

Identification of ticks and tick-borne pathogens of wildlife necropsy cases submitted to the SANBI National Zoological Gardens, South Africa

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Highlights

- A total of 48 ticks were collected from 13 host species coming from nine localities in South Africa, Namibia, and Botswana.
- Ticks identified belonged to *Amblyomma*, *Hyalomma*, *Rhipicephalus*, *Haemaphysalis*, *Ixodes*, *Rhipicentor*, and *Otobius*
- *Amblyomma* species were the most prevalent (22.9 %)
- Eight COI barcodes were generated in this study.
- *Hepatozoon fitzsimonsi* and *Hepatozoon ingwe* showed 100% and 99.39 % similarity respectively with public sequences.

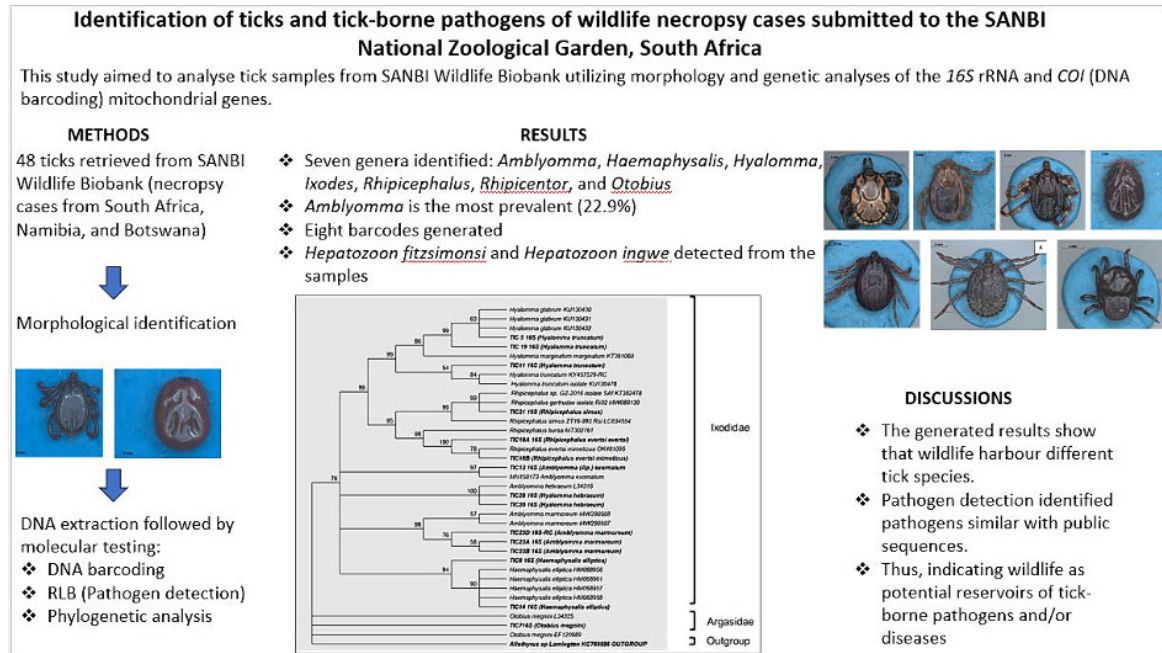
Abstract

Ticks are arachnid blood-feeding parasites, which infest livestock, wildlife, and humans, transmitting medically and veterinary significant pathogens. Their biodiversity and distribution in wild animals remains complex. This study analysed archived tick samples ($n = 48$) from the South African Biodiversity Institute (SANBI) Wildlife Biobank utilizing morphology and genetic analyses of the *16S* rRNA and *COI* (DNA barcoding) mitochondrial genes to identify ticks collected among 13 vertebrates, avian, reptilian, and mammalian host species. The specimens came from nine localities including nature reserves and captive facilities (zoological garden) in South Africa, Namibia, and Botswana. These ticks were also assessed for associated pathogens with the reverse line blot (RLB) hybridization assay. Seven tick genera, *Amblyomma*, *Hyalomma*, *Haemaphysalis*, *Ixodes*, *Rhipicephalus*, *Rhipicentor*, and *Otobius* were identified, with *Amblyomma* being the most prevalent (22.9 %) in our sample set.

Obtained sequences were 95–100 % similar to published records of tick species collected from wild and domestic animals, as well as those collected from vegetation, from different southern African areas. However, tick specimens ($n = 3$) identified morphologically as *Hyalomma truncatum*, *Rhipicephalus e. evertsi*, and *R. simus*, were, on a molecularly level, more closely related to their sister taxa (*H. glabrum*, *R. e. mimeticus*, and *R. gertrudae*, respectively) suggesting a need for taxonomic verification. With the RLB hybridization assay, six samples reacted with the *Ehrlichia/Anaplasma* genus-specific probe, while two reacted

with the *Theileria/Babesia* genus-specific probe. Sequencing of the RLB amplicons targeting the 18S rRNA gene ($n = 2$) indicated 100 % similarity to *Hepatozoon fitzsimonsi*, while one was closely related to *He. ingwe* with 99.39 % similarity. The results show that wildlife harbour different tick species, and pathogen detection identified novel genotypes, indicating wildlife as potential pathogens reservoirs. This study enhances our understanding of tick biodiversity, distribution and highlights wildlife's role in harbouring diverse tick species and novel pathogens.

Graphical abstract



Keywords: Ticks; 16S rRNA; COI; Barcoding; Wildlife; Tick-borne pathogens

1. Introduction

Ticks are small arachnids that feed on the blood of terrestrial vertebrate hosts including humans and animals (Parola and Raoult, 2001a). They have a global distribution, with approximately 900 tick species described from three families, namely the Ixodidae, Argasidae, and Nuttalliellidae (Dantas-Torres, 2018; Machado-Ferreira et al., 2015; Guglielmo et al., 2010; Jongejan and Uilenberg, 2004). Ticks are the second-largest group of vectors of human diseases after mosquitoes and the most significant in transmitting disease-causing pathogens in domestic and wild animals (de la Fuente et al., 2008; Jongejan and Uilenberg, 2004; Parola and Raoult, 2001b). They transmit a wide range of pathogens such as bacteria, viruses, and protozoa, some of which are zoonotic (Chitanga et al., 2014). Although, southern African wildlife harbour a diverse array of ticks (Horak et al., 2018), the diversity of ticks and tick-borne and tick-borne diseases (TBDs) including their interactions with hosts has not been fully explored in South Africa (Ledwaba et al., 2022). Ongoing anthropogenic effects such as habitat changes that have affected the variation of ecosystem and community compositions of vertebrates, including changing climatic conditions have influenced the distribution of ticks

and their patterns of risk (Cutler et al., 2021). Furthermore, human social-recreational changes (e.g. urbanization and human migration), translocation of animals, as well as the illegal wildlife trade, contribute significantly to TBD transmissions that affect the health of domestic animals and humans (Estrada-Peña et al., 2012; Gubler, 2009). For example, wild animals kept for captive breeding or conservation purposes pose a risk of transmission of TBDs to the animal handlers, public, and other susceptible animals during translocations (Tonetti et al., 2009).

According to Chitanga et al. (2014), the prevalence of vector-borne diseases in southern Africa experienced a steady decline in the 20th century, solely due to the implementation of extensive vector control programs. However, in the 21st century, the authors observed the re-emergence of tick-borne diseases. Identification of ticks has relied mostly on morphologically distinct taxonomic characteristics for species delimitation (Brahma et al., 2014; Sonenshine and Sonenshine, 1991). However, accurately identifying specimens with damaged body parts, similar or conserved structural organismal traits of closely related taxa, and immature life stages remains challenging (Couper and Sweit, 2018). Under-studied invertebrate fauna such as ticks have also been noted to have high levels of cryptic diversity with most morphological characters being uninformative (or even subjective), plastic, and/or difficult to study or describe (Hebert et al., 2003). Studies aimed at identifying tick species have therefore adopted the use of DNA taxonomy using DNA barcoding, a method that uses a short section of DNA from a specific gene region to delineate species boundaries based on genetic distances (Hebert et al., 2003). These DNA barcodes are managed through the Barcode of Life Data System (BOLD) - <http://www.barcodinglife.org>, a database that clusters barcode sequences algorithmically into operational taxonomic units (OTUs) and putative species. DNA taxonomy has been successful as species are separately evolving lineages of populations or metapopulations, that accumulate changes in taxonomic characters over time (e.g., DNA or morphology) that will have measurable intraspecific or interspecific signals. An integrative taxonomic approach that uses methods and data from both disciplines (morphology and genetics) for tick identification, should therefore enhance and increase the reliability of the tick identification process.

