replicates/tree. The genetic distances between the sequences were estimated by determining the number of nucleotide differences between sequences using MEGA7. The 18S rDNA sequence *Plasmodium*

falciparum (JQ627152) was included as outgroup. All positions containing gaps and missing data were eliminated. The final data set comprised a total of 1385 positions. Evolutionary analyses were conducted in MEGA7.

The 18S rRNA gene sequences of the sequences identified in this study were submitted to GenBank under accession numbers MT903276-MT903307.

2.4. Statistical analysis

The chi-square test was used to compare the prevalence of infection in various populations (www. https://www.socscistatistics.com/tests/chisquare2/default2.aspx).

3. Results

3.1. RLB

In total 57/156 (36.3%) specimens subjected to PCR amplification and RLB hybridization tested positive for the presence of piroplasms: 18/80 (23%) from *D. b. bicornis* and 39/76 (51%) from *D. b. minor* (Table 2). The difference is significant (χ^2 =13.9566; p=0.00019; df=1). When results from *D. b. bicornis* in Addo Elephant NP (8/32 positive) were compared with results from *D. b. minor* in Great Fish River NR (11/25 positive), where vegetation type and ecological conditions are comparable, the difference was not statistically significant (χ^2=2.28; p=0.13105; df=1).

RLB detected *B. bicornis* in 9/156 (5.8%) and *T. bicornis* in 37/156 (23.7%) of specimens. *Theileria bicornis* occurred in significantly more of the *D. b. minor*

population (36/76; 47%) than the *D. b. bicornis* population (1/80; 1%) (χ^2=45.817; p<0.00001; df=1). With *B. bicornis* the difference was not significant: *D. b. bicornis* 5/80 (6%) and *D. b. minor* 9/76 (11%) (χ^2=1.492; p=0.22191; df=1).

Table 2. Piroplasms detected by the Reverse Line Blot assay in South African *Diceros b.bicornis* and *D. b. minor* metapopulations.

Empty Cell	n	Pos specimens	*B. bicornis*	*T. bicornis*	*T. equi*	*T. annulata*	T/B catch-all[d]	*Babesia* 1 catch-all	E/A catch-all
				Diceros b. bicornis					
AENP	42	11	4	1	1	5	–	–	–
MoNP	20	3	–	–	–	1	2	–	–
PC*	6	3	–	–	–	3	–	–	2
MZNP	4	2	1	–	1	–	–	1	–
Other**	8	0	–	–	–	–	–	–	–
subtotal	**80**	**18**	**5**	**1**	**2**	**9**	**2**	**1**	**2**
				Diceros b. minor					
GFNR	26	11	4	11	–	–	–	4	–
MaNP	28	17	5	15	1	–	–	8	1
KNP	16	8	–	8	–	–	–	7	2
PNP	3	1	–	–	–	–	1	–	–
MaNR	3	2	–	2	–	–	–	2	–
subtotal	**76**	**39**	**9**	**36**	**1**	–	**1**	**21**	**3**
TOTAL	**156**	**57**	**9**	**37**	**3**	**9**	**3**	**22**	**5**

(AENP = Addo Elephant National Park; GFRNR = Great Fish River Nature Reserve; KNP = Kruger National Park; MaNP = Marekele National Park; MaNR = Maremani Nature Reserve; MoNP = Mokala National Park; MZNP = Mountain Zebra National Park; PC = Private Conservancy; PNP = Pilanesberg National Park).

*Private Conservancy, Northern Cape Province.
**Karoo National Park and Tswalu Private Game Reserve.
dCatch-all = genus-specific RLB probe.

RLB detected faint signals for *Theileria equi* and *Theileria annulata* in three and nine animals, respectively. PCR products from three individuals hybridized with the *Babesia*/*Theileria* genus-specific probe only, while PCR products from 21 of 37 *T. bicornis*-positive individuals hybridized with the *Babesia* genus-specific probe 1, suggesting the presence of novel species or variants of species. PCR products from five

individuals hybridized with the *Ehrlichia*/*Anaplasma* genus-specific probe as well as with other probes.

3.2. Molecular characterisation

To confirm the RLB results obtained, 13 specimens that tested positive for the presence of *T. bicornis* (n=10), *B. bicornis* (n=2) and *T. equi* (n=1) DNA were selected for further molecular characterization of the 18S rRNA gene. The parasite near full-length 18S rRNA gene of all 13 specimens was successfully amplified, cloned and 32 recombinants were sequenced. The sequences were assembled, edited and aligned with sequences of related *Theileria* and *Babesia* 18S rRNA sequences from GenBank. BLASTn homology searches showed that the obtained sequences had 98-99% sequence identity to published sequences of *T. bicornis* (n=25, obtained from 10 rhinoceroses), *B. bicornis* (n=4, obtained from two rhinoceroses), and *T. equi* (n=3, obtained from one rhinoceros). Of the 32 recombinant sequences obtained, five sequences from five rhinoceros specimens were short (< 1400 bp) and were excluded from the subsequent phylogenetic analysis. The origin of the specimens, RLB assay results and phylogenetic classification for the obtained sequences are listed in Table 3.

