

# Occurrence of tick-borne haemoparasites in South African rodent species and evidence of *Babesia microti*-like sequence variants in two *Rhabdomys* species

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## ABSTRACT

In recent years, vector-borne viral, bacterial and parasitic diseases have emerged or re-emerged in many geographic regions, causing health and economic problems. Wildlife can act as reservoirs for many human and livestock diseases and that arthropods are often involved in the transmission of these pathogens. Little is known about the zoonotic pathogens that naturally occur in wild rodent and insectivorous shrew species in South Africa. The aims of the study were to: 1) record the tick-borne haemoparasite diversity associated with rodents and shrews at several localities in South Africa, and 2) characterise *Babesia microti* that occur in rodents using Sanger sequencing of the parasite 18S ribosomal RNA (rRNA) gene and internal transcribed spacer region 1 (ITS1). Nine wild rodent genera (species: n = 412) and two insectivorous shrew genera (species: n = 11) were simultaneously screened for the presence of *Theileria*, *Babesia*, *Ehrlichia* and *Anaplasma* species using the Reverse Line Blot (RLB) hybridization assay. The RLB PCR amplicons obtained from *Mastomys* spp., *Micaelamys namaquensis*, *Mus musculus*, *Rhabdomys dilectus* and *R. pumilio* hybridized with the following RLB species-specific probes: *Babesia microti* (11.3%), *Anaplasma bovis* (3.5%), *Anaplasma phagocytophilum* (1.8%) and *Ehrlichia ruminantium* (3.5%). The near full-length 18S rRNA gene and partial ITS1 spacer region of 12 of the specimens were cloned and the recombinants sequenced. *Babesia microti*-like 18S rDNA and ITS1 sequence variants were recorded in two rodent species (*Rhabdomys dilectus* and *Rhabdomys pumilio*). The *B. microti*-like 18S rDNA sequence variants, obtained in this study, clustered phylogenetically within the established *B. microti*-like clade (Clade I). Nevertheless, they formed a distinct South African group that seems to differ significantly from *B. microti sensu stricto* and could potentially represent a fifth distinct lineage within the *B. microti*-like clade. The study highlights the potential of small mammals acting as important reservoirs for vector-borne haemoparasites in South Africa.

## 1. Introduction

Wildlife are major sources of zoonotic diseases (Raoult and Roux, 1997; Taylor et al., 2001). In particular, rodents and the parasites they harbour play an important role in the maintenance and transmission of zoonotic pathogens, and may be involved in the transmission of diseases to domestic and wild animals (Walker, 1991; Parola and Raoult, 2001; Colwell et al., 2011; Otranto and Eberhard, 2011). Rodents are reservoirs for several zoonotic pathogens including at least 40 protozoa,

bacteria, viruses and rickettsias (Meerburg et al., 2009). *Babesia microti*, a parasite found in rodents, is one of the main causes of human babesiosis (Western et al., 1970; Krause, 2019). The white-footed mouse (*Peromyscus leucopus*) and several other wild rodent species are reservoirs for *B. microti* in the United States of America (USA), Europe and Asia (Van Peen et al., 1977; Watkins et al., 1991; Burkot et al., 2000; Duh et al., 2003; Siński et al., 2006; Beck et al., 2011). Other zoonotic *Anaplasma* and *Ehrlichia* species (causing human granulocytic anaplasmosis and human monocytic ehrlichiosis, respectively) are known to

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occur concurrently in the same reservoir as *B. microti* and share the same tick vectors (Barbour, 1998).

Rodent associated vector-borne diseases are a global cause for concern (Mills and Childs, 1998; Cleaveland et al., 2001; Meerburg et al., 2009; Luis et al., 2013; Islam et al., 2021; Herrera-Mares et al., 2022). For example, human ehrlichiosis (Chapman et al., 2006) and babesiosis (Herwaldt et al., 2012) have been added to the list of notifiable diseases in the USA. In addition, the incidence of *Anaplasma phagocytophilum* infections has increased considerably in Lyme disease-endemic areas in the USA, particularly in regions where the geographic range and abundance of *Ixodes scapularis* have expanded, such as the Northeast and upper Midwest (e.g., Wisconsin, Minnesota, and New York) (Paddock et al., 2016). The increase of tick-borne encephalitis virus cases in Europe and especially Sweden (Lindgren and Gustafson, 2001) has been associated with a northern range expansion by the tick vector *Ixodes ricinus* (Tälleklint, and Jaenson, 1998). In recent years, cases of human babesiosis, primarily caused by *Babesia microti* and predominantly transmitted by ticks of the genus *Ixodes*, have also increased in the USA (Westblade et al., 2017) and Europe (Hunfeld et al., 2002; Kumar et al., 2021).

The reservoir status of rodents for zoonotic pathogens is poorly studied in South Africa and other African countries. The few studies that are available have mainly focussed on leptospirosis and *Yersinia pestis* infections using serological detection methods (Kilonzo et al., 2005; Makundi et al., 2008; Laudisoit et al., 2009). In Tanzania, *Yersinia pestis* was confirmed in both domestic and wild rodents as well as in dogs in several districts (Kilonzo et al., 2005). Katakweba et al. (2012) used blood smears and serology to investigate the prevalence of potentially zoonotic haemoparasite infections in rodents and shrews in Namibia, Tanzania and Swaziland. In their study, only *Trypanosoma lewisi* parasites were recorded and only in Tanzania and Swaziland. Katakweba (2018) later recorded *Babesia*, *Bacillus*, *Plasmodium* and *Trypanosoma* species from rodent blood smears in Tanzania. Microscopic examination of blood smears was also used to record *Trypanosoma* and *Plasmodium* species in rodents from western Kenya (Makokha et al., 2011). There have been reports of the detection of *A. phagocytophilum* in Africa, mainly identified using nucleic acid-based detection methods. *Anaplasma phagocytophilum* DNA has been detected in horses, ticks and cattle from Tunisia (M'Ghirbi et al., 2012; M'Ghirbi et al., 2016), vervet monkeys and baboons in Zambia (Nakayima et al., 2014), lions, African wild cats and servals in Zimbabwe (Kelly et al., 2014), and dogs and cattle in Algeria (Azzag et al., 2015; Dahmani et al., 2015). In South Africa, Mtshali et al. (2015) reported on the detection of *A. phagocytophilum* DNA in ticks collected from cattle, sheep and goats, however, Kolo et al. (2020) demonstrated that the finding could not be considered valid as the polymerase chain reaction (PCR) primers used in that study could amplify any *Anaplasma* species. While the preceding studies suggest that *A. phagocytophilum* is present throughout Africa, this conclusion may not be valid, since it is possible that previously unrecognized *Anaplasma* spp. might have been detected in these studies. Subsequently, Kolo et al. (2020) provided the first report of the detection of *A. phagocytophilum* DNA in humans (patients with acute febrile illnesses), rodents and dogs in Mnisi, a rural community in South Africa.