Most studies have demonstrated the use and efficiency of the mitochondrial (mtDNA) Cytochrome C oxidase subunit I (*COI*) for DNA barcoding of ticks and other animals (Hajibabaei et al., 2006; Hebert et al., 2004; 2003; Halajian et al., 2015). However, the universal *COI* primers are not effective in identifying all tick species (Smit et al. 2023), therefore, other studies have explored nuclear genes and other mtDNA markers such as *12S* or *16S* ribosomal RNA (Lv et al., 2014; Roth et al., 2019) and NADH dehydrogenase subunit 5 (*nad5*) genes (Chitimia et al., 2010) for tick identification. These markers have been proven to work efficiently in differentiating tick species and genera. The use of morphology and molecular methods is essential as both methods complement each other in defining boundaries for classifying species that can be followed up by taxonomists. This study aimed to identify ticks and tick-borne pathogens from wild animals submitted for necropsy at the South African National Biodiversity Institute (SANBI) National Zoological Garden (NZG) using morphological and molecular techniques. The study contributes to our understanding of TBDs of wildlife and the host-vector-pathogen relationships which are important in the management of TBDs in captive animals in the region.

2. Materials and methods

2.1. Tick collection and mining of the National Wildlife Disease Database

The ticks included in this study were all collected opportunistically from individual carcasses submitted to the SANBI National Zoological Gardens (NZG) for necropsy between 2006 and 2017. Ticks of different life stages were collected during necropsy procedures that were performed by a pathologist using forceps from the carcasses. The clinical case details of each necropsy were uploaded on the National Wildlife Disease Database managed by SANBI. The associated metadata included information on the host species, location, age, date of death, date of necropsy, and type of biomaterial collected from the carcasses. All specimens were preserved in 70 % ethanol and archived at the SANBI Wildlife Biobank.

2.2. Morphological identification of ticks

Ticks were morphologically identified to species, life stage, and sex by a taxonomist at Gertrud Theiler Tick Museum, ARC/OVR (South Africa) using a Zeiss Discovery V20 Stereomicroscope (Zeiss Research Microscopy Solutions, Zeiss Group). Tick identification was done using established taxonomic characters described by (Banks, 1915; Keirans et al., 1993; Walker et al., 2000; Horak et al., 2018).

2.3. DNA barcoding and molecular detection of TBDs

Before DNA extraction, individual ticks (previously morphologically identified at the genus level) were subjected to surface sterilization using phosphate-buffered saline (PBS) containing Tween 20 to remove surface contamination. This was followed by crushing each tick using a sterilised mortar and pestle covered with foil. For DNA extraction, we used the ZymoBIOMICS™ Miniprep DNA (Zymo Research, Freiburg im Breisgau, Germany) kit according to the manufacturer's instructions with minor modifications (incubation at 55 °C on a heating block for 48 h). For tick species identification, the *COI* and *16S* rRNA genes were amplified using the primers HCO2198 forward (5' -TAA ACT TCA GGG TGA CCA AAA AAT CA- 3') and LCO1490 reverse (5' -GGT CAA CAA ATC ATA AAG ATA TTG G-3') and *16S* + 1 forward primer (5'-CCG GTC TGA ACT CAG ATC AAG T-3') and reverse primer *16S*-1 (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') and cycling conditions described by (Folmer et al., 1994) and (Black and Piesman, 1994) respectively, with minor modifications. Amplicons with the expected band size of 710 bp (*COI* gene) and 460 bp (*16S* rRNA gene) were submitted to Inqaba Biotechnologies (South Africa) for bi-directional sequencing using the PCR primers.

DNA extracts from 20 tick samples selected based on the identified species and location were tested for tick-borne pathogens (*Theileria*, *Babesia*, *Ehrlichia*, and *Anaplasma* spp.) (Table 1), using the reverse line blot (RLB) hybridization assay as described by Bekker et al. (2002) and Gubbels et al. (1999). Samples that were positive by the RLB assay were further subjected to amplification and sequencing using a conventional PCR targeting the *16S* rRNA gene using the primers Ehr-Forward (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG -3') and Ehr-Reverse (5'-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3') (Gubbels et al., 1999) and *18S* rRNA gene using the primers RLB-Forward (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-Reverse (5'-CTA AGA ATT TCA CCT CTG ACA GT-3') (Bekker et al., 2002). For the amplification of

Ehrlichia/Anaplasma spp., cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 35 cycles with denaturation at 95 °C for 15 s, annealing at 53 °C for 30 s, extension at 72 °C for 15 s and a final extension at 72 °C for 5 min. The amplification of *Theileria/Babesia* spp., cycling conditions included an initial denaturation at 94 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C for 1 min and extension at 72 °C for 10 min.

2.4. DNA sequence analysis

All DNA sequences were assembled, edited, and aligned using the CLC main workbench (<https://qiagen.com/>). Consensus sequences were compared to published sequences from GenBank using the Basic Local Search tool (BLASTn) (Baulcombe et al., 1998), and these sequences were added to the analysis (Table 3). Additionally, reference sequences of all identified species that could not be amplified with the *COI* gene, were downloaded and added to the analysis. Furthermore, consensus sequences were deposited into the BOLD database where they were to obtain Barcode Index Numbers (BINs) by the system. For verification of sequences, *COI* gene sequences were compared to other *COI* sequences deposited in BOLD. The *16S* rRNA gene was verified by cross-referencing the sequences with records on GenBank (<https://www.ncbi.nlm.nih.gov/genbank>). Phylogenetic trees using the neighbour joining (NJ) method, based on the Kimura 2-parameter model (Kimura, 1980), were done for the *COI* gene and maximum likelihood was done for the *16S* rRNA gene. We used the closest related species, *Allothyrus sp Lamington* (KC769586) belonging to the superorder parasitiformes, as an outgroup in both trees. The evolutionary divergence between species was estimated by determining the number of base differences per site from estimation of the net average between groups of sequences (Table 4).

Nucleotide sequence data reported in this paper are available in the GenBank database under accession numbers: **COI-5P ($n = 9$): PP835208 - PP835216; 16S ($n = 15$): PP835217-PP835231; 18S ($n = 3$): PP835669 - PP835671.**

3. Results

3.1. Tick species

A total of 48 ticks collected among 13 vertebrates (avian, reptilian, and mammalian) host species, from nine localities including one national park, three nature reserves, and five captive facilities (zoological garden). These localities were from five biomes (Grassland, Nama-Karoo, Succulent Karoo, Savannah, and Semi-arid Savanna) in Botswana, Namibia, and South Africa (Table 1; Fig. 1). The ticks were morphologically identified and assigned to seven genera: hard ticks (*Amblyomma*, *Hyalomma*, *Rhipicephalus*, *Haemaphysalis*, *Ixodes*, *Rhipicentor*), and soft ticks (*Otobius*) (Table 1, Fig. 1, Fig. 2). The highest number of tick species were mostly collected from mammals (32 %), followed by reptiles (14 %), and birds (2 %). These ticks were mostly collected from grassland habitats, followed by the Savanna and Nama-Karoo. The Succulent Karoo and Semi-arid Savanna had the lowest number of ticks (Appendix A). *Amblyomma* spp. were the most abundant tick species identified, representing 22,9 % of the sample size. In terms of age (life stage) adults ($n = 13$) were the most prevalent, followed by nymphs ($n = 6$) and larvae ($n = 5$), (Table 1). Larvae and nymphs of *A. marmoratum*

Table 1. Host and geographical origin of the tick species morphologically identified in the study. Samples highlighted in bold were analysed using the RLB assay.

Province	Locality/Biome	Host Species	Tick Sample ID	Tick Species	Life Cycle Stage
BOTSWANA					
Kweneng*	Khutse game reserve (Savanna)	Leopard (<i>Panthera pardus</i>)	TIC 15	<i>Hyalomma truncatum</i>	adult
NAMIBIA					
Otjozondjupa	Africat (semi-arid savanna)	Cheetah (<i>Acinonyx jubatus</i>) Leopard (<i>Panthera pardus</i>)	TIC 6	<i>Hyalomma rufipes</i>	adult
			TIC 10	<i>Rhipicephalus evertsi evertsi</i>	adult
			TIC 18a	<i>Rhipicephalus evertsi evertsi</i>	adult
			TIC 18b	<i>Rhipicephalus evertsi mimeticus</i>	adult
SOUTH AFRICA					
Eastern Cape*	Cat Conservation Trust Karoo (Succulent Karoo)	African black-footed cat (<i>Felis nigripes</i>) Banded mongoose (<i>Mungos mungo</i>) Bosc's monitor lizard (<i>Varanus exanthematicus</i>) White rhino (<i>Ceratotherium simum</i>) Cheetah (<i>Acinonyx jubatus</i>) Hog deer (<i>Axyls porcinus</i>)	TIC 30a	<i>Amblyomma marmoreum</i>	nymph
			TIC 30c	<i>Amblyomma hebraeum</i>	larvae
			TIC 2	<i>Ixodes</i> spp.	larvae
			TIC 13	<i>Amblyomma (Aponomma) exornatum</i>	adult
			TIC 3 (364)	<i>Hyalomma truncatum</i>	adult
			TIC 8	<i>Haemaphysalis elliptica</i>	adult
			TIC 7	<i>Otobius megnini</i>	nymph
			TIC 4	<i>Amblyomma marmoreum</i>	adult
			TIC 16	<i>Amblyomma marmoreum</i>	male
			TIC 22a	<i>Amblyomma marmoreum</i>	adult
			TIC 22 b	<i>Amblyomma nuttalli</i> *	nymph
			TIC 23a	<i>Amblyomma marmoreum</i> *	larvae
			TIC 23b	<i>Amblyomma marmoreum</i>	adult
Gauteng*	National Zoological Garden (Grassland)	Leopard tortoise (<i>Stigmochelys pardalis</i>)	TIC 23c	<i>Amblyomma nuttalli</i> *	nymph
			TIC 23d	<i>Amblyomma marmoreum</i> *	nymph