Table 3. Origin and results of the black rhinoceros specimens selected for 18S rRNA gene characterization.

Sample nr	Place of Origin (Locality)	RLB results	Clone nr	Sequence length (bp)	18S rDNA sequence type	Phylogenetic classification	*T. bicornis* haplotype	GenBank ID
280	**MZNP***	*T. equi*	280/2	1585	Teq1	*T. equi*	N/A	MT903276
			280/3	1585	Teq1	*T. equi*	N/A	MT903278
			280/4	1585	Teq1	*T. equi*	N/A	MT903277
332	**MaNR**	*T. bicornis*	332/1	1610	Tb3	*T. bicornis*	H3	MT903289
			332/2	1610	Tb3	*T. bicornis*	H3	MT903297
334	**MaNP**	*T. bicornis*	334/1	1610	Tb3	*T. bicornis*	H3	MT903294
			334/2	1610	Tb3	*T. bicornis*	H3	MT903291
			334/3	1610	Tb3	*T. bicornis*	H3	MT903290
335	**KNP**	*T. bicornis*	335/1	1613	Tb2	*T. bicornis*	H4	MT903283
			335/2	1159	(Tb3)**	*T. bicornis*	H3	MT903303
			335/3	1610	Tb3	*T. bicornis*	H3	MT903295
336	**KNP**	*T. bicornis*	336/1	1613	Tb2	*T. bicornis*	H4	MT903282
			336/2	1613	Tb2	*T. bicornis*	H4	MT903284
			336/3	1160	(Tb2)**	*T. bicornis*	H4	MT903306
338	**KNP**	*T. bicornis*	338/1	1616	Tb1	*T. bicornis*	H1	MT903281
			338/2	1616	Tb1	*T. bicornis*	H1	MT903280
			338/3	1616	Tb1	*T. bicornis*	H1	MT903279
339	**MaNP**	*T. bicornis*	339/1	1611	Tb3	*T. bicornis*	H3	MT903286
			339/2	1611	Tb3	*T. bicornis*	H3	MT903287
			339/3	1611	Tb3	*T. bicornis*	H3	MT903288
343	**MaNR**	*T. bicornis*	343/1	994	(Tb3)**	*T. bicornis*	H3	MT903305
			343/2	1159	(Tb3)**	*T. bicornis*	H3	MT903304
			343/3	1610	Tb3	*T. bicornis*	H3	MT903292
348	**KNP**	*T. bicornis*	348/2	1616	Tb1	*T. bicornis*	H1	MT903302
			348/3	1613	Tb4	*T. bicornis*	H4	MT903285
372	**AENP**	*B. bicornis*	372/1	1565	Bb1	*B. bicornis*	N/A	MT903300
			372/2	1565	Bb1	*B. bicornis*	N/A	MT903299
			372/3	1565	Bb1	*B. bicornis*	N/A	MT903298
384	AENP	*B. bicornis*	384/2	1566	Bb2	*B. bicornis*	N/A	MT903301
405	**GFRNR**	*T. bicornis*	405/1	1382	(Tb3)**	*T. bicornis*	H3	MT903307
			405/2	1610	Tb3	*T. bicornis*	H3	MT903296
427	GFRNR	*T. bicornis*	427/2	1610	Tb3	*T. bicornis*	H3	MT903293

(AENP = Addo Elephant National Park; GFRNR = Great Fish River Nature Reserve; KNP = Kruger National Park; MaNP = Marekele National Park; MaNR = Maremani Nature Reserve; MZNP = Mountain Zebra National Park).

(N/A = Not applicable).

*This individual had been translocated to MZNP from AENP.
**18S rDNA genotype could not be assigned with certainty due to shorter sequence length.

The estimated evolutionary divergence between the observed gene sequences and those of closely related *Theileria* and *Babesia* 18S rRNA sequences was subsequently compared by determining the number of base differences per near full-length 18S rRNA gene sequence. All ambiguous positions were removed for each sequence pair. There was a total of 1919 positions in the final dataset. We found four (Tb1, Tb2, Tb3 and Tb4) 18S rDNA sequence types for *T. bicornis*, two for *B. bicornis* (Bb1 and Bb2) and one for *T. equi* (Teq1) (Table 3). The *T. bicornis* 18S rDNA sequences obtained differed by 2-16 bp from the *T. bicornis* Umfana (MF536661) and *T. bicornis* Aluka (MF536660) sequences described in captive white rhinoceroses in Australia (Yam et al., 2018). They furthermore differed by 4-18 bp from the *T. bicornis* (AF499604) type sequence described in a black rhinoceros from the Great Fish River NR, South Africa (Nijhof et al., 2003). The *B. bicornis* 18S rDNA sequences differed by 2-20 bp from the *B. bicornis* (AF419313) type sequence as described from black rhinoceroses that had died in wildlife areas in Tanzania and South Africa (Nijhof et al., 2003). The obtained *T. equi* 18S rDNA sequences differed by 1 bp from the *T. equi* (Z15105) sequence described from a horse in South Africa (Allsopp et al., 1994) and from a mule (KY952226) in Brazil (Braga et al., 2017).