Although *B. microti* is primarily associated with rodents, some studies reported the presence of *B. microti* from wild and domestic animals in several African countries, including South Africa (Maamun et al., 2011; Bloch et al., 2018; Bosman et al., 2019). In particular, the presence of *Babesia* and *B. microti*-like parasites were reported in baboons (*Papio* spp.) and African green monkeys (*Chlorocebus* spp.) in Kenya (Maamun et al., 2011), as well as in baboons from Zambia (Nakayima et al., 2014). More recently, a *B. microti* 18S rRNA gene sequence (MK095342), showing 100% sequence identity over a 1281 base-pair region to the published *B. microti* Otsu strain 18S rDNA sequence (AB119446), was detected in a single domestic cat in South Africa (Bosman et al., 2019). Based on these studies, *Babesia microti* and *B. microti*-like parasites are present in Africa and may occur in several other wildlife species or

domestic animals (Siński et al., 2006).

Human babesiosis cases is rarely diagnosed in Africa, likely due to the widespread occurrence of malaria, which shares significant clinical similarities with babesiosis (Bloch et al., 2018). Differentiating between the two diseases can be difficult as *Babesia* parasites closely resemble *Plasmodium* species on peripheral blood smears. As a result of this overlap in clinical presentation and diagnostic features, it is likely that human babesiosis cases may be misdiagnosed or underreported (Homer et al., 2000; Bloch et al., 2018). To date, three human babesiosis cases caused by uncharacterized babesias have been documented in Egypt (Michael et al., 1987; El-Bahnasawy and Morsy, 2008; El-Bahnasawy et al., 2011), and two suspected cases have been reported in South Africa (Bush et al., 1990). Additionally, a pilot seroprevalence study in children from the Kilosa district of Tanzania provided serological evidence for *B. microti* infection (Bloch et al., 2018).

South Africa is known for its high biodiversity that includes 103 extant small mammals and insectivore species (Skinner and Chimimba, 2005), with more than 40 ixodid tick species associated with small mammals (Horak et al., 2018). At present, little is known regarding the protozoan diversity that naturally circulates within wild rodent species in South Africa and thus no information is available on the associated risk to livestock and humans. The aims of the study were to: 1) record the tick-borne haemoparasite diversity associated with rodents and shrews using the Reverse Line Blot (RLB) hybridization assay, and 2) characterise the *B. microti* strain(s) associated with the rodents using gene sequence and phylogenetic analysis. In this study, no sequencing or phylogenetic analysis was performed on the Anaplasmataceae-positive samples identified through the RLB assay.

## 2. Material and methods

### 2.1. Sample collection and host data

Blood samples were collected during various studies that focused on macroparasites of small mammals in South Africa between 2007 and 2012 (Froeschke and Matthee, 2014; Matthee et al., 2007, 2010). Rodents and insectivores were trapped at several localities across South Africa, although the main sampling regions were the Western and Northern Cape provinces (Table 1). The animals were trapped using Sherman-type live traps that were baited with peanut butter and oats and left out for 4–7 days per locality, depending on the trapping success. The traps were checked twice daily.

Only adult animals were selected for this study (based on the weight range as listed in Skinner and Chimimba (2005)). Rodents and insectivores were identified by means of field guides (e.g. Stuart and Stuart, 2001; Skinner and Chimimba, 2005) and euthanized using intra-peritoneal injection with sodium pentobarbitone (200 mg/kg). Blood samples were collected after the last heartbeat, either from the heart or from the tongue onto FTA filter paper (Merck, South Africa).

### 2.2. DNA extraction

Genomic DNA was extracted from five punched circles (3 mm diameter) of dried blood spots on the FTA filter paper using the QIAamp® DNA mini kit (Qiagen) according to the manufacturer's instructions. The DNA was eluted into 100 µl of elution buffer and stored at -20 °C.

### 2.3. PCR amplification and Reverse Line Blot (RLB) hybridization assay

DNA samples were subjected to the RLB hybridization assay as described by Nijhof et al. (2005). *Theileria* and *Babesia* genus-specific primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3') (Nijhof et al., 2005), were used to amplify the V4 hypervariable region of the parasite 18S rRNA gene. For the simultaneous detection of *Anaplasma* and

**Table 1**

Species, locality information and sample sizes of the rodents and shrews included in the study.

Species	Province	Locality
<b>Rodents (Order Rodentia)</b>		
<i>Desmodillus auricularis</i> (n = 4)	Northern Cape (n = 4)	Springbok (n = 4)
<i>Mastomys</i> spp. (n = 10)	Eastern Cape (n = 3)	East London (n = 3)
	KwaZulu-Natal (n = 7)	Oribi Gorge (n = 4) Vernon Crookes N.R (n = 3)
<i>Micaelamys namaquensis</i> (n = 25)	Northern Cape (n = 20)	Kuruman (n = 4) Springbok (n = 16)
	Western Cape (n = 5)	Beaufort West (n = 5)
<i>Mus musculus</i> (n = 20)	Eastern Cape (n = 20)	East London (n = 20)
<i>Otomys irroratus</i> (n = 2)	Western Cape (n = 2)	StellenboschA (n = 2)
<i>Otomys unisulcatus</i> (n = 14)	Western Cape (n = 4)	Beaufort West (n = 4)
	Northern Cape (n = 9)	Springbok (n = 7) Groblershoop (n = 2)
	Eastern Cape (n = 1)	East London (n = 1)
<i>Parotomys brantsii</i> (n = 4)	Northern Cape (n = 4)	Springbok (n = 3) Kuruman (n = 1)
<i>Rhabdomys dilectus</i> (n = 32)	Eastern Cape (n = 14)	East London (n = 14)
	KwaZulu-Natal (n = 18)	Oribi Gorge (n = 2) Vernon Crookes N.R (n = 16)
<i>Rhabdomys pumilio</i> (n = 298)	Western Cape (n = 162)	Beaufort West (n = 33) Franschhoek (n = 21) Gordon's Bay (n = 23) StellenboschA (n = 29) StellenboschB (n = 29) Somerset West (n = 16) Wellington (n = 11)
	Northern Cape (n = 136)	Groblershoop (n = 16) Springbok (n = 120)
	Northern Cape (n = 3)	Kuruman (n = 3)
<b>Insectivorous shrews (Orders Macroscelidea/Soricomorpha)</b>		
<i>Crociodura</i> spp. (n = 9)	KwaZulu-Natal (n = 9)	Oribi Gorge (n = 4) Vernon Crookes N.R (n = 5)
<i>Elephantulus</i> spp. (n = 2)	Northern Cape (n = 2)	Springbok (n = 2)