			TIC 26a	<i>Amblyomma nuttalli</i>	adult
			TIC 26b	<i>Amblyomma marmoreum</i>	adult
			TIC 26c	<i>Amblyomma marmoreum*</i>	nymph
			TIC 26d	<i>Amblyomma marmoreum</i>	adult
			TIC 27	<i>Amblyomma marmoreum*</i>	larvae
		Porcupine (<i>Erethizon dorsatum</i>)	TIC 12	<i>Rhipicephalus simus</i>	adult
			TIC 25a	<i>Amblyomma hebraeum</i>	adult
		Roan antelope (<i>Hippotragus equinus</i>)	TIC 25b	<i>Rhipicephalus</i> spp. cf. sp. nr <i>pravus</i>	nymph
			TIC 25c	<i>Rhipicephalus evertsi evertsi</i>	larvae
			TIC 25d	<i>Rhipicephalus decoloratus</i>	adult
		Vervet monkey (<i>Chlorocebus pygerythrus</i>)	TIC 28	<i>Amblyomma hebraeum</i>	larvae
	Rietvlei Nature Reserve (Grassland)	Cheetah (<i>Acinonyx jubatus</i>)	TIC 14a	<i>Haemaphysalis elliptica</i>	adult
Limpopo*	Mokopane Conservation Centre (Savanna)	Brown snake eagle (<i>Circaetus cinereus</i>)	TIC 29a	<i>Amblyomma marmoreum</i>	nymph
			TIC 29b	<i>Amblyomma marmoreum</i>	larvae
Mpumalanga*	Hoedspruit Endangered Species Centre (Savanna)	Cheetah (<i>Acinonyx jubatus</i>)	TIC 21a	<i>Amblyomma hebraeum</i>	adult
			TIC 21b	<i>Haemaphysalis elliptica</i>	nymph
			TIC 21c	<i>Rhipicephalus simus</i>	adult
			TIC 5a	<i>Hyalomma truncatum</i>	adult
			TIC 5b	<i>Rhipicephalus foliis</i>	male
Northern Cape*	Carnarvon (Tankwa Karoo National Park) (Nama-Karoo)	Tankwa goat (<i>Capra hircus</i>)	TIC 11	<i>Hyalomma truncatum</i>	adult
			TIC 17a	<i>Hyalomma truncatum</i>	adult
			TIC 17b	<i>Rhipicephalus foliis</i>	adult
			TIC 17c	<i>Rhipicephalus simus</i>	adult
			TIC 19	<i>Hyalomma truncatum</i>	male
			TIC 1	<i>Haemaphysalis elliptica</i>	adult
North-West*	Ann Van Dyk Cheetah Centre (Savanna)	Cheetah (<i>Acinonyx jubatus</i>)	TIC 20	<i>Amblyomma hebraeum</i>	adult
			TIC 24	<i>Haemaphysalis elliptica</i>	larvae
		Leopard (<i>Panthera pardus</i>)	TIC 9	<i>Rhipicentor nuttalli</i>	adult

Note: *Tick samples with an asterisk are classified as *Amblyomma Marmoreum* complex.

were collected from a Brown snake eagle. Additionally, nymphs of *A. marmoreum* and larvae of *A. hebraeum*, as well as fleas, were collected from an African black-footed cat (Eastern Cape). Two adult ticks from the *Amblyomma* and *Rhipicephalus* genera, as well as a *Haemaphysalis* nymph, were collected from a cheetah from Mpumalanga. Ticks collected from a cheetah and leopard from Namibia were identified as *R. e. evertsi* and *H. rupifes*, as well as the *R. e. mimeticus*, and *R. e. evertsi* respectively.

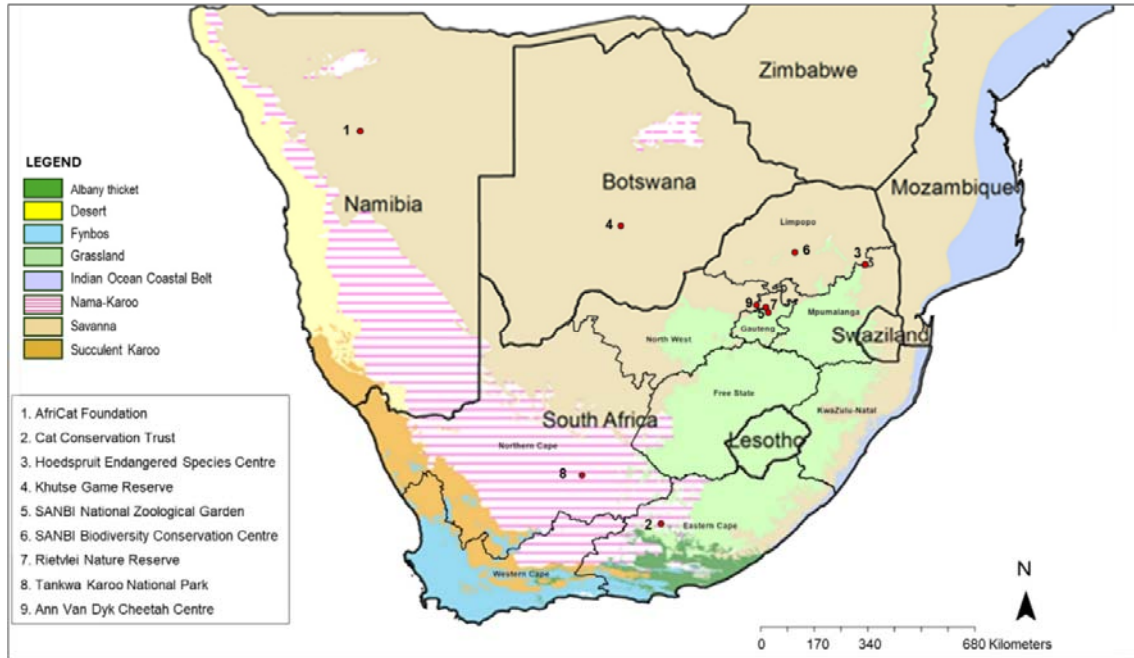


Fig. 1. Map showing the origin of hosts and the different tick species (Generated using the ESRI 2020, ArcGIS Desktop: ArcMap 10.8.1 software).



A) *Amblyomma marmoreum* and B) *Rhipicephalus e. mimeticus* (Female (♀) and male (♂) specimens with dorsal and ventral views.



C) *Rhipicentor nuttalli* and D) *Hyalomma truncutum* (Female (♀) and male (♂) specimens with dorsal and ventral views.



E) *Haemaphysalis elliptica* (Female (♀) and male (♂) specimens with dorsal and ventral views. F) *Ixodes* larvae with dorsal and ventral views and G) *Otobius megnini* nymph with dorsal and ventral views.

Fig. 2. Images of representative specimens of the identified tick species.

The results further showed that *Amblyomma marmoreum* was associated with different hosts including the African black-footed cat (*Felis nigripes*), the Brown snake eagle *Circaetus cinereus*) with more ticks (10) collected from Leopard tortoise (*Stigmochelys pardalis*) (Table 1, Fig. 3). Immature stages of *A. hebraeum* were isolated from the African black-footed cat,

porcupine, and vervet monkey while adult stages were collected from cheetah coming from Hoedspruit Endangered Species Centre and the Ann Van Dyk Cheetah Centre. The results further showed that *Hyalomma truncatum* infested a wide range of hosts, including leopard, white rhino, Tankwa goat, and cheetah. While species of the genus *Rhipicephalus* were associated with felids and a roan antelope. *Haemaphysalis elliptica*, showed host specificity, as it was mostly collected from cheetah.