Tb3, the most common *T. bicornis* 18S rRNA sequence genotype obtained, was found at all study sites (except Addo Elephant NP, where only *B. bicornis* sequences were detected). The *T. bicornis* Tb1, Tb2 and Tb4 genotypes were obtained only from the Kruger NP. Two rhinoceroses (335 and 348) were co-infected with two *T. bicornis* 18S rDNA genotypes (Tb2 and Tb3; Tb1 and Tb4) (Figs. 3 and 4).

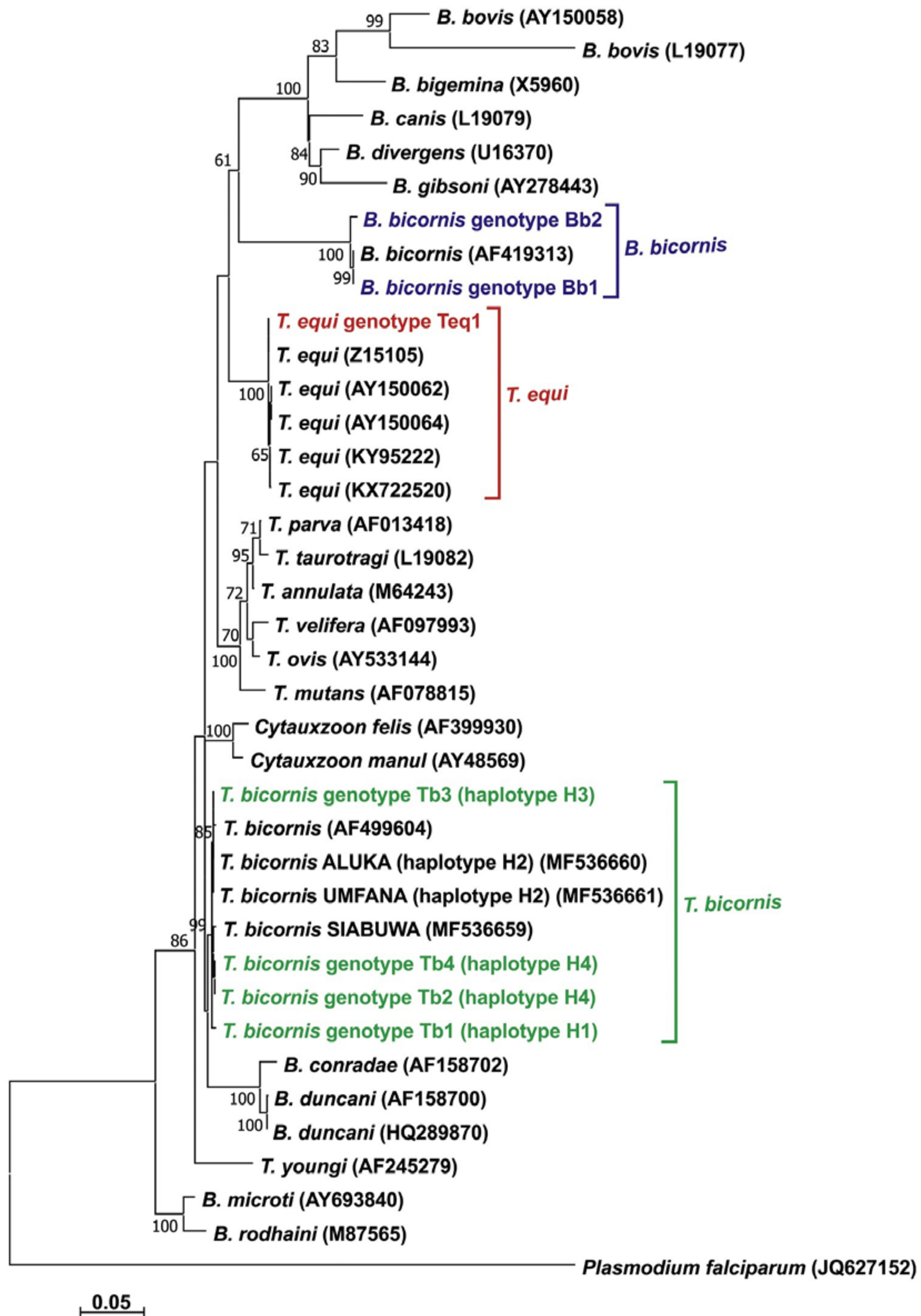

Fig. 2. Maximum likelihood tree showing the evolutionary relationships of the *Theileria* and *Babesia* 18S rDNA genotypes obtained from South African rhinoceroses, with published sequences. A single representative of each genotype was used to construct the tree. The evolutionary history was inferred by

using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3936)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 38.42% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. All positions containing gaps and missing data were eliminated. There was a total of 1385 positions in the final dataset.