*Ehrlichia* spp., primers Ehr-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') and 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 hyper-variable region of the 16S rRNA gene for *Anaplasma* and *Ehrlichia*. Positive and negative controls were included in each batch of samples processed. The positive control consisted of *Babesia bovis* DNA extracted from the *B. bovis* vaccine (Onderstepoort Biological Products, South Africa), while water served as the negative control. Amplification was performed using a touchdown PCR thermocycler programme (Nijhof et al., 2005). Known *Babesia*, *Theileria*, *Ehrlichia*, and *Anaplasma* genus- and species-specific oligonucleotide probes were used at predetermined optimized concentrations.

#### 2.4. Amplification, cloning and sequencing of the 18S rRNA gene and the internal transcribed spacer region 1 (ITS1)

Selected samples that hybridized to the *B. microti* species-specific probe during the RLB hybridization assay were chosen for amplification of an approximately 1800 bp fragment of the parasite 18S rDNA gene. Phusion Flash High-Fidelity (Thermo Scientific) PCR master mix was used for amplification of the 18S rRNA gene by means of the primers NBab\_1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and T/B Rev (5'-AATAAT TCA CCG GAT CAC TCG-3') (Matjila et al., 2008; Oosthuizen et al., 2008). The parasite internal transcribed spacer region 1 (ITS1) of the same sample set, was also amplified using the Phusion Flash High-Fidelity (Thermo Scientific) PCR master mix, but with

primers ITS1for (5'-CGA GTG ATC CGG TGA ATT ATT C-3') and ITS1rev (5'-CCT TCA TCG TTG TGT GAG CC-3') as described by Blaschitz et al. (2008). Five separate reactions were prepared per sample, pooled (to avoid Taq polymerase-induced errors) and purified using the High Pure PCR Product Purification KIT (Roche, South Africa). *Babesia bovis* DNA extracted from the *B. bovis* vaccine (Onderstepoort Biological Products, South Africa) and water were used as positive and negative controls, respectively, for the PCR amplification.

Purified products were cloned using the pJET vector system (Thermo Fischer Scientific) and transformed into competent *E. coli* JM109 High Efficiency Competent cells (Promega, USA). At least five colonies per sample were selected for subsequent Sanger sequencing using the vector primers pJET1.2.F (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJET1.2.R (5'-AAG AAC ATC GAT TTT CCA TGG CAG-3'). Sequencing reactions were analysed on an ABI 3100 genetic analyser at Inqaba Biotech Systems, Pretoria, South Africa.

#### 2.5. Phylogenetic analysis

The obtained recombinant sequences were assembled and edited using the GAP4 programme of the Staden Package (Staden et al., 1999) (<https://staden.sourceforge.net>). GenBank BLASTn homology searches (Altay et al., 2008) ([ncbi.nlm.nih.gov/blast](https://ncbi.nlm.nih.gov/blast)) was done using the consensus sequences. These were then aligned using MAFFT version 7 (<https://mafft.cbrc.jp/alignment/server/gotomafft.html>); each alignment was manually examined and truncated to the size of the shortest sequence using BioEdit Version 7.2.5 (Hall, 1999). For each alignment, the estimated evolutionary divergence was calculated by determining the number of nucleotide differences between similar sequences using MEGA X (Kumar et al., 2018) (<https://www.megasoftware.net>). A model test was performed to determine the most suitable nucleotide substitution model to infer phylogenetic relationships. Phylogenetic trees were constructed using both Neighbour joining and Maximum Likelihood methods, in combination with the bootstrap method (Felsenstein, 1985) with 1000 replicates per tree, using MEGA X. The 18S rRNA gene sequences identified in this study were submitted to GenBank (PQ578023-PQ578028).

### 3. Results

#### 3.1. Reverse Line Blot hybridization assay

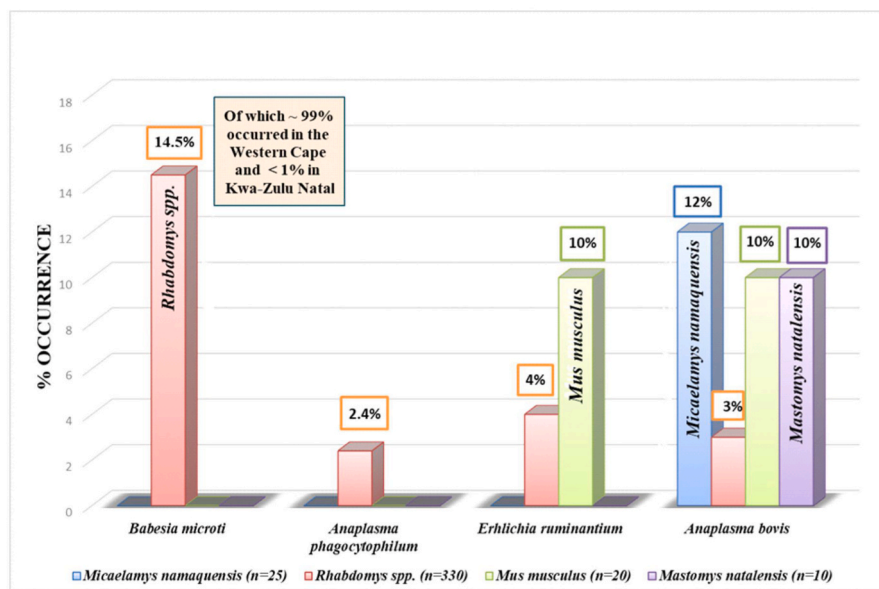
A total of 423 blood samples from rodents (n = 412) and insectivores (n = 11) were simultaneously screened for the presence of *Theileria*, *Babesia*, *Ehrlichia* and *Anaplasma* species using the RLB hybridization assay; a valuable screening tool for the simultaneous detection of infections using species- and genus-specific probes (Bekker et al., 2002; Gubbels et al., 1999). The probes used in the RLB assay are generally highly specific and accurate, being carefully designed and validated. However, it is important to acknowledge that their performance can be influenced by factors such as genetic variability in the targeted pathogens, hybridization conditions, and the quality of the samples being analysed. Ultimately, gene sequence analysis of the pathogen of interest should be done to verify the results.