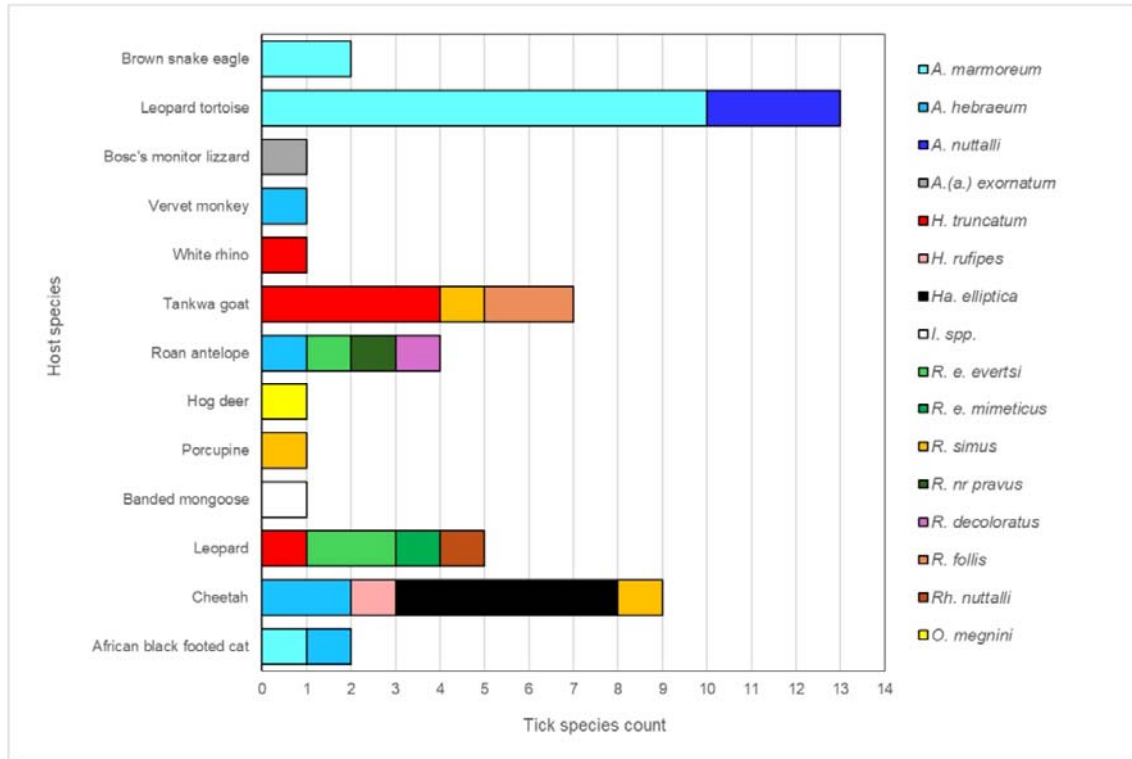


Fig. 3. Graph showing the number of identified ticks per species collected from each host.

3.2. Molecular analysis of ticks

Of the 48 collected ticks, 41 were characterized using the *COI* and *16S* rRNA mtDNA gene data, while the remaining seven samples were preserved and accessioned in the SANBI Wildlife Biobank tick collection as species reference voucher specimens. DNA sequencing results for both *COI* and *16S* rRNA genes corresponded with the morphological identifications, showing genetic differences among the identified species. The obtained sequences exhibited had 95–100 % similarity (in terms of genetic distances) to the corresponding species sequences published on GenBank. However, for two species, the BLAST matches showed lower similarity levels compared to published sequences (Table 2). Firstly, sequences from the two ticks morphologically identified as *Hyalomma truncatum* were identical or closely matched to submitted *H. glabrum* sequences (KU130596 and KU130432: 99.2–100 %) for both gene regions, while *R. e. evertsi* sequences were closely matched to a banded-legged tick *R. e. mimeticus* *16S* rRNA sequence (OK481095: 99.75 %). Five and seven distinct clades were obtained from the *COI* and *16S* rRNA by the Neighbour-Joining analyses, respectively (Fig. 4 and Fig. 5). All *COI* sequences verified on BOLD systems matched those published in the

Table 2. DNA sequencing BLAST results.

Species or taxon name	Sample ID	CO1 gene			16S rRNA gene		
		BLAST Match	GenBank ID	% Identity	BLAST Match	GenBank ID	% Identity
<i>Amblyomma (a.) exornatum</i>	TIC 13	<i>A. (a) exornatum</i>	MN150167	95.12 %	<i>A. (a) exornatum</i>	MN150173	96.17 %
<i>Amblyomma hebraeum</i>	TIC 20	–	–	–	<i>A. hebraeum</i>	L34316	100 %
<i>Amblyomma hebraeum</i>	TIC 28	–	–	–	<i>A. hebraeum</i>	L34316	100 %
<i>Amblyomma marmoreum</i>	TIC 23a	<i>A. marmoreum</i>	KY457515	100 %	<i>A. marmoreum</i>	MW290508	97.71 %
<i>Amblyomma marmoreum</i>	TIC 23b	<i>A. marmoreum</i>	KY457515	100 %	<i>A. marmoreum</i>	MW290508	97.71 %
<i>Amblyomma marmoreum</i>	TIC 23d	–	–	–	<i>A. marmoreum</i>	MW290508	97.71 %
<i>Haemaphysalis elliptica</i>	TIC 8	<i>Ha. elliptica</i>	MZ351133	100 %	<i>Ha. elliptica</i>	HM068961	98.99 %
<i>Haemaphysalis elliptica</i>	TIC 14	<i>Ha. elliptica</i>	MZ351133	97.65 %	<i>Ha. elliptica</i>	HM068961	100 %
<i>Hyalomma truncatum</i>	TIC 5	<i>H. glabrum</i>	KU130596	99.10 %	<i>H. glabrum</i>	KU130432	100 %
<i>Hyalomma truncatum</i>	TIC 11	<i>H. truncatum</i>	OK481109	99.64 %	<i>H. truncatum</i>	KU130478	98.99 %
<i>Hyalomma truncatum</i>	TIC 19	<i>H. glabrum</i>	KU130596	99.82 %	<i>H. glabrum</i>	KU130432	99.75 %
<i>Otobius megnini</i>	TIC 7	<i>O. megnini</i>	MG582606	100 %	<i>O. megnini</i>	L34325	95.87 %
<i>Rhipicephalus e. evertsi</i>	TIC 18a	–	–	–	<i>R. e. mimeticus</i>	OK481095	99.75 %
<i>Rhipicephalus e. mimeticus</i>	TIC 18b	–	–	–	<i>R. e. mimeticus</i>	OK481095	100 %
<i>Rhipicephalus simus</i>	TIC 21	–	–	–	<i>R. gertrudae</i>	MW080139	99.73 %
<i>Rhipicephalus simus</i>	TIC 21	–	–	–	<i>R. simus</i>	LC634554	99.46 %
<i>Rhipicephalus simus</i>	TIC 21	–	–	–	<i>R. sp</i>	KT382478	99.00 %

database with no discrepancies. The deposited sequences were for eight barcoded specimens, representing six unique barcode index numbers (BINS) for six species.