The observed sequence similarities were subsequently confirmed by phylogenetic analyses and demonstrated the close relationship of the obtained 18S rDNA genotypes to *T. bicornis*, *B. bicornis* and *T. equi*, respectively (Fig. 2). The *T. bicornis* group clustered separately from the transforming and non-transforming *Theileria* species, which was in concordance with previous studies (Nijhof et al., 2003; Otiende et al., 2015; Yam et al., 2018). *Babesia bicornis* and *T. equi* clustered in distinct, well-supported clades, in contrast to findings by Schnittger et al. (2012), who stated that *B. bicornis* and *T. equi* are close relatives, belonging to a distinct group within the piroplasmid order (corresponding to Clade IV as described by Schnittger et al., 2012).

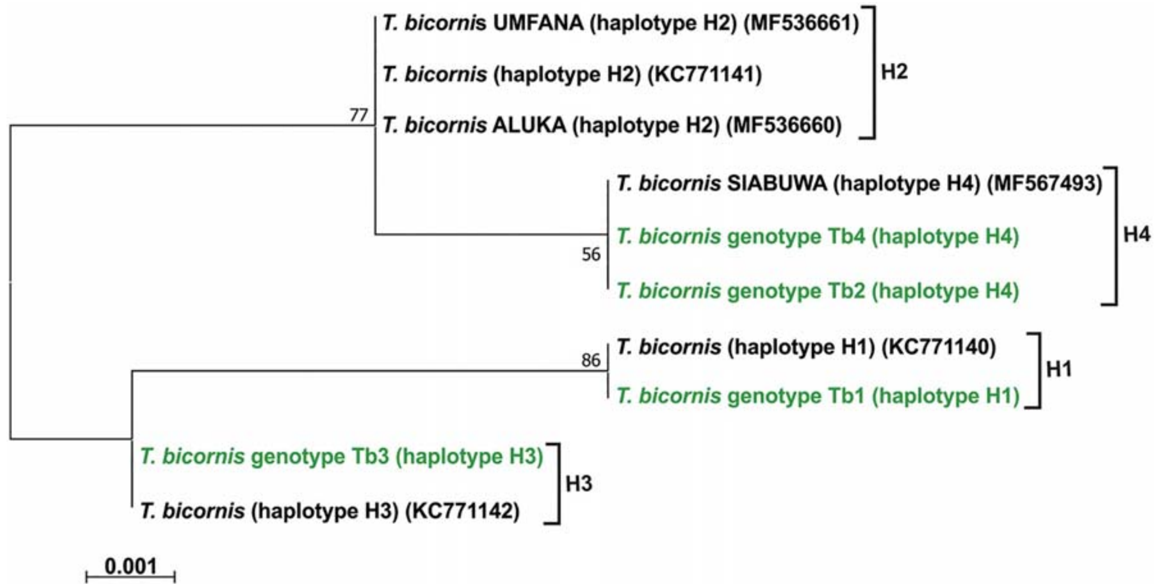

Fig. 3. Maximum-likelihood tree based on a 398 bp fragment of the obtained *T. bicornis* 18S rDNA genotypes to demonstrate the *T. bicornis* haplotypes circulating in South Africa. A single representative of each genotype was used to construct the tree. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0500)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 49.49% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. All positions containing gaps and missing data were eliminated. There was a total of 394 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