The RLB hybridization assay results (Table 2) indicated the presence of a variety of tick-borne blood parasites either as single or mixed infections. PCR products hybridized with the *Theileria/Babesia* genus-specific probe in 41.6% (n = 176) of the samples and 21.7% (n = 92) hybridized with the *Ehrlichia/Anaplasma* genus-specific probe. A total of 200 samples (47.3%) tested negative (or below the detection limit of the assay).

*Babesia microti*, *Anaplasma bovis*, *A. phagocytophilum* and *Ehrlichia ruminantium* occurred either as single or mixed infections in *Rhabdomys dilectus*, *R. pumilio*, *Mus musculus*, *Micaelamys namaquensis* and *Mastomys* spp. (Table 2) (Fig. 1). Furthermore, *B. microti* DNA was only present in the two *Rhabdomys* species of which the highest incidence was recorded

**Table 2**  
Occurrence of tick-borne haemoparasites in rodent and insectivore species from various localities in South Africa as determined using the RLB.

Species	Genus-specific				Species-specific		Genus-specific	Species-specific		
	<i>Theileria/Babesia</i>	<i>Theileria</i>	<i>Babesia</i> 1	<i>Babesia</i> 2	<i>Babesia microti</i>	<i>Babesia bovis</i>	<i>Ehrlichia/Anaplasma</i>	<i>Anaplasma bovis</i>	<i>Anaplasma phagocytophilum</i>	<i>Ehrlichia ruminantium</i>
<b>Rodents</b>										
<i>Rhabdomys pumilio</i> (n = 298)	145 (48.6%)	25 (8.3%)	131 (66%)	110 (36.9%)	47 (15.7%)	4 (1.3%)	72 (24.1%)	6 (2%)	7 (2.3%)	13 (4.3%)
<i>Rhabdomys dilectus</i> (n = 32)	9 (28%)	5 (15.6%)	6 (18.7%)	5 (15.6%)	1 (3.1%)	0 (0%)	6 (18.7%)	3 (9.3%)	1 (3.1%)	0 (0%)
<i>Saccostomus campestris</i> (n = 3)	1 (33.3%)	0 (0%)	2 (66.6%)	2 (66.6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<i>Micaelamys namaquensis</i> (n = 25)	3 (12%)	1 (4%)	6 (24%)	3 (12%)	0 (0%)	0 (0%)	5 (20%)	3 (12%)	0 (0%)	0 (0%)
<i>Desmodillus auricularis</i> (n = 4)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<i>Mus musculus</i> (n = 20)	5 (25%)	4 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (15%)	2 (10%)	0 (0%)	2 (10%)
<i>Mastomys natalensis</i> (n = 10)	1 (10%)	0 (0%)	2 (20%)	2 (20%)	0 (0%)	0 (0%)	1 (10%)	1 (10%)	0 (0%)	0 (0%)
<i>Otomys irroratus</i> (n = 2)	2 (100%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<i>Myotomys unisulcatus</i> (n = 14)	7 (50%)	3 (21.4%)	3 (21.4%)	5 (35.7%)	0 (0%)	0 (0%)	2 (14.2%)	0 (0%)	0 (0%)	0 (0%)
<i>Parotomys brantsii</i> (n = 4)	1 (25%)	1 (25%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<b>Insectivores</b>										
<i>Crocidura</i> spp. (n = 9)	0 (0%)	0 (0%)	1 (11.1%)	1 (11.1%)	0 (0%)	0 (0%)	2 (22.2%)	0 (0%)	0 (0%)	0 (0%)
<i>Elephantus</i> spp. (n = 2)	2 (100%)	0 (0%)	2 (100%)	1 (50%)	0 (0%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	0 (0%)
<b>Total (n = 423)</b>	<b>176 (41.6%)</b>	<b>39 (9.2%)</b>	<b>154 (36.4%)</b>	<b>129 (30.4%)</b>	<b>48 (11.3%)</b>	<b>4 (0.94%)</b>	<b>92 (21.7%)</b>	<b>15 (3.5%)</b>	<b>8 (1.8%)</b>	<b>15 (3.5%)</b>



**Fig. 1.** Occurrence (%) of tick-borne haemoparasites in rodents from various localities in South Africa based on the Reverse Line Blot hybridization assay results.

in *R. pumilio* (47/298, 15.7%), followed by *R. dilectus* (1/32, 3.1%). *Anaplasma bovis* DNA was mostly detected in *M. namaquensis* (3/25, 12%), followed by *Mastomys* spp. (1/10, 10%), *M. musculus* (2/20, 10%), *R. dilectus* (3/32, 9.3%) and *R. pumilio* (6/298, 2%). *Anaplasma phagocytophilum* DNA was only detected in *R. dilectus* (1/32, 3.1%) and *R. pumilio* (7/298, 2.3%). *Ehrlichia ruminantium* DNA was detected in *M. musculus* (2/20, 10%) and *R. pumilio* (13/298, 4.3%). Less than 1% of

the PCR products (n = 4) hybridized with both the *B. bovis* and *Theileria buffeli* species-specific probes. PCR products of four samples hybridized to the *B. bovis* species-specific probe and one sample's PCR product hybridized to the *Anaplasma* spp. Omatjenne species-specific probe.

### 3.2. *B. microti*-like 18S rRNA gene sequence analysis

Twelve *R. pumilio* blood samples collected in different localities in the Western Cape province were selected for further analysis based on their positive *B. microti* RLB and SYBR green real-time PCR results (M Troskie, 2017). Near full-length parasite 18S rRNA genes were successfully amplified from eight of the 12 selected *Rhodomys* samples, cloned and sequenced. Four partial and 23 almost full-length recombinant 18S rRNA sequences were obtained. The sequences were assembled, edited and aligned with sequences of related genera from Genbank. BLASTn homology searches showed that the obtained sequences had 96–97% sequence identity to published sequences of *B. microti* (USA-type Gray) (AY693840) isolated from congeneric species in a human in the USA, *B. leo* (AF244911) previously identified from a lion (*Panthera leo*) in South Africa's Kruger National Park and *B. lengau* (KC790444) detected in two domestic cat cases in South Africa (Bosman et al., 2010). The obtained sequences showed 95–97% sequence identity with *B. felis* (AF244912) detected in a lion in the Kruger National Park, South Africa. Furthermore, it showed 91–96% sequence identity to published sequences of *B. rodhaini* (M87565, AB049999) previously identified in rodents.