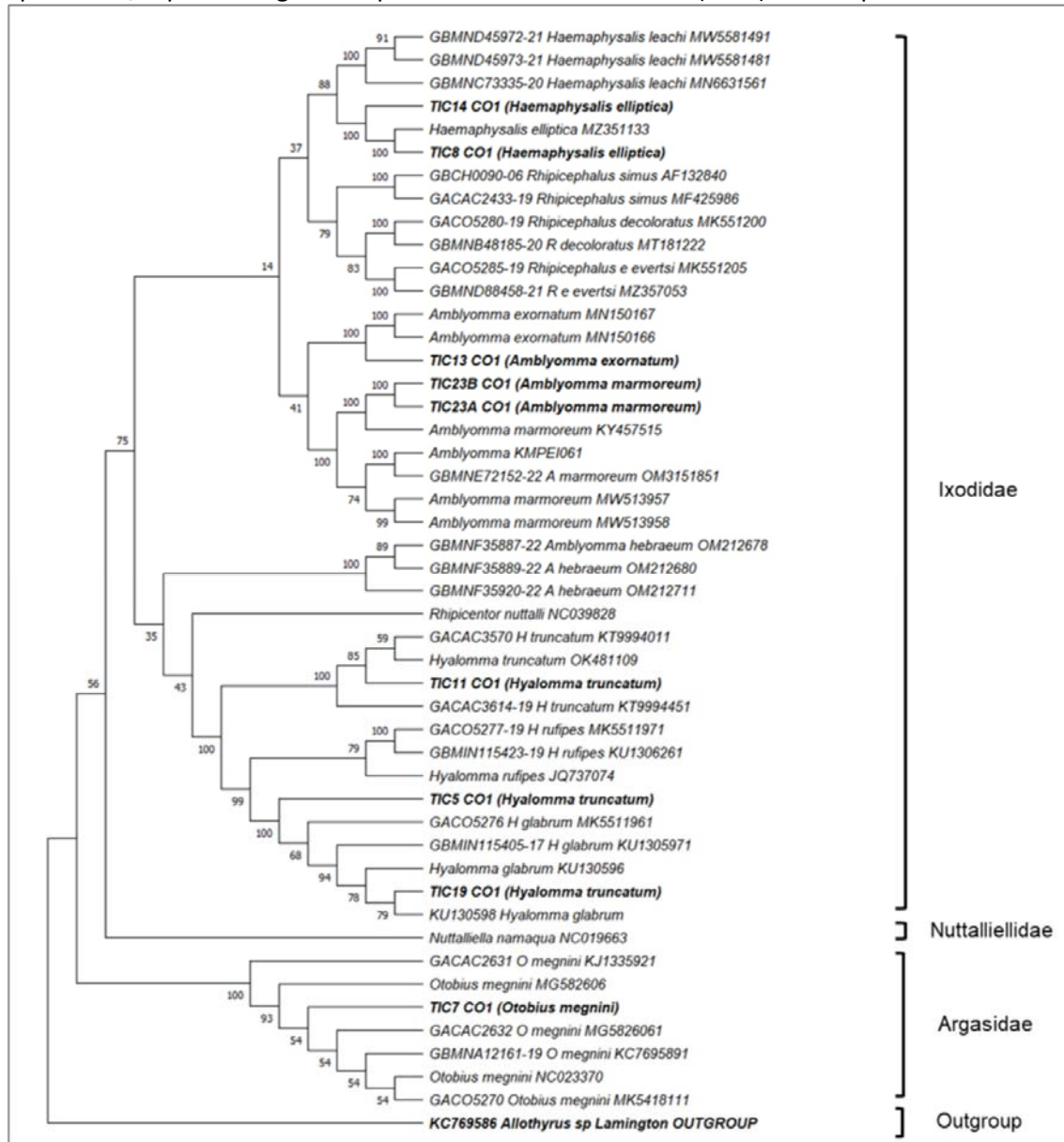


Fig. 4. Neighbour-Joining (NJ) phylogenetic tree based on the COI gene, expected band size of 710. Evolutionary analyses were conducted in MEGA 10. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura,1980). This analysis involved 48 nucleotide sequences. For every sequence pair, all ambiguous positions were removed for each sequence pair (pairwise deletion option). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Sequences generated from this study are highlighted in BOLD, along with the Outgroup used.

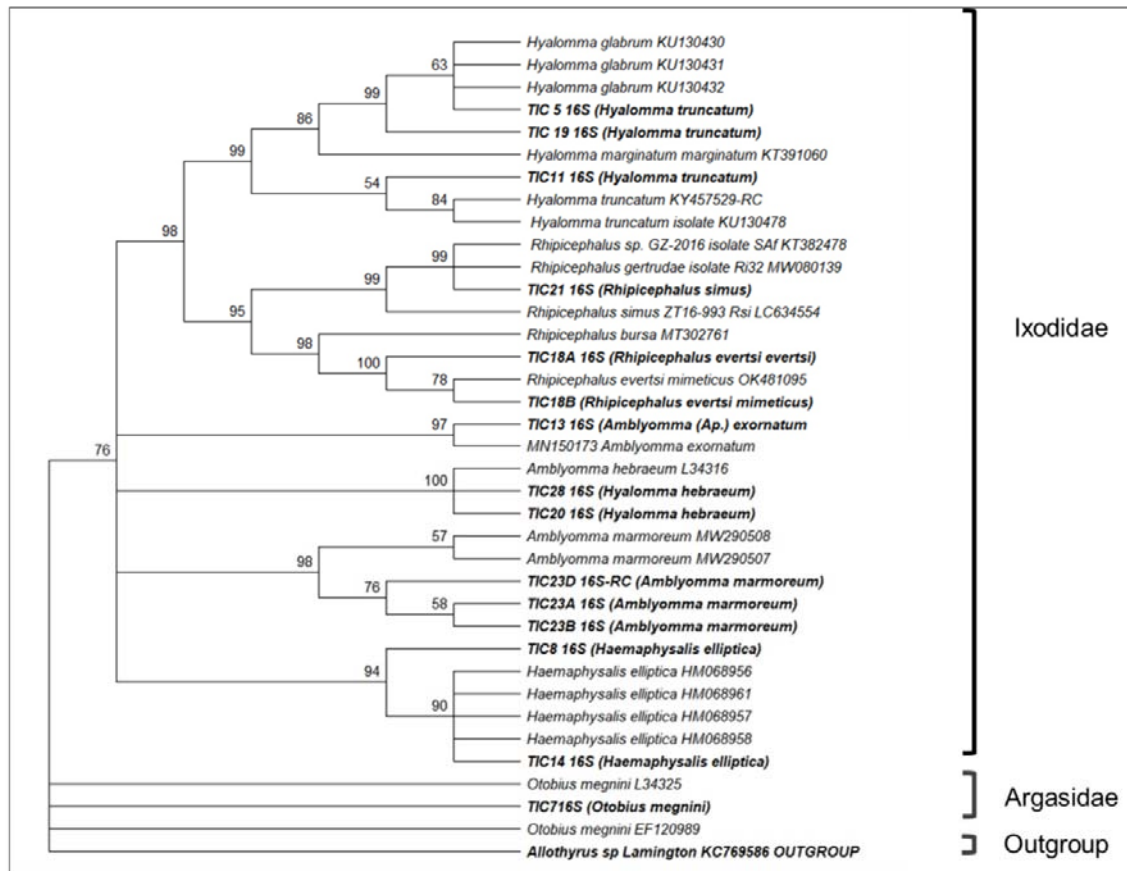


Fig. 5. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [Kimura,1993]. The tree with the highest log likelihood (-2957.80) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) 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. This analysis involved 37 nucleotide sequences. There were a total of 538 positions in the final dataset. Evolutionary analyses were conducted in MEGA 10. Sequences generated from this study are highlighted in BOLD, along with the Outgroup used.

Table 3. Reference sequences used for phylogenetic analyses

Species	Genbank Accession Number	Host	Country	Reference
COI gene				
<i>Haemaphysalis elliptica</i>	MZ351133	Vegetation	Eswatini	Ledger et al., 2021
<i>Hyalomma truncatum</i>	OK481109	Cattle	Angola	Palomar et al., 2021
	KU130597	Different livestock and wildlife species	South Africa	Sands et al., 2017
<i>Hyalomma glabrum</i>	KU130596	Different livestock and wildlife species	South Africa	Sands et al., 2017
<i>Hyalomma rufipes</i>	JQ737074	Not indicated*	China	Gou et al., 2012
<i>Amblyomma (a.) exornatum</i>	MN150166	Rock monitor	South Africa	Hornok et al., 2020
<i>Amblyomma (a.) exornatum</i>	MN150167	Rock monitor	South Africa	
<i>Amblyomma marmoreum</i>	MW513958	Reptiles	South Africa	Mofokeng et al., 2021
<i>Amblyomma marmoreum</i>	MW513957	Reptiles	South Africa	
<i>Amblyomma marmoreum</i>	KY457515	Not indicated*	South Africa	Mans et al., 2019
<i>Otobius megnini</i>	MG582606	Cattle	Iran	Chegeni et al., 2018
16S rRNA gene				
<i>Hyalomma truncatum</i>	KY457529	Not indicated*	South Africa	Mans et al., 2019
<i>Hyalomma truncatum</i>	KU130478	Different livestock and wildlife species	South Africa	Sands et al., 2017
<i>Hyalomma marginatum</i>	KT391060	Sheep	Not indicated*	Erster et al., 2015
<i>Hyalomma glabrum</i>	KU130432	Different livestock and wildlife species	South Africa	Sands et al., 2017

<i>Hyalomma glabrum</i>	KU130431	Different livestock and wildlife species	South Africa	Sands et al., 2017
<i>Amblyomma (a.) exornatum</i>	MN150173	Rock monitor	South Africa	Hornok et al., 2020
<i>Rhipicephalus</i> sp.	KT382478	Dogs	South Africa	Zemtsova et al., 2016
<i>Rhipicephalus simus</i>	KY457542	Not indicated*	South Africa	Mans et al., 2019
<i>Rhipicephalus e. mimeticus</i>	OK481095	Cattle	Angola	Palomar et al., 2021
<i>Rhipicephalus</i> sp. GZ-	KT382478	Dogs	South Africa	Zemtsova et al., 2016
<i>Rhipicephalus gertrudae</i>	MW080139	Vegetation	South Africa	Bakkes et al., 2021
<i>Rhipicephalus simus</i>	LC634554	Vegetation	South Africa	Kobayashi et al., 2021
<i>Rhipicephalus bursa</i>	MT302761	<i>Capra aegagrus</i>	Turkey	
<i>Amblyomma marmoreum</i>	MW290508	Reptiles	South Africa	Mofokeng et al., 2021
	MW290507	Reptiles	South Africa	
<i>Amblyomma (a.) exornatum</i>	MN150173	Rock monitor	South Africa	Hornok et al., 2020
	EF120989	Bovine	Argentina	Nava et al., 2009b
<i>Otobius Megnini</i>	L34325	Not indicated*	Not indicated	Black and Piesman, 1994
<i>Haemaphysalis elliptica</i>	HM068957 HM068956	Cheetah	South Africa	Golezardy et al., (unpublished)

Table 3. Reference sequences used for phylogenetic analyses.

