Identification of selected South African ticks using morphological traits and DNA barcoding

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Dissertation submitted in partial fulfilment of the requirements

for the degree Master of Science in

Wildlife Health, Ecology and Management at the

Faculty of Veterinary Science

University of Pretoria

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July 2022

ACKNOWLEDGMENTS

I would like to firstly thank Dr. Mamohale Chaisi for welcoming me into her team and introducing me to the world of molecular biology and guiding me throughout the process. Additionally, I take this opportunity to express my gratitude to her as my supervisor and Prof Marinda Oosthuizen for offering me the opportunity to do this degree, and for her advice, encouragement and their contribution to this project. They jumped in when everything was halted and provided full support till the end of the project. Again, great appreciation goes to Dr. Maphuti Ledwaba (Post-doctoral fellow, SANBI) who helped me with laboratory work and gave guidance on the write-up, as well as Dr. Deon Bakkes (taxonomist, ARC-OVI) for assisting with the identification of the ticks and offering to train me in the field. Thanks to Mr. Nkhumeleni Ramavhunga (SANBI) for assisting with the map, and to the SANBI Biobank staff for allowing me to use the samples in their collection. Last but not least, I would like to thank Prof Emily Mitchell for developing and maintaining the Wildlife Disease database that has produced excellent data for research purposes as well as for emotionally supporting me throughout my studies.

Mostly, I would like to thank the molecular epidemiology team at the National Zoological Garden, for being patient with me and offering advice where needed. Lastly, I give special thanks to my son Aliko, who has been very understanding that I had to spend less time with him to complete my project.

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ABBREVIATIONS AND ACRONYMS

16S:	16S ribosomal ribonucleic acid
ARC:	Agricultural Research Council
BLAST:	Basic Local Alignment Search Tool
COI:	Cytochrome oxidase subunit 1
ddH20:	Double Distilled water
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
IUCN:	International Union for Conservation and Nature
MEGA:	Molecular Evolutionary Genetics Analysis
ML:	Maximum likelihood
NBA:	National Biodiversity Act
NCBI:	National Centre for Biotechnical Information
NZG:	National Zoological Garden
PCR:	Polymerase chain reaction
PBS:	Phosphate Buffered Saline
rDNA:	Ribosomal Deoxyribonucleic Acid
SANBI:	South African National Biodiversity Institute
RESC:	Research Ethics & Scientific Committee
TAE:	Tris Acetate EDTA
12S:	12S ribosomal ribonucleic acid
18S:	18S ribosomal ribonucleic acid

ABSTRACT

The study aimed to identify and characterise ticks collected from selected wildlife species from selected areas in South Africa using morphological traits and DNA barcoding. The ticks were collected during necropsy and stored at the South African National Biodiversity Institution-National Zoological Gardens (SANBI/NZG) Biobank. A total of 48 individual tick specimens (adult, engorged and nymphs) from 13 hosts of captive wildlife were morphologically identified using appropriate morphological keys. DNA was extracted from whole ticks, followed by amplification of the *COI* and *16S* rRNA genes. Amplification was confirmed by gel electrophoresis, and the amplicons were sequenced. The following tick species were morphologically identified from different species of wild animals: *Amblyomma marmoreum, Haemaphysalis elliptica, Amblyomma hebraeum, Amblyomma nuttalli, Ixodes* spp., *Hyalomma truncatum, Hyalomma rufipes, Otobius megnini, Rhipicentor nuttalli, Rhipicephalus simus, Rhipicephalus evertsi evertsi, Amblyomma (Aponomma) exornatum, Rhipicephalus evertsi mimeticus* and *Rhipicephalus* spp *cf.* sp nr *pravus. Amblyomma* spp. were the most common species, and represented 22.9% of the identified ticks. Sequencing results confirmed the morphologically results, and indicated that the new sequences were 95 – 100% similar to published sequences of ticks from wild and domestic animals, and vegetation in different parts of southern Africa. However, sequences of three tick species that were morphologically identified as *Rhipicephalus evertsi evertsi*, *Hyalomma truncatum and Rhipicephalus simus,* were closely similar to published sequences of *Rhiphicephalus mimeticus, Hyalomma glabrum and Rhipicephalus gertrudae,* respectively. The findings of this study confirm previous reports that wildlife in South Africa harbour a wide diversity of ticks of veterinary and public health importance, and that *COI* and *16S* rRNA genes are suitable markers for