Evolutionary divergence between the 23 near full-length 18S rRNA gene sequences and those of closely related *Babesia* sequences was established based on the number of base differences over a 1585 bp region of the 18S rRNA gene. Six sequence variants were obtained, differing by 1–4 bp from each other; equating to 99.94–99.75% sequence similarity over a 1585 bp region of the 18S rRNA gene.

The observed sequence similarities were confirmed by phylogenetic analyses. Neighbour-joining and Maximum Likelihood techniques were used to reveal the phylogenetic relationships between the six near full-length 18S rDNA sequence variants obtained to related *Babesia* species. The topologies of both trees were similar. The 18S rDNA Maximum Likelihood tree (Fig. 2) and the Neighbour-joining trees for both the 18S rDNA and ITS1 region is shown in Fig. 3(a) and (b), respectively. The obtained sequences clustered phylogenetically within the established *B. microti*-like clade (Clade I) (Jalovecka et al., 2019), however, as a separate South African group that appears to be significantly different from *B. microti* sensu stricto.

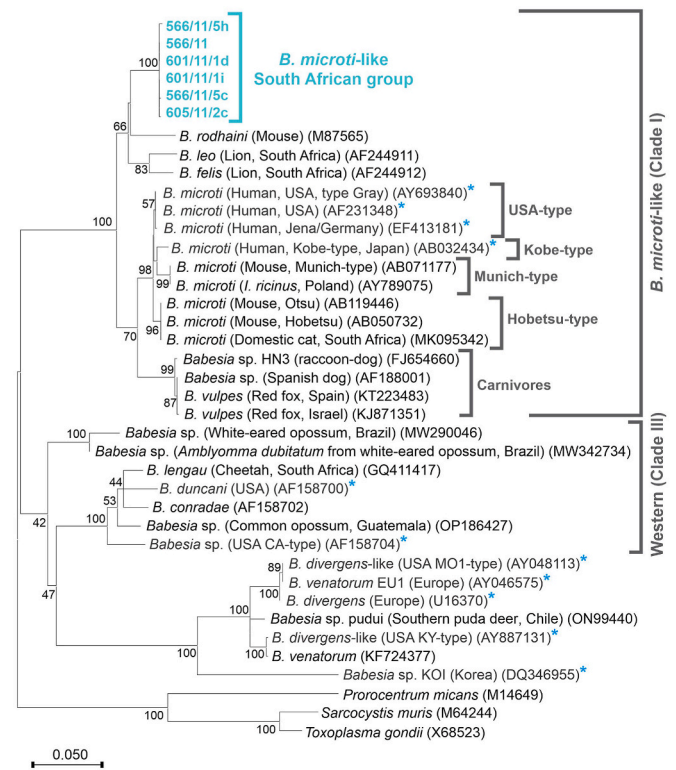
### 3.3. *B. microti*-like ITS1 spacer region sequence analysis

The ITS1 spacer region was successfully amplified from six of the *Rhodomys* samples, which were cloned and sequenced. Eleven (11) recombinant sequences were obtained, all being identical to each other (representing one sequence variant). BLASTn homology searches showed that the obtained sequence had 94–86% sequence identity to several published sequences of *B. microti*, and 95% with *B. rodhaini*. This was also evident by the Neighbour-joining and Maximum Likelihood phylogenetic analyses. The topologies of both trees were similar; the Neighbour-joining trees are shown in Fig. 3. As in the case of the 18S rDNA sequence analysis, the obtained sequences clustered within Clade I, the *B. microti*-like group (Schnittger et al., 2012; Jalovecka et al., 2019), but as a separate South African group (Fig. 3).

## 4. Discussion

The present study confirms the presence of several haemoparasite species in rodents and insectivores in South Africa. In particular, several rodent species were found to be carriers of *Babesia*, *Anaplasma*, and/or *Ehrlichia* species. Furthermore, the widely distributed and commensal rodents *R. pumilio* and *R. dilectus* were positive for *B. microti*-like parasites.

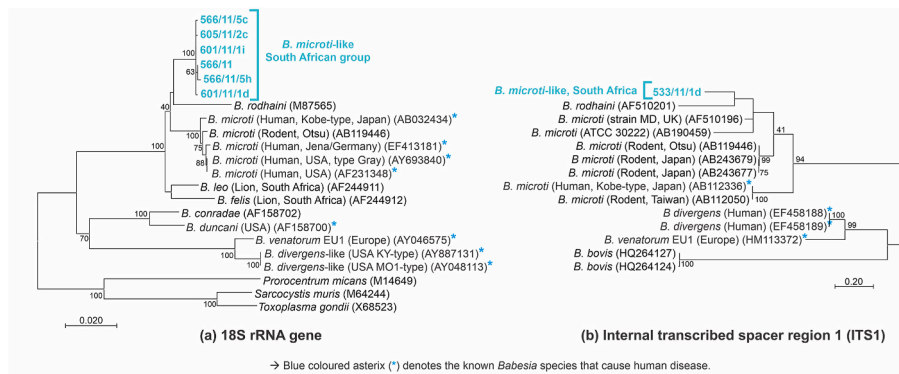
In the present study, *B. microti* parasites were recorded in *R. pumilio* (n = 47) and *R. dilectus* (n = 1) samples using the RLB hybridization assay. The presence of *B. microti*-like parasites was confirmed by 18S rRNA gene and internal transcribed spacer region 1 (ITS1) sequence and



→ Clade numbers correspond to 18S rDNA phylogenetic lineages described by Jalovecka et al. (2019).  
→ Blue coloured asterisk (\*) denotes the known *Babesia* species that cause human disease.