3.3. Molecular detection of tick-borne pathogens

DNA samples from 20 ticks, selected as representatives of each identified species and locality were screened for tick-borne pathogens (*Theileria*, *Babesia*, *Ehrlichia*, and *Anaplasma*) using the RLB hybridization assay (Table 1). The results indicated the presence of infections in six samples from Namibia and South Africa (Gauteng and Northern Cape province). Four PCR amplicons hybridized with the *Ehrlichia/Anaplasma* (bacteria genera of order Rickettsiales) genus-specific probe only and not with any of the species-specific probes, indicating the presence of novel species or species variants. One amplicon hybridized with the *Theileria/Babesia* (both genera of protozoan parasites that belong to the phylum Apicomplexa) genus-specific probe only, and one amplicon hybridized with *Babesia* genus-specific probe only. Out of the six positive samples, we amplified and sequenced them further, obtaining three sequences. The 18S rDNA sequence obtained from *A. marmoreum* parasitizing the leopard tortoise was identical (100 % similarity) over a 750 bp region to published sequences of *Hepatozoon* species, namely *He. fitzsimonsi* (KR069084.1) (Cook et al., 2015). Additionally, pathogen sequences obtained from *Rh. e. evertsi* parasitizing a Namibian leopard were similar (99.36 %) to the published sequence of *He. ingwe* (MN793001.1) (van As et al., 2020).

4. Discussion

This study identified tick species parasitizing various wildlife species from nature reserves and captive breeding facilities in southern Africa and detected bacteria and protozoan pathogens associated with them. Ticks were identified by morphology and then confirmed by PCR amplification targeting two mitochondrial markers, *COI* and *16S* rRNA genes through genetic diversity estimates. Ticks belonging to the Ixodidae family were prevalent in the savanna and grassland biomes in Botswana, Namibia, and South Africa and a minority were from the succulent (known for its abundance of succulent plants) and Nama-Karoo (characterized by grassy, dwarf shrubland vegetation) respectively. A high diversity of ticks was mostly found in captive facilities, compared to those collected in Nature reserves. These findings are to be expected as captive facilities do translocations from different localities that have different tick species. Most of the adult tick specimens were accurately identified with morphology, however, immature life stages of some of the specimens hindered accurate morphological verification. Therefore, the use of molecular analysis as an additional taxonomic tool was valuable in assisting with resolving the identifications of these life stages. According to Lv et al., (2014), the *COI* gene may be paired with other genes including *12S* rRNA, *16S* rRNA, and *18S* rRNA to yield better outcomes when identifying organisms. The genetic diversity of tick species analysed in this study using Neighbour-Joining method for the *COI* and maximum likelihood for the *16S* rRNA gene was well supported with topologies that had 99–100 % bootstrap support. It should however be noted that, in the *COI* tree topology, members of the genus *Rhipicephalus* were not included because of poor *COI* sequence amplification for this genus. The most common tick species collected belonged to the *Amblyomma* genus, with the *16S* rRNA tree revealing three different polyphyletic lineages. These results are supported by a study looking at the mitochondrial genome phylogeny using 120 species of ticks that was conducted by Kelava et al., (2021), which also observed that *Amblyomma* spp. are

Table 4. The number of base differences per site from the estimation of the net average between groups of sequences. The number of base differences per sequence from between sequences is shown. This analysis involved 38 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 535 positions in the final dataset. Evolutionary analyses were conducted in MEGA.

Tick species	1	2	3	4	5	6	7	8	9	10	10	12	13
1 <i>N. namaqua</i>													
2 <i>O. megnini</i>	0.25												
3 <i>Ha. leachi</i>	0.25	0.23											
4 <i>Ha. elliptica</i>	0.23	0.21	0.15										
5 <i>Rh. nuttalli</i>	0.24	0.23	0.20	0.18									
6 <i>H. truncatum</i>	0.24	0.25	0.19	0.17	0.17								
7 <i>H. glabrum</i>	0.23	0.24	0.20	0.18	0.16	0.10							
8 <i>H. rufipes</i>	0.22	0.22	0.18	0.16	0.15	0.09	0.03						
9 <i>R. simus</i>	0.25	0.22	0.17	0.16	0.17	0.18	0.19	0.16					
10 <i>R. decoloratus</i>	0.26	0.25	0.18	0.17	0.18	0.21	0.19	0.18	0.15				
11 <i>R. e. evertsi</i>	0.25	0.24	0.19	0.18	0.18	0.18	0.17	0.15	0.14	0.12			
12 <i>A. (a.) exornatum</i>	0.20	0.16	0.16	0.13	0.12	0.14	0.14	0.12	0.12	0.13	0.12		
13 <i>A. marmoreum</i>	0.24	0.19	0.18	0.15	0.16	0.18	0.16	0.14	0.15	0.16	0.15	0.06	

polyphyletic, and monophyly can only be supported within the genus when *A. transversale* and *A. fimbriatum* are excluded from the analysis.

The highest number of ticks were found among mammals, this finding is in agreement with previous studies on tick distribution and host preferences. Because of their diversity, and abundance, mammals often serve as primary hosts for ticks (Estrada-Peña and de la Fuente, 2014). The significant proportion of tick species identified on reptiles highlights their importance as hosts. The findings correspond with the increasing knowledge of reptiles' role in tick ecology and disease transmission (Mofokeng et al., 2021, Mofokeng et al., 2022; a KJFDC/ Omondi et al., 2017). The most prevalent tick species identified was *A. marmoreum*, commonly known as the African tortoise tick or tortoise tick due to its preferred host, the leopard tortoise (Horak et al., 2006b). Horak et al. (2000) also reported the collection of *A. marmoreum* from humans. In susceptible hosts, the species transmits pathogens such as *Ehrlichia ruminantium*, and Crimean-Congo haemorrhagic fever (CCHF) virus. The species is morphologically similar to the Pan-African tortoise tick *Amblyomma nuttalli* making it difficult to distinguish the two species (Horak et al., 2006a) as they are typically similar in sizes and colour. Immature tick life stages of *A. nuttalli* have been reported in reptiles, birds, and sometimes in mammals, while adults have been reported from reptiles such as the Bell's hinged tortoise (*Kinixys belliana*), monitor lizards, as well as snakes (Horak et al., 2006a). Larval and nymph stages of *A. marmoreum* commonly parasitize reptiles, birds, and mammals, whereas adults are parasites of reptiles (tortoises and snakes) (Norval, 1975; Horak and Cohen, 2001). In the current study, adult ticks were found on the leopard tortoise (from a captive facility) while suspected nymphs were collected from a Brown snake eagle and an African black-footed cat. The nymphs could not be accurately identified to species as *A. marmoreum* and *A. nuttalli* are morphologically very similar. Furthermore, previous studies (Mohammed et al., 2022; Cotes-Perdomo et al., 2023) have suggested that a more comprehensive dataset might aid in resolving the taxonomic structures of the *A. marmoreum* complex and its relationships with related species. Both *A. marmoreum* and *A. nuttalli* were identified from the leopard tortoise in this study. However, there was no PCR amplification of *A. nuttalli* to generate DNA sequence data for genetic comparison. The amplified *A. marmoreum* was also similar and or closely related to published GenBank sequences (97.71 %).

The second commonly identified species was *A. hebraeum*, the South African Bont tick, which is widely distributed in South Africa, Mozambique, and Swaziland (Horak et al., 2009). This tick species was previously collected from hosts in four South African provinces (Mpumalanga, Gauteng, Eastern Cape, and North West Province), which are known to have warm and moist temperatures (Horak et al., 2009; Theiler and Salisbury, 1959). This finding supports those of Nyangiwe et al., 2011), who indicated that this species prefers regions with stable temperature ranges.

Mostly parasitizing cattle, domestic animals, and wildlife, *A. hebraeum* is said to provide an interface for the emergence of human diseases (Horak et al., 2018; Iweriebor et al., 2020). Furthermore, the species is known as a vector of *Ehrlichia ruminantium*, the causative agent of heartwater disease, that mostly affects domestic ruminants, although wild ruminants continue to be susceptible (Allsopp et al., 1999). This tick species is also a primary vector of *Rickettsia africae*, a species that can cause African tick-bite fever (ATBF) in humans

(Mazhetese et al., 2022; Pillay and Mukaratirwa, 2020). In the current study, *A. hebraeum* was collected from cheetah, vervet monkey, African black footed cat, and roan antelope. Cheetah and roan antelope are among the most illegally traded species, and they are also popular in legal trade among nature reserves and zoos (Tricorache et al., 2021; Havemann et al., 2016). This increases the possibility of disease transmission through the introduction of ticks and tick-borne pathogens in new areas. Lastly, the least common tick *A. (a.) exornatum*, a tick species known as the tick of varanid lizards (Horak et al., 2006a) commonly found in monitor lizards, was collected from a Bosc monitor lizard. The species has also been reported in mammals such as the Rock dassie (*Procavia capensis*), Namaqua rock mouse (*Micaelamys namaquensis*), and Scrub hare (*Lepus saxatilis*) and avian hosts such as Helmeted guineafowl (*Numida meleagris*), Grey-winged francolin (*Scleroptila afra*) and Crested francolin (*Ortygornis sephaena*) (Elbl and Anastos, 1966; Horak et al., 2006b).