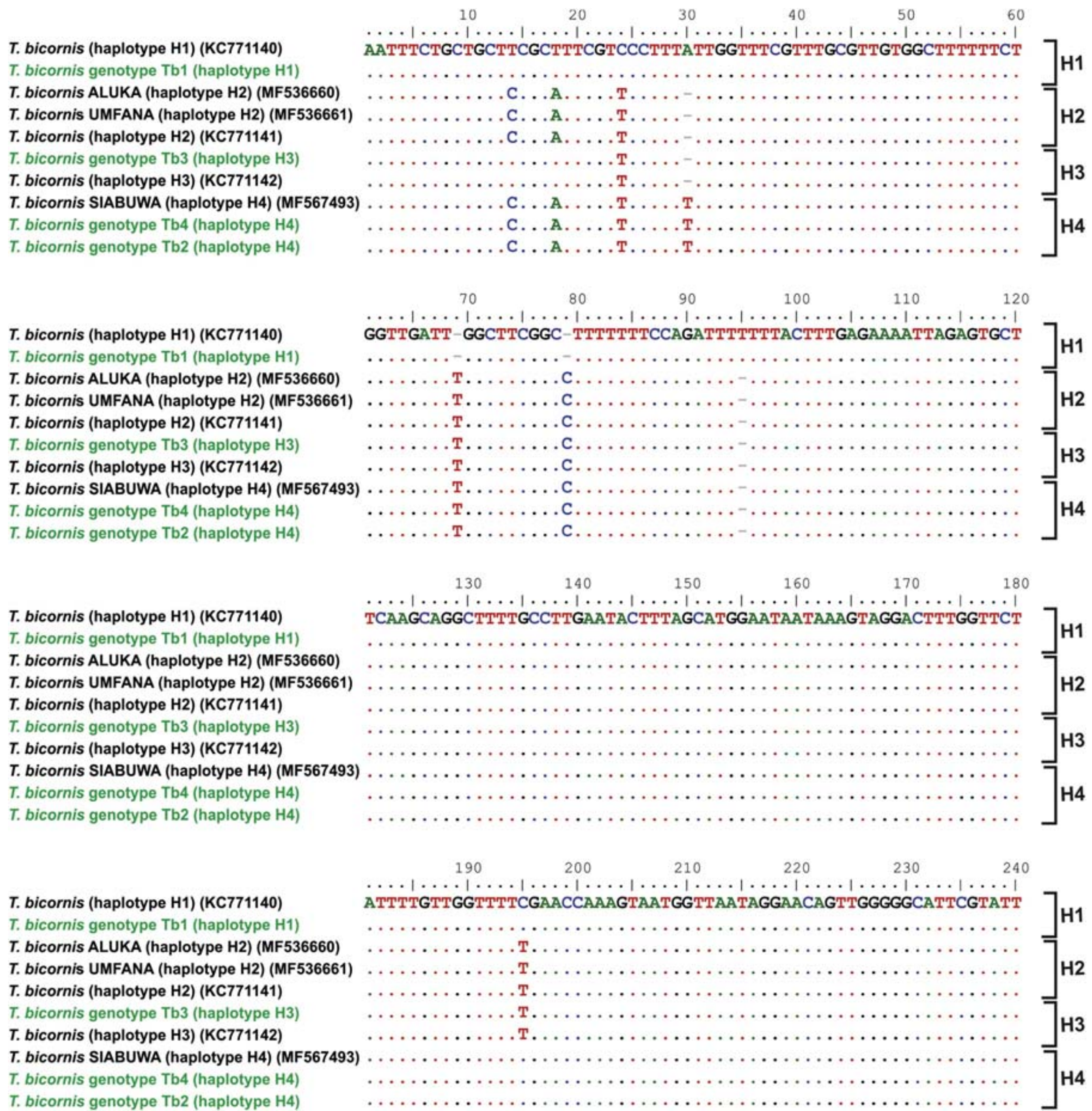

Fig. 4. Truncated alignment (showing 240 bp of the 396 bp alignment) of the 18S rRNA *T. bicornis* haplotypes to highlight the differences between the four *T. bicornis* haplotypes (H1-H4).

3.2.1 Theileria bicornis haplotypes

To determine which *T. bicornis* haplotypes are circulating in South Africa, a 396 bp fragment of the obtained *T. bicornis* 18S rDNA genotypes and the previously described haplotypes from white (H1 and H3) and black (H2) rhinoceroses in Kenya (Otiende et al.,

2015) and captive black rhinoceroses (H4) in Australia (Yam et al., 2018) were aligned and compared (Fig. 3 and 4). Results showed that haplotypes H1, H3 and H4 were present in South Africa; genotype Tb1 corresponded to haplotype H1 and Tb3 to haplotype H3. Interestingly, Tb2 and Tb4 both corresponded to haplotype 4; differing by only 1 bp from each other (over a 1613 bp region).

In a recent study, King'ori et al. (2019) described six partial *T. bicornis* 18S rRNA gene sequences (468 bp) from the elephant bont-tick (*Amblyomma tholloni*) in Kenya. They referred to these sequences as haplotypes H2-H7; but did not correlate them to the *T. bicornis* haplotypes H1-H4 as described in rhinoceroses in Kenya and Australia (Otiende et al., 2015; Yam et al., 2018). We subsequently aligned these sequences with the *T. bicornis* haplotypes H1-H4; results revealed that the *T. bicornis* haplotypes H3, H5 and H7 as described from *A. tholloni* corresponded to the *T. bicornis* haplotype H3, while H2 and H6 corresponded to H4, and H4 from *A. tholloni* corresponded to the *T. bicornis* haplotype H1 (data not shown).

4. Discussion

Our study confirmed the presence of *B. bicornis*, *T. bicornis* and *T. equi* in both black rhinoceros metapopulations in South Africa, with individuals in the *D. b. minor* metapopulation at a significantly higher risk of being infected with haemoparasites than those in the *D. b. bicornis* metapopulation. Faint RLB signals were also detected for *T. annulata* (5.7%); however, since *T. annulata* does not occur in South Africa, the faint

signals were most probably as a result of cross-reactions to the other RLB probes with previously unknown targets. The report of *T. annulata* from salivary glands of *Rhipicephalus decoloratus* and *Rhipicephalus evertsi evertsi* in South Africa, which was based on RLB and not confirmed by cloning and sequencing, may be a similar case (Berggoetz et al., 2014).

We used molecular characterization of the near full-length parasite 18S rRNA gene of 13 selected specimens to confirm the RLB results obtained. We identified four (Tb1, Tb2, Tb3 and Tb4) 18S rDNA sequence types for *T. bicornis*, two for *B. bicornis* (Bb1 and Bb2) and one for *T. equi* (Teq1). We furthermore identified *T. bicornis* haplotypes H1, H3 and H4 in ten rhinoceroses; H3 was the most common haplotype identified and common to all the study sites except the Addo Elephant NP. Although haplotype H1 and H4 were only identified in rhinoceroses from Kruger NP, our sample set was too small to correlate geographical location with a specific haplotype. Two rhinoceroses were found to each be co-infected with two *T. bicornis* haplotypes. Whether any of these haplotypes contributes to disease remains unknown, and further studies should be done to determine haplotype specificity for a particular host or whether the haplotypes play a pathogenic role in infected animals.