Fig. 2. Maximum Likelihood phylogenetic tree showing the relationship of the obtained parasite 18S rDNA sequence variants (n = 6) from *Rhodomys pumilio* to related *Babesia* species based on the near full-length 18S rRNA gene sequences. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (−5710,73) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0,3772)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 44,20% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Prococtum micans*, *Sarcocystis muris*, and *Toxoplasma gondii* were used as outgroups. There was a total of 1208 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

phylogenetic analysis. Before the advent of molecular technologies, *Babesia* and *Theileria* species were traditionally taxonomically classified based on biological and morphological characteristics (Levine, 1971; Mehlhorn et al., 1994; Reichard et al., 2005). During a molecular phylogenetic study (Schnittger et al., 2012) using new advanced technologies based on the 18S rRNA gene, a Bayesian phylogenetic tree was established of 600 nearly complete 18S rRNA gene sequences using a secondary structure model. Six major monophyletic piroplasmid lineages were deduced, and referred to as Clade I to Clade VI. Clade I was well supported being the sister to all the other piroplasmid species. It is referred to as the *B. microti*-group, containing *Babesia* parasites with supposedly ancient features (e.g. lower vertebrate host specificity, reflecting an ancestral state before more specialized host-parasite relationships evolved; basal position in phylogenetic trees of *Babesia* species, suggesting that it diverged earlier from the common ancestor of *Babesia* parasites compared to other groups), with many originating from Africa. Notably, this clade contains the feline-infecting *B. felis* and *B. leo* and rodent-infecting *B. rodhaini* and *B. microti* parasites.



**Fig. 3.** Visual representation showing similar Neighbour-Joining phylogenetic tree topologies obtained for both the (a) 18S rDNA and (b) internal transcribed spacer region 1 (ITS1) sequences obtained in the study. (a) Neighbour-Joining phylogenetic tree showing the relationship of the obtained parasite 18S rDNA sequence variants ( $n = 6$ ) from *Rhabdomys pumilio* to related *Babesia* species. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). All positions containing gaps and missing data were eliminated (complete deletion option). *Prorocentrum micans*, *Sarcocystis muris*, and *Toxoplasma gondii* were used as outgroups. There were a total of 1474 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. (b) The evolutionary history based on the ITS1 region was inferred using the Neighbour-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 267 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

The current thought on phylogenetic relationships of piroplasmids (Jalovecka et al., 2019) now recognises at least ten principal lineages of which *Babesia* parasites constitute four clades: *Babesia* sensu stricto known as ‘true’ *Babesia* (Clade X, which includes the bovine- and equid-infecting species, and the sheep/goat- and carnivorous parasites), and three lineages that are made up of Percei (Clade V, which includes exclusively *Babesia* species infecting birds), Western (Clade III, infecting several herbivores or carnivores, and humans as accidental hosts) and the *B. microti*-like group (Clade I, which branches into at least four lineages), collectively referred to as *Babesia* sensu lato (Jalovecka et al., 2019). Within these new proposed lineages, the *B. microti*-like group remain in Clade I, and it is thought that felines and rodents are their primordial evolutionary hosts (Penzhorn et al., 2001; Criado-Fornelio et al., 2003), suggesting that piroplasmid lineages evolved by cospeciating with their principal mammalian host taxon (Jalovecka et al., 2019).

*Babesia microti* is a genetically diverse species complex comprising of several geographically distinct clusters which includes at least four zoonotic lineages (USA, Munich, Kobe and Otsu/Hobetsu). Notably, a *B. microti* 18S rDNA gene sequence with 100% sequence identity with the published *B. microti* Otsu strain (over a 1281 base-pair region), was detected in a single domestic cat in South Africa (Bosman et al., 2019). In this study, the phylogenetic analysis showed that the six South African sequence variants clustered in the *B. microti*-like group (Clade Ia), but separately from the other four zoonotic lineages. Due to the distinct characteristics of the *B. microti* and *B. microti*-like group compared to the *Babesia* sensu stricto group, it has been proposed that future taxonomic revisions should elevate the *B. microti* and *B. microti*-like group to a separate genus (Puri et al., 2021). Until a taxonomic revision is undertaken, it remains difficult to determine whether the parasite 18S rDNA sequence variants detected in *Rhabdomys* species in South Africa represent *B. microti*-like sequences or an as-yet undescribed *Babesia* species representing a fifth distinct lineage within the *B. microti*-like clade.

In the present study, *B. microti*-like parasites were only recorded in the genus *Rhabdomys*, which may suggest that *Rhabdomys* species are the primary hosts for *B. microti*-like in South Africa, similarly to the white-

footed mouse (*Peromyscus* spp.) being the primary host for *B. microti* in the United States (Healy et al., 1976; Spielman et al., 1981). However, this will need to be confirmed in future studies. The study also revealed that *R. pumilio* and *R. dilectus* were concurrently infected with multiple haemoparasite species using the RLB hybridization assay (*A. bovis*, *A. phagocytophilum* and *E. ruminantium*). This finding confirms reports that *B. microti*-like organisms often co-exists with other zoonotic pathogens in the same reservoir host (Magnarelli et al., 2006; Abrams, 2008; Beck et al., 2011). High haemoparasite diversity can only be possible if the host is exposed to multiple ectoparasite species as is the case for *R. pumilio* (Matthee et al., 2007, 2010). *Rhabdomys pumilio* and *R. dilectus* are locally abundant and regionally widespread species that have successfully adapted to human-modified landscapes, increasing their exposure to a larger parasite diversity. This is supported by Froeschke et al. (2013) who recorded higher ectoparasite diversity and abundances in *R. pumilio* individuals that occur in remnant fragments of natural vegetation within an agricultural mosaic compared to extensive natural areas. In particular, the study showed that the tick *Haemaphysalis elliptica* was significantly more abundant on *R. pumilio* in the remnant fragments close to houses (Froeschke et al., 2013).

The potential vector for the transmission of *B. microti*-like parasites in *R. pumilio* in South Africa is unknown, although at least 13 ixodid tick species, representing five genera were recovered from *R. pumilio* in the Western Cape (Matthee et al., 2007). In the latter study, *Rhipicephalus gertrudae* was the most abundant and prevalent ticks (prevalence of 82.2%), while *Hyalomma truncatum*, *Ixodes bakeri* and *H. elliptica* were recorded in lower, but comparable numbers from the rodents (Matthee et al., 2007). In the same province, *H. elliptica* was also previously recovered from *R. pumilio* at other localities (Horak et al., 1987; Horak and Boomker, 1998). In the Western Cape, *H. elliptica* is the only economically important species complex within its genus. *Haemaphysalis elliptica* transmits *B. rossi*, the causative organism of canine babesiosis in domestic dogs, *Rickettsia conorii*, the cause of human tick-bite fever as well as the causative agent of Q fever (*Coxiella burnetii*) in humans and animals (Walker, 1991). It is thus quite plausible that *H. elliptica* may be considered as a potential vector for *B. microti*-like parasites in the Western Cape province.