Under the genus *Haemaphysalis*, the only species that was identified in this study was *Ha. elliptica*, commonly known as the southern African yellow dog tick that is known to transmit *Babesia rossi* which causes canine babesiosis in domestic dogs (*Canis familiaris*) (Penzhorn et al., 2020). According to (Birkenheuer, 2021; Collett, 2000), babesiosis infects dogs throughout the year, with cases rising during the summer season. In addition, (Schoeman, 2009) reported that disease incidences are mostly detected in young dogs, however, mature dogs can also be infected. *Ha. elliptica* is widespread in southern Africa and parasitizes various wild and domestic carnivores including domestic dogs and cats as well as lion (*Panthera leo*), leopards, and cheetah (Horak et al., 2018; Horak et al., 2010). In the current study, *Ha. elliptica* was collected from a cheetah from Rietvlei Nature Reserve, Gauteng, and is supported by findings reported by (Horak et al., 2010) as the most dominant species infesting domestic cats and wild felids in South Africa. The threat of disease spill-over from Cheetah to domestic animals (such as dogs and cattle) is high in African countries such as Botswana where there are areas where domestic animals, humans, and wildlife have no clear boundaries (Raboloko et al., 2020; Thompson et al., 2020). Identifying the threat of disease spill-over from tick-borne pathogens is therefore critical. According to Apanaskevich et al. (2007) the species has been misidentified as the yellow dog tick *Ha. leachi* in most studies in South Africa, a species that only occurs in the Northern regions of Africa. Sequence analyses revealed that *Ha. elliptica* is comparable to or closely related to the *COI* and *16S* rRNA (100%–98.99% respectively) gene sequences submitted in GenBank. However, verification of the *COI* genes on the BOLD systems showed that the species is closely related to *Ha. leachi* sequences from Nigeria (100% match).

Under the genus *Hyalomma*, two species were identified namely *H. rufipes* and *H. truncatum*. *Hyalomma rufipes*, known as the coarse Bont-legged tick is the most commonly distributed species in Africa (Hornok and Horváth, 2012) having been reported in South Africa, Botswana, Zimbabwe, Northern Namibia, Kenya, Ethiopia, and Somalia as well as the southern regions of West Africa (Apanaskevich and Horak, 2008; Apanaskevich and Horak, 2006). This species was collected from a cheetah from Namibia. On the contrary, (Turner et al., 2017) have reported the nymphs and adults of this species from birds and large mammalian herbivores respectively. This finding supports the report by Hornok and Horváth (2012) which indicated that both larval and nymphal stages of this species prefer small mammals and bird hosts, while Chitimia-Dobler et al., (2019) reported that adults of this species are mainly found on cattle, sheep, goats, and wild ungulates, as well as horses. Another study by (Horak et al.,

2010), reported a single collection of this tick species from a lion in Botswana, thus supporting the fact that the species does infest felids. The tick also has a wide distribution in the Afrotropical region (Apanaskevich and Horak, 2008). In addition, the species is known as a vector of Crimean Congo haemorrhagic fever (CCHF) virus in humans (Gargili et al., 2017; Dunster et al., 2002) as well as other pathogens such as *Rickettsia aeschlimannii*, *Anaplasma marginale* and *Babesia occultans* (Chitimia-Dobler et al., 2019; Papa et al., 2015).

Hyalomma truncatum, known as the shiny *Hyalomma* or smooth Bont-legged tick is generally found in regions with high temperatures. We collected the species from a Tankwa goat that came from the Northern Cape province, which is known for its high temperatures and dryness, thus confirms reports in literature on this ticks' preferred environment. The species also transmits zoonotic diseases such as Crimean–Congo hemorrhagic fever (CCHF) virus and *Rickettsia conorii* as well as diseases of veterinary importance such as anaplasmosis, babesiosis, and sweating sickness in livestock (Hoogstraal, 1956a). Diseases of veterinary importance cause substantial economic losses in the livestock sector (Bram et al., 2002). Although morphological identification showed that two specimens (TIC5 and TIC19) were *H. truncatum*, DNA sequence analysis indicated that these individuals are closely related to *H. glabrum* based on available published data. There is therefore a need for morphological and genetic analysis to verify the diversity of these two species.

In the *Rhipicephalus* genus, five tick species were identified, namely *R. e. evertsi*, *R. e. mimeticus*, *R. follis*, *R. simus*, and *R. sp. cf. nr pravus*. The species of this genus have been reported as the most dominant tick species infesting cattle in central-western regions of South Africa (Dreyer et al., 1998; Nyangiwe et al., 2017). *Rhipicephalus e. evertsi*, is the most common tick species distributed throughout sub-Saharan Africa, with all life stages of this species being recorded to feed on large animals such as horses (*Equus caballus*), cattle (*Bos taurus*), zebras (*Equus quagga*), and eland (*Taurotragus oryx*). However, it has been reported that domestic equids and wild zebras appear to be the preferred hosts (Hoogstraal, 1956b). The tick species is believed to transmit protozoan parasites causing equine piroplasmiasis (*Babesia caballi* and *Theileria equi*), anaplasmosis, CCHF virus as well as *Ehrlichia ruminantium* (Hoogstraal, 1956a). This species has also been collected from other animals such as sheep (*Ovis aries*), goats (*Capra aegagrus*), impala (*Aepyceros melampus*), African buffalo (*Syncerus caffer*), blue wildebeest (*Connochaetes taurinus*), and greater kudu (*Tragelaphus strepsiceros*) (Spickett et al., 1992). In this study, *R. evertsi evertsi* (TIC 18 A) was collected from a Namibian leopard with its sequence being closely related to a sequence of *R. evertsi mimeticus* (99.75 %) that originated from domestic cattle in Angola (Palomar et al., 2021). On the other hand, *R. e. mimeticus* was also collected from Namibia. This species is indigenous to Namibia and Angola and is believed to have possibly been introduced into South Africa through translocations of animals (Nyangiwe et al., 2017), thus the presence from the host species in this study is not surprising. Sequence analysis showed that the species is closely related to the reference sequence *R. e. mimeticus* (100 %) collected from cattle in Angola (Palomar et al., 2021) with 100 % identity. *Rhipicephalus e. mimeticus* has minimal economic significance (Spickett et al., 2011), and Gothe et al. (1986) reported that this is attributed to the spread of CCHF fever virus but with less pathogenic effect. *Rhipicephalus simus* was collected from cheetah, Tankwa goat as well as porcupine. The black-pitted ticks are distributed in the northern, eastern, and south-eastern regions of South Africa. This species has been reported to feed on monogastric animals such as zebras, warthogs, rhinoceroses, as well as the larger

carnivores, buffaloes, elands, and cattle (Olwoch et al., 2007), however nymphal stages are reported to prefer murid rodents as hosts. Reports show that the species is a vector of anaplasmosis and bovine babesiosis in cattle (Booyesen et al., 2017; Olwoch et al., 2007). Additionally, Kelly (2001) reported that the tick species is a vector of bacteria *Rickettsia conorii* which is the causative agent for Mediterranean spotted fever in humans. Although the tick species was morphologically identified as *R. simus*, 16S rRNA gene sequence analysis showed that it was similar to *R. gertrudae* (MW080139; 99.73 %); *R. simus* (LC634554; 99 %) and identified *Rhipicephalus* sp. (KT382478; 99 %) deposited on Genbank. *Rhipicephalus follis* was collected from a Tankwa goat from the Northern Cape Province. This tick species has been reported to occur in the mountainous or higher altitude regions mainly in the eastern half of South Africa (Olwoch et al., 2007). *Rhipicephalus spp* cf. *sp nr pravus* nymphs were collected from a cheetah from the Mpumalanga province. In Ethiopia, adult ticks of this species are most abundant during the rainy season, thus its presence in the highveld region may be attributed to the similar temperature conditions.