Since we generated near full-length 18S rRNA gene sequences in our study, we could correlate the *T. bicornis* genotypes identified with the *T. bicornis* haplotypes previously described and based on partial 18S rDNA sequences (396 b). The *T. bicornis* genotype Tb1 corresponded to haplotype H1, while Tb3 corresponded to haplotype H3.

Interestingly, Tb2 and Tb4 both corresponded to haplotype H4. This also emphasizes the value of generating near full-length or full-length gene sequences, especially when describing new species.

Theileria bicornis is apparently not specific for rhinoceroses. It was reported from 53% of 97 apparently healthy free-ranging nyalas (*Tragelaphus angasii*) in northern KwaZulu-Natal, South Africa, based on RLB (Pfitzer et al., 2011), and also from impalas (*Aepyceros melampus*), an eland (*Tragelaphus oryx*) and a sable antelope (*Hippotragus niger*) (Clift et al., 2020).

Theileria equi, which had previously been reported from white rhinoceroses in Kruger NP, based on RLB (Govender et al., 2011), was confirmed in both black rhinoceros metapopulations, albeit at low prevalences. Plains zebras (*Equus quagga burchelli*) and Cape mountain zebras (*Equus zebra zebra*), which co-exist with *D. b. minor* and *D. b. bicornis* metapopulations, are subclinical carriers of *T. equi* (Bhoora et al., 2010, 2020). *Rhipicephalus e. evertsi*, the main tick vector of *T. equi* in South Africa, commonly infests equids but has not been reported from black rhinoceroses (Horak et al., 2017). Tapirs (*Tapirus terrestris*), which like equids and rhinoceroses belong to the Order Perissodactyla, can also harbour *T. equi* (Da Silveira, 2017; Gonçalves, 2020). There is mounting evidence that *T. equi* can occur in other mammal orders. It has, for instance, been incriminated in causing clinical signs in domestic dogs in South Africa (Rosa et al., 2014) and has also been reported from dogs in Paraguay (Inácio, 2019) and Saudi Arabia (Salim et al., 2019).

Two rhinoceroses, from Addo Elephant NP and Mokala NP, respectively, tested positive for both *T. bicornis* and *T. equi.* Subsequent phylogenetic analysis revealed that *T. bicornis* clustered with *T. equi*, suggesting a close relationship between the two piroplasms (Nijhof et al., 2003). Our results showed that *T. bicornis* and *T. equi* clustered in distinct clades, however, which was in concordance with the findings of Schnittger et al. (2012). From the RLB probe sequences, it is unlikely that they would cross react. The most likely explanation is that this was a dual infection with both *T. equi* and *T. bicornis*. Of 195 white rhinoceroses sampled in Kruger NP, 71 (36.4 %) tested positive for *T. bicornis* on RLB, with no significant change in the haematological parameters measured, while 18 (9.2 %) tested positive for *T. equi* (Govender et al., 2011). None of these white rhinoceroses had tested positive for *B. bicornis*.

Indication of possible presence of rickettsias, i.e. *Anaplasma* and/or *Ehrlichia* spp., was found in five rhinoceroses of both subspecies, but none of the specimens reacted with species-specific probes. Due to financial constraints, sequencing of these specimens could not be performed. Although 18 of 32 (56%) black rhinoceroses from the lower Zambezi Valley, Zimbabwe, were seropositive to *Ehrlichia ruminantium* (Kock et al., 1992), no other references to occurrence of rickettsias in black rhinoceroses could be traced.

4.1 Vectors

The vectors of *B. bicornis* and *T. bicornis* have not been confirmed. Based on RLB, *T. bicornis* was reported from salivary glands of *R. e. evertsi* from South Africa, which

suggests that it may be a vector (Berggoetz et al., 2014); this tick has not been reported from black rhinoceroses, however (Horak et al., 2017). *Amblyomma rhinocerotis* and *Dermacentor rhinocerinus*, which prefer feeding on black and white rhinoceroses (Horak et al., 2017), are possible candidates. *Dermacentor rhinocerinus* occurs in the Kruger NP as well as in KwaZulu-Natal nature reserves, while *A. rhinocerotis* is restricted to the latter only (Horak et al., 2017). At least one other vector is therefore involved in transmission.