Furthermore, the detection of *B. microti*-like DNA in South Africa is a significant finding as it may have important health implications for human populations in close contact with commensal rodents, such as *Rhodomys pumilio* and *R. dilectus*. Further studies are also needed to investigate the presence of *B. microti*-like organisms in other commensal rodent species (e.g., *Mastomys* spp. and *Rattus* spp.), particularly in the malaria-endemic north-eastern summer rainfall regions of South Africa, where environmental conditions may support the overlap of vector-borne infections. This is especially relevant because the potential role of *Babesia* infections in patients presenting with acute febrile illness (AFI) at community clinics in Africa remains unknown. Acute febrile illnesses are common clinical presentations in Africa, and South Africa and can be caused by various infectious agents, including *Plasmodium* spp. (the causative agent of malaria) (Maze et al., 2018). *Plasmodium falciparum* accounts for most malaria cases in South Africa and can lead to severe complications if untreated. Although *Babesia* infections have not been widely reported in South Africa, *Babesia microti* and other *Babesia* spp. are known to cause human babesiosis, which presents with symptoms similar to malaria, such as fever, chills, fatigue, and anaemia. This clinical overlap complicates diagnosis, especially in resource-limited settings where malaria is often presumed in febrile patients. However, the therapeutic regimens for these parasitic infections differ significantly. Given the potential for misdiagnosis and the public health significance of zoonotic diseases, further research is crucial to understand the prevalence, distribution, virulence and transmission dynamics (to name a few) of *B. microti*-like organisms and their possible contribution to AFI cases in South Africa and across Africa, particularly in malaria-endemic regions.

We recorded *A. bovis* DNA, the causal agent of bovine anaplasmosis, in five rodent species (15/423, 3.5%) using the RLB hybridization assay, namely: *M. namaquensis* (12%), *Mastomys* spp. (10%), *M. musculus* (10%), *R. dilectus* (9.3%) and *R. pumilio* (2%). Similar to *Rhodomys* spp., *M. namaquensis* and *Mastomys* spp. are widespread commensal rodent species in South Africa (Skinner and Chimimba, 2005). Although *A. bovis* infection is mainly reported in cattle, little is known about the epidemiology of this agent in South Africa.

*Anaplasma bovis*, a small gram-negative obligatory intracellular bacterium, infects the monocytes (Rar and Golovljova, 2011) of cattle and buffalo in Africa, South America and Asia (Donatien and Lestoquard, 1936; Dumler et al., 2001) although it has not been reported to cause clinical disease (Dreher et al., 2005; Hofmann-Lehmann et al., 2004). *Anaplasma bovis* DNA was also found in cottontail rabbits from North America (Goethert and Telford, 2003), racoons from Japan (Sashika et al., 2011), water deer from Korea (Kang et al., 2011) and deer from South Korea and Japan (Lee et al., 2009; Kawahara et al., 2006). Reports of human infection with *A. bovis* are limited, with cases recorded in China (Lu et al., 2022) and the USA (Karpathy et al., 2023) thus far. The study in China reported clinical symptoms associated with febrile illness (Lu et al., 2022).

*Rhipicephalus appendiculatus* and *Amblyomma variegatum* in Africa, *A. cajennense* in Brazil and *Hyalomma* spp. in Iran are considered possible tick vectors for *A. bovis* (Dumler et al., 2001; Uilenberg, 1993). Molecular techniques have also identified *A. bovis*-like parasites in *Dermacentor andersoni* in Canada (Dergousoff and Chilton, 2011), *Haemaphysalis concinna* in the Russian Far East (Shpynov et al., 2006), *Haemaphysalis longicornis* from Korea and Japan (Kim et al., 2003; Kawahara et al., 2006) and *Haemaphysalis lagrangei* in Thailand (Parola et al., 2003). Furthermore, a unique genetic variant, distinct from other *A. bovis* isolates worldwide, was detected in *Amblyomma triguttatum* subsp. ticks from southwest Western Australia (Gofton et al., 2017). In South Africa, *A. bovis* has been identified not only in cattle and goats, but also in several wildlife species. It has been reported in nyala (*Tragelaphus angasii*) (Pfitzer et al., 2011); and in a *Rhipicephalus evertsi* tick collected from a gemsbok (*Oryx gazella*) at the Sandveld Nature Reserve (Tonetti et al., 2009). Makgabo et al. (2023) identified *A. bovis* 16S rRNA sequences in kudu (*Tragelaphus strepsiceros*) and leopard (*Panthera pardus*)

samples from the Kruger National Park, which were 100% identical to the *A. bovis* type strain sequence (U03775) previously described in cattle from South Africa. Additionally, *A. bovis* was detected in a population of eastern rock sengis (*Elephantulus myurus*), suggesting that sengis may serve as natural reservoir hosts (Harrison et al., 2011, 2013). These findings suggest that other South African rodent species could potentially act as reservoir hosts for *A. bovis* and possibly other *Anaplasma* species. To the best of our knowledge, this study represents the first report of *A. bovis* infections in wild rodent species in South Africa. Sequencing and phylogenetic analysis of the 16S rRNA gene will, therefore, be crucial to confirm our findings, as such analyses were not conducted on the Anaplasmataceae-positive samples due to being beyond the scope of this study. The results will help establish whether the obtained sequences correspond to the classic *A. bovis* or if genetic variants are present in the rodent species. Currently, the 16S rRNA gene remains the primary genetic marker for detecting and characterizing *A. bovis*. However, molecular studies using additional genetic loci are recommended to identify the reservoir hosts and tick vectors of *A. bovis* more comprehensively (Kolo, 2023).

*Anaplasma phagocytophilum* DNA was recorded in *R. dilectus* (1/32, 3.1%) in the Eastern Cape and *R. pumilio* (7/298, 2.3%) in the Western Cape using the RLB hybridization assay. As in the case of *A. bovis*, sequencing and phylogenetic analysis were not conducted on these Anaplasmataceae-positive samples. A recent study conducted across a wildlife-human-domestic animal interface provided the first report of the detection of the zoonotic agent *A. phagocytophilum* in humans, rodents, and dogs in Mnisi, a rural community in South Africa using gene sequence and phylogenetic analysis (Kolo et al., 2020). While *A. phagocytophilum* is a well-documented cause of human granulocytic anaplasmosis in regions like North America, Europe, and parts of Asia, there is a scarcity of confirmed human cases from Africa. (El-hamiani Khatat et al., 2016; 2017). In South Africa, there have been no reported *A. phagocytophilum* infections in humans. In this study, Kolo et al. (2020) reported the presence of two 16S rRNA gene sequence variants and one *gltA* gene sequence variant of *A. phagocytophilum* in dogs, rodents and an AFI patient in the community. The authors obtained *A. phagocytophilum* near full length 16S rRNA gene sequences from three dogs and a rodent (*Mastomys natalensis*), while partial 16S rRNA gene sequences (fragment lengths between 690 and 693 bp) were also obtained from two rodents (*M. natalensis* and *Rattus tanezumi*), and an AFI patient from the Mnisi community. The significance of detecting *A. phagocytophilum* DNA in humans, dogs and rodents, and the potential role of *A. phagocytophilum* as a cause of AFI in South Africa, is not known. In another study, Van Wyk et al. (2022) reported on the detection of *A. phagocytophilum* DNA from a pool of *Haemaphysalis elliptica* ticks collected from urban stray dogs in and near Potchefstroom, North-West Province, South Africa using 16S rRNA gene sequence analysis.