Other identified ticks with no sequences and small numbers of samples include the species of *Ixodes* sp. and *Rhipicentor nuttalli*. *Ixodes* is the largest genus in the Ixodidae family, including over 245 species and is highly complex morphologically and physiologically. This genus is found all over the world and is known to infect various vertebrate species, including mammals and birds, being found occasionally in reptiles (Ash et al., 2017). An *Ixodes* tick was collected from a banded mongoose in Gauteng. *Rhipicentor nuttalli*, on the other hand, was collected from a leopard from the North West Province, and is confined to the African continent based on published reports (Fourie et al., 2002). In South Africa, it has been recorded in the Eastern and Western Cape, Free State, Gauteng, Limpopo, and North West Province provinces (Fourie et al., 2002). Fourie et al. (2002) reported that the species causes paralysis in dogs.

Otobius megnini commonly known as the spinose ear tick, was the one species from the Argasidae family identified from a hog deer from the SANBI-NZG in Gauteng. According to Walker (2003), only the nymphal stages of *O. megnini* cause parasitic infections. Currently, there are no reports of pathogen transmission (Barker and Walker, 2014), however it has been reported to induce paralysis in horses (Miller, 2020). The occurrence of *O. megnini* infestations in zoo animals may cause mini outbreaks of ear infections as animals stay in groups in confined spaces. This tick species feeds on a wide variety of animals, including ungulates, sheep, goats, cattle, horses, and dogs, as well as humans. DNA sequence analysis of both markers showed that *O. megnini* (TIC 7) collected from a hog deer from the SANBI-NZG in Gauteng is identical (16S rRNA) or closely similar (COI) to *O. megnini* (100–95.87 % respectively) that were collected from cattle from Iran and Argentina (Black and Piesman, 1994; Nava et al., 2009a).

In this study, RLB assay was used to successfully identify pathogens to species level., Sequences of *Hepatozoon fitzsimonsi* were obtained from DNA extracted from *A. marmoreum* tick collected from a leopard tortoise from the SANBI-NZG in Gauteng. The obtained sequence showed 100 % similarity with sequences deposited in Genbank. *He fitzsimonsi* is classified as a protozoan parasite, that was first described by Dias (1953) under the genus *Haemogregarina*. A subsequent study by Cook et al. (2014), focusing on pathogens in tortoises, showed the presence of *He fitzsimonsi* in an Eastern-hinged tortoise infested with adult species of *A. marmoreum*, therefore, supporting the findings in the current study.

Additionally, we obtained a sequence from DNA extracted from *R. evertsi evertsi* ticks collected from a leopard from Namibia, showing a close similarity of 99.36 % to *He ingwe*. This pathogen species was recently described by [van As et al. \(2020\)](#) from leopards in Mpumalanga and Northern Limpopo. These findings suggest that captive leopards are susceptible to this pathogen.

One of the limitations of the current study was the smaller sample size. This was because the tick specimens available from the necropsy that had preserved and archived in the SANBI biobank were low. And this was because not all host animals that were brought in for necropsy had ticks. Moreover, due to budget constraints, a limited number of tick samples were submitted for RLB testing. Regardless of these limitations, the number of ticks utilized, provided sufficient data to establish and draw preliminary conclusions about tick-host relationships. This research is also new, and the data obtained should provide valuable insights into the tick species harbored by the wildlife hosts studied, as well as their potential role in disease transmission.

5. Conclusion

Although the study looked at selected tick species infesting wildlife, the results obtained here demonstrate the diversity of vectors that southern African species hosts' harbour. Wildlife therefore serve as reservoirs and intermediate hosts for TBDs (Kock, 2005), which cause significant economic impacts ([Uilenberg, 1995](#)) as the continuous interaction of humans, livestock, and wildlife (e.g. birds) may perpetuate the spread of zoonotic diseases including TBPs ([Gortázar et al., 2007](#); [Uilenberg, 1995](#)). Thus, the findings from the study indicate that captive wildlife species harbour a variety of ticks of veterinary and public health importance and that both morphology and molecular techniques using *COI* and *16S* rRNA genes can be used to identify these tick species. Additionally, wildlife species are potential role players in the transmission of these diseases and can be possible reservoirs.

Ethical clearance

The study was approved by the SANBI Animal Research and Scientific Committee (RESC) [Project No: [SANBI/RES/2020-12](#)]; the University of Pretoria, Faculty of Veterinary Science, Research Ethics Committee ([REC 167-20](#)) as well as the Animal Ethics Committee (AEC). Section 20 permit was granted by the Department of Agriculture, Land Reform and Rural Development (DALRRD) permitting office [Ref- [12/11/1/1/18/1599](#)(HP)].

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CRedit authorship contribution statement

Nozipho Khumalo: Writing – original draft, Project administration, Formal analysis, Data curation, Conceptualization. **Maphuti Betty Ledwaba:** Writing – review & editing, Conceptualization. **Kim Labuschagne:** Writing – review & editing. **Ilse Voster:** Writing – review & editing. **Marinda Oosthuizen:** Writing – review & editing, Supervision. **Monica Mwale:**

Writing – review & editing. **Mamohale Chaisi:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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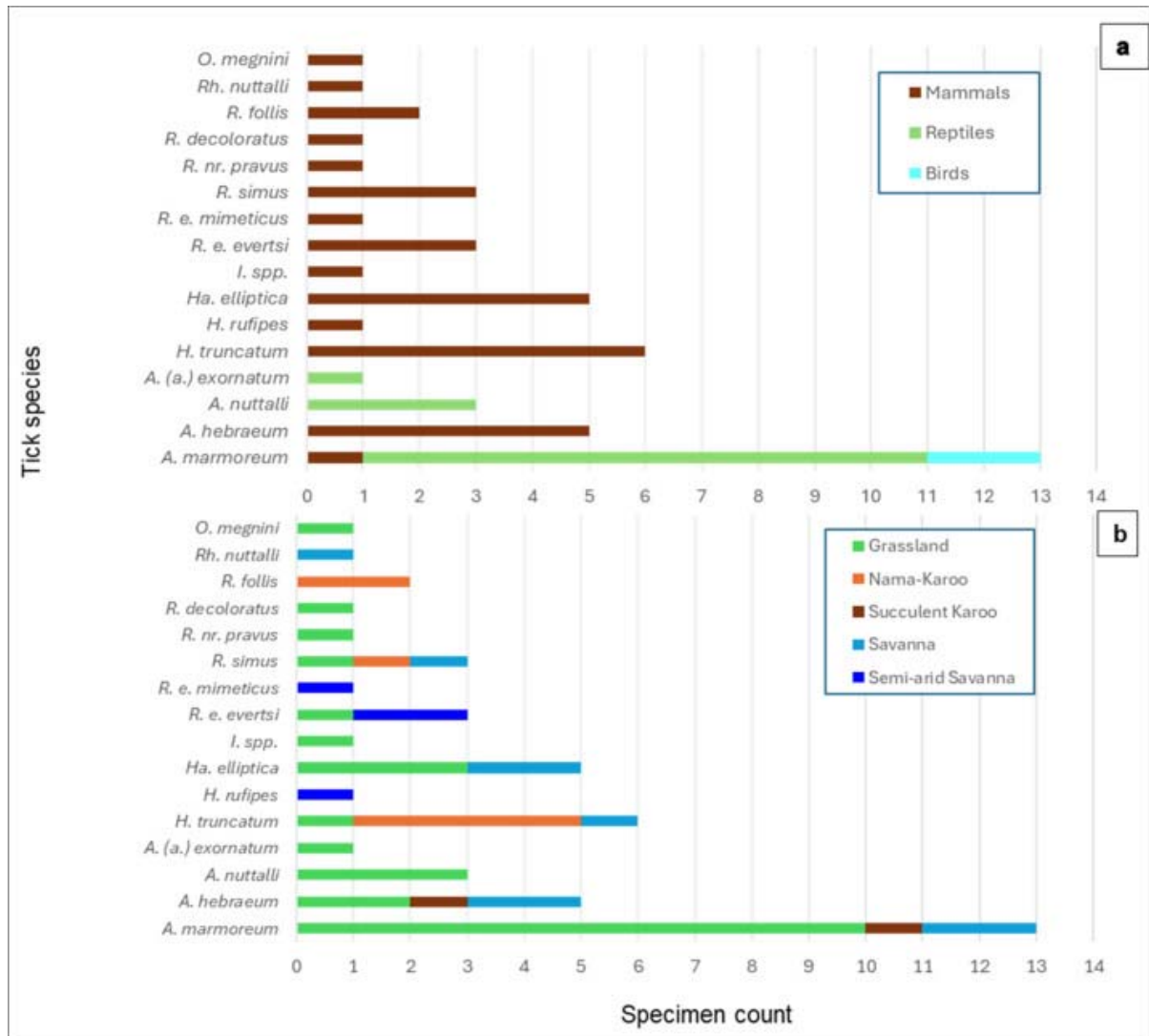


Fig. 4: The number of tick species and specimens collected from different vertebrate classes (a) and biome from southern Africa (b).

Data availability

Sequence data is available on BOLD <https://www.boldsystems.org/index.php/Login/page>

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