The absence or dearth of piroplasm infections in rhinoceros populations in arid areas reflects the low levels of tick infestation, rather than an innate resistance to the piroplasms. Occurrence of piroplasms in *D. b. bicornis* in Addo Elephant NP did not differ significantly from that in *D. b. minor* in the nearby Great Fish River NR, where general climatic conditions and vegetation are similar. One or more of the following ticks, that occur in both of these conservation areas, could possibly be vectors of *B. bicornis* and/or *T. bicornis*: *Amblyomma hebraeum*, *Hyalomma rufipes*, *Hyalomma truncatum*, *Rhipicephalus follis*, *Rhipicephalus simus* and *Rhipicephalus zumpti* (Horak et al., 2017). Of these, *A. hebraeum* is by far the most prevalent and common, infesting all but one of 90 black rhinoceroses examined in Southern Africa (Horak et al., 2017). In a total collection from one rhinoceros in Addo Elephant NP, 293 *A. hebraeum* larvae, 394 nymphs and 1,211 adults were recovered (Knapp et al., 1997). *Rhipicephalus simus* was the next most prevalent species, recovered from 46% of 90 black rhinoceroses (Horak et al., 2017).

4.2 Black rhinoceros metapopulation management in South Africa

Most of Africa's black rhinoceroses currently occur in Southern Africa, but this was not always the case. By the end of the 19th century, black rhinoceroses had been hunted virtually to extinction in South Africa, surviving in small but viable populations only in Hluhluwe GR (now Hluhluwe-iMfolozi Park) and Mkuzi GR in north-eastern KwaZulu-Natal (Fig. 1). These were of the subspecies *D. b. minor*. A few individuals had also survived in Kruger National Park (NP), but the last positive record was in 1936 (Stevenson-Hamilton, 1929, 1947). With the exception of those in Hluhluwe and Mkuzi, therefore, all black rhinoceros populations in South Africa have been reintroduced.

For conservation purposes, the two black rhinoceros subspecies in South Africa are managed as two distinct metapopulations: the south-western *D. b. bicornis* and the south-eastern *D. b. minor*.

4.2.1. Diceros bicornis bicornis

Due to strenuous conservation efforts, the overall number of south-western subspecies, *D. b. bicornis* is increasing, and this subspecies is currently regarded as Near-threatened (Emslie, 2020a). South African populations of *D. b. bicornis* originate from Etosha NP, Namibia. The first reintroductions were to Augrabies NP and Vaalbos NP (Northern Cape Province) during the 1980s (Hall-Martin and Knight, 1994; Hall-Martin, 1986; Raath and Hall-Martin, 1989). For various reasons, these two populations were later relocated elsewhere, including Karoo NP (Western Cape Province) and Mokala NP (Northern Cape Province) (Fig. 1) (Hall-Martin and Knight, 1994). Animals from Etosha NP were also

translocated to Tswalu Private GR (Northern Cape Province) (Hall-Martin and Knight, 1994). Populations have also been reintroduced to private conservancies.

Black rhinoceros conservation at Addo Elephant NP (Eastern Cape Province) is a special case. The initial population, of the eastern subspecies *D. b. michaeli* imported from Kenya in the early 1960s (Carter, 1965; Hall-Martin and Penzhorn, 1977; Penzhorn, 1971), was augmented by three *D. b. minor* males from Hluhluwe in 1977 (de Vos and Braack, 1980). Two of the latter were removed to Kruger NP in 1981 (Hitchins, 1984). The entire population was subsequently removed. The pure-bred *D. b. michaeli*, a subspecies listed as critically endangered (Emslie, 2020b), were repatriated to East Africa [e.g., Ngorongoro Crater, Tanzania (Fyumagwa et al., 2007)], while the possible *D. b. minor* x *D. b. michaeli* crosses were relocated to a zoo. Addo Elephant NP was subsequently restocked with south-western *D. b. bicornis*. In line with metapopulation management guidelines, some *D. b. bicornis* individuals from Addo NP have been translocated Mountain Zebra NP (Fig. 1).

The environment in these parks varies from a very dry, semi-desert/Nama-Karoo habitat in the Northern Cape to more mesic thicket and fynbos habitats in the southern areas of the Eastern and Western Cape Provinces (Fig. 1). The exposure to various diseases and potential vectors, such as ticks, also varies according to the environment and habitat type.

Black rhinoceroses originating from Namibia are apparently not infected with piroplasms (Penzhorn et al., 2008) and therefore did not pose a risk to the destination ecosystems.

This would also suggest that any rhinoceroses that have tested positive for piroplasms would have been exposed to a tick vector, and had become infected, since their reintroduction to South Africa from Namibia.

In this study, blood samples from black rhinoceros populations in semi-arid/Nama-Karoo area the (Vaalbos NP, Karoo NP and Tswalu GR) tested negative for both *T. bicornis* and *B. bicornis*, indicating that they had not been exposed to these piroplasms and/or vectors do not occur in the area. Blood specimens from two rhinoceroses from Vaalbos NP gave a positive *Theileria/Babesia* genus-specific signal, without a species-specific signal, suggesting a possible novel species or variant of a species in the area. Both these animals were born at Vaalbos NP and were presumably exposed to the parasite and vector while in that ecosystem.

The only specimens that tested positive for *B. bicornis* or *T. bicornis* were from Addo Elephant NP and Mountain Zebra NP. The *B. bicornis*-positive individual at Mountain Zebra NP had been translocated from Addo Elephant NP. Since the blood specimen was collected soon after his arrival, it is likely that he had become infected in Addo Elephant NP.