Rodents appear to be competent hosts for *A. phagocytophilum* and other closely related *Anaplasma* species in Africa (Mangombi et al., 2021; Selmi et al., 2021; Mosha et al., 2024). In Gabon, a closely related *Anaplasma* species was recorded in *Rattus rattus* and other rodent species (Mangombi et al., 2021). In Tunisia, *A. phagocytophilum* and *A. phagocytophilum*-like DNA were recorded in two *R. rattus* individuals (Selmi et al., 2021), while a recent study recorded *A. phagocytophilum* in *Arvicanthus niloticus* and several other rodent species in Kenya (Mosha et al., 2024). Globally, ticks in the genus *Ixodes* have mainly been implicated as vectors for transmitting *A. phagocytophilum*, while *Amblyomma*, *Dermacentor*, and *Rhipicephalus* ticks can also act as vectors (Woldehiwet, 2010; Dehghani et al., 2019; Karshima et al., 2022). In South Africa the immature stages of several *Ixodes* and *Rhipicephalus* ticks were recorded on *R. pumilio* and two species in particular (*Ixodes bakeri* and *Rhipicephalis gertrudae* group) were recorded on more than 20% of *R. pumilio* in the Western Cape (Matthee et al., 2007, 2010; Apanaskevich et al., 2011; Horak et al., 2018). Although no comparable studies have been conducted on *R. dilectus* as yet, it is quite possible that *Ixodes* and *Rhipicephalus* spp. occur on this rodent species, given their

overlap in geographic distributions (Horak et al., 2018), and can thus play a role in the transmission of *A. phagocytophilum*.

The RLB hybridization assay revealed the presence of *E. ruminantium* DNA, the causative agent of heartwater, in *R. pumilio* (13/298, 4.3%) and *M. musculus* (2/20, 10%). Unfortunately, sequencing and phylogenetic analysis were not conducted at the time of the study to confirm the findings. Heartwater is a tick-borne disease of sheep, goats, and cattle caused by *Ehrlichia ruminantium* and transmitted in South Africa by the tick *Amblyomma hebraeum*. The immature stages of *A. hebraeum* are known to feed on various hosts, including several rodent species such as *R. pumilio* (Theiler, 1962; Horak et al., 2018). Although *R. pumilio* may be fed on by immature ticks, a study by Howell et al. (1989) confirmed that *R. pumilio* is not an effective reservoir host for *E. ruminantium* and is therefore unlikely to contribute to the transmission cycle of heartwater.

Based on the RLB hybridization assay, both *B. bovis* and *T. buffeli* DNA were detected in less than 1% of the rodent species. *Babesia bovis* (an Apicomplexa protozoon) causes a haemolytic disease in cattle worldwide and is transmitted mainly by *Rhipicephalus microplus* ticks in South Africa (Uilenberg, 1993). *Rhipicephalus microplus* is a one-host tick (tick larvae attach to a host and remain on the host until after engorgement as an adult) and is associated with larger-bodied vertebrates such as antelope, buffalo and cattle. *Theileria buffeli* belongs to a group of closely related benign parasites infecting cattle and buffalo in Asia, Africa, Australia, the United States and Europe (Chansiri et al., 1999; Chae et al., 1998; Cossio-Bayugar et al., 2002; Aktas et al., 2007; Altay et al., 2008; Gimenez et al., 2009; Chaisi et al., 2011; Mans et al., 2011). The tick vectors in Africa and the United States are unknown but ticks in the genus *Haemaphysalis* act as vectors in Europe, Australia and Asia (M'ghirbi et al., 2008). The low incidence of *B. bovis* and/or *T. buffeli* in the present study is possibly an indication that rodents, and/or the particular rodent species included in the study are not natural hosts of these haemoparasites.

To conclude, this study provides baseline data on the diversity of selected haemoparasites in terrestrial small mammals in South Africa, emphasizing their potential role as significant reservoirs for (zoonotic) vector-borne parasites. Expanding sampling efforts and thoroughly characterizing relevant species using molecular approaches are expected to provide deeper insights. The data generated can be utilized to map pathogen and vector distributions, model transmission dynamics, and identify key reservoir hosts and vectors. It supports the development of improved diagnostic tools, informs public health and conservation strategies, and enhances vector management programs. Furthermore, it establishes a foundation for long-term disease surveillance and contributes to comparative phylogenetic analyses. Collectively, these efforts advance understanding, proactive control, and management of zoonotic and vector-borne diseases.

### Ethics and permits

The study was approved by Animal Care and Use Ethics Committee of Stellenbosch University (reference numbers: 2006B01007 and SU-ACUM11-00004 (P)), Animal Ethics Committee of the University of Pretoria (document reference number: V032-14) and Animal Ethics Committee of the Tshwane University of Technology (reference number: AREC2014/06/002). A Section 20 permit was obtained from the Department of Agriculture, Forestry and Fisheries (Reference number V032-14), as well as collection permits from provincial Nature Conservation authorities: Eastern Cape, CRO37/11CR; Western Cape, 0035-AAA007-00423; Northern Cape, FAUNA 1076/2011 and KwaZulu-Natal, OP4990/2010).

### Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGTP in order to improve readability and language in certain sections of the

manuscript. After using this tool/service, the author(s) responsibly reviewed and edited the content as needed.

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### CRediT authorship contribution statement

**Milana Troskie:** Writing – original draft, Methodology, Investigation, Formal analysis. **Sonja Matthee:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Barend L. Penzhorn:** Writing – review & editing, Resources, Funding acquisition. **Raymond Jansen:** Writing – review & editing, Supervision, Conceptualization. **Marinda Oosthuizen:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

### Declarations of interest

None.

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