Although the current black rhinoceros population in Addo Elephant NP is *D. b. bicornis*, both *D. b. michaeli* (from Kenya) and *B. b. minor* (from KwaZulu-Natal, South Africa) had been present previously (De Vos and Braack, 1980; Hall-Martin and Penzhorn, 1977; Penzhorn, 1971). It is suspected that both *B. bicornis* and *T. bicornis* must have been

introduced to Addo Elephant NP, along with their black rhinoceros host, from KwaZulu-Natal and/or Kenya, where piroplasms had been reported earlier (Bigalke et al., 1970; Brocklesby, 1967; McCullough and Achard, 1969; Mugera and Wandera, 1967).

The apparent absence of piroplasms from *D. b. bicornis* populations in arid or semi-arid regions has important implications for conservation managers performing a disease risk assessment. Everything that happens up until the point of releasing individuals should be done to maximize the chances that these individuals will survive and establish or re-enforce a wild population without damage to either source or release ecosystems (Ewen et al., 2012). In most natural ecosystems, a dynamic relationship is maintained between parasitic organisms, their indigenous hosts, and the environment (Penzhorn, 2006). If these naïve animals are translocated into a known infected area, they are at risk of becoming infected and developing clinical disease. This was likely the case of the only confirmed black rhinoceros mortality recorded as a result of babesiosis in the Addo Elephant NP population, which was soon after the reintroduction of the *D. b. bicornis* population (Nijhof et al., 2003). Diligently monitoring the rhinoceroses post-release would therefore be prudent. Prophylactic treatment with an anti-*Babesia* compound such as diminazene aceturate could also be considered (Fyumagwa et al., 2004).

Rhinoceroses translocated from the Addo Elephant NP population also pose a risk of introducing the piroplasms to destination ecosystems. The consequences of such translocations could be potentially disastrous for the destination ecosystem, in the event that the disease of concern infects the susceptible hosts, resulting in failure of the overall

translocation effort. At this stage it is unknown whether ticks occurring in the arid areas of the country would be vectors of the piroplasms. This could be a major setback for the metapopulation management program and it would therefore be important to treat relocated individuals prophylactically with an anti-*Babesia* compound, such as diminazene aceturate or imidocarb, and monitor the whole population post-release for a potentially infected animal.

4.2.2. Diceros bicornis minor

Populations of Hluhluwe and Mkuzi stock (*D. b. minor*) have been reintroduced into various game reserves and conservancies within southern Africa. In some cases, such as the Great Fish River NR (Eastern Cape Province) and Addo Elephant NP, introductions were outside the natural range of this subspecies (Fig. 1) (Brooks, 1989; Hitchins, 1984; Hitchins et al., 1972). The Kruger NP population was augmented by some animals from Zimbabwe (Brooks, 1989; Hall-Martin and Knight, 1994). Later, the population in the newly established Marekele NP (Limpopo Province) was founded with Kruger NP animals, as well as two captive-bred black rhinoceroses from the Frankfurt Zoo, Germany.

The number of *D. b. minor* is stable, but this subspecies is still regarded as Critically Endangered (Emslie, 2020c). With the exception of Pilanesberg NP, where only three rhinoceroses had been sampled, *T. bicornis* was found in all conservation areas. *Babesia bicornis* was found only at Great Fish River NR and Marakele NP. Since the Marakele NP population was established with Kruger NP stock, the apparent absence of *B. bicornis*

in the latter probably reflects a relatively small sample size (n=16) and is not necessarily a true reflection of the current situation. The presence of the piroplasms suggests that they are, or have become, endemic to these areas. One of the rhinoceroses that tested positive for *T. bicornis* was born in the Frankfurt Zoo, Germany, and had been translocated to Marakele NP. The rhinoceros would first have been exposed to *T. bicornis* after translocation, which suggests a degree of innate resistance. This supports the contention that *T. bicornis* is not pathogenic to rhinoceroses.

From a disease risk point of view, there is a high probability that individuals in the *D. b. minor* metapopulation have been exposed to piroplasms. Therefore, the magnitude of risk during translocations between various populations is reduced. There is more of a risk of the translocated animals succumbing to babesiosis due to stress-related immunosuppression. Prophylactic administration of anti-*Babesia* compounds at translocation is therefore warranted.

5.Conclusion

Based on findings to date, *B. bicornis* and *T. bicornis* are relatively widespread in black rhinoceros populations in South Africa and pose a potential risk to the success of metapopulation management programs. They should therefore be considered in a disease risk analysis prior to any relocation of black rhinoceroses. Of the two black rhinoceros subspecies that occur in South Africa, *D. b. bicornis* is at greater risk due to their apparently *Babesia/Theileria*-naïve status in certain areas, when compared to the subspecies *D. b. minor*. Conservation managers need to carefully evaluate methods and

procedures during the translocation of black rhinoceroses, especially when relocating from geographically and climatically diverse ecosystems and more so when dealing with *D. b. bicornis*.

This study (V071/07) was approved by the Animal Use and Care Committee of the University of Pretoria and the Research Committee of the Faculty of Veterinary Science, University of Pretoria.

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