characterisation of ticks. This study also highlights the risk of transmission of ticks and tick-borne diseases to new areas and hosts during translocation of wild animals. Future work should assess the risk of these ticks as vectors of tick-borne infections of human and livestock in order to inform management of tick-borne diseases, including tick-borne zoonoses, in captive facilities in South Africa.

Key words: ticks, microscopy, *16S* rRNA gene, *COI*, DNA barcoding

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Ticks (Order: Ixodida) are blood ectoparasites of all terrestrial vertebrates (de la Fuente *et al*., 2002). They feed on domestic, wild animals and humans and spread a variety of infections such as bacteria, viruses and protozoa (Jongejan and Uilenberg, 2004). As a result, ticks are considered pests. Ticks and other ectoparasites, on the other hand, are an important element of a healthy ecosystem, as reported by (James *et al.*, 2006). When compared to non-parasitized animals, parasites create evolutionary pressure on host populations and are partly responsible for keeping greater levels of genetic variation in their hosts (Dawkins, 1990; Klein and O'huigin, 1997). The presence of infections preserves a healthier host population because weak or susceptible hosts are eliminated in nature (Potts *et al*., 1994). Ticks are food for many species of birds and reptiles. The decline in the population of these animals, such as oxpeckers (*Buphagus* spp.), which feed mostly on ticks of rhinoceroses and other large mammals in southern Africa has been attributed to the reduction of ticks due to the application of acaricides. If these ticks become extinct, oxpecker and other tick-feeding bird populations are most likely to decline (Durden and Keirans, 1996) .

Ticks spread several pathogens, including bacteria, viruses, and protozoa(Sauer *et al.*, 1995). They are significant vectors of emerging and re-emerging zoonotic pathogens (Jones *et al.*, 2008). In domestic ruminants, ticks are significant vectors for illnesses such as babesiosis, anaplasmosis, and ehrlichiosis. They have the potential of exacerbating non-specific illness symptoms such as anaemia, toxicosis, and paralysis. Most tick species infect several host species, while some of them are limited to specific taxa hosts. Generally, ticks tend to be limited to particular ecosystems, but the translocation of wildlife, climate change, ecotourism, urbanization, as well as the

trade of live wildlife animals provide the possibility of spreading outside their natural ranges. The range and the number of wildlife species are immense all over the world and is supporting a wide variety of diseases caused by ticks (Bengis *et al.*, 2004; De Meneghi, 2006). Furthermore, wild herbivores are believed to be important reservoirs of tick-borne pathogens which affect humans and animals because they support a large population of species of tick (Tonetti *et al.*, 2009). Removal of ticks and treatment of external parasites, as part of the conservation efforts of threatened vertebrates, has resulted in a significant decline in tick populations (Durden and Keirans, 1996). As a result, host-specific ticks are endangered alongside their vertebrate hosts.

Tick species such as *Amblyomma rhinocerotis, Dermacentor rhinocerinus, and Cosmiomma hippopotamensis* plus severalseveral other parasites, feed mostly, or almost so, on white, *Ceratotherium simum* as well as a variety of other parasitic ticks, and/or black *Diceros bicomis* rhinoceroses. The recent drastic reduction in populations of the four tick species has been associated with declines of the rhinoceroses, and it has been suggested that *A. personatum* may soon become extinct (Horak *et al.*, 2017). Coextinctions of ticks following the extinction of their hosts have also been documented (Mihalca *et al.*, 2011). Durden and Keirans, (1996) and Mihalca *et al.* (2011) have provided lists of rare ticks – those that are parasites of only one or a few, endangered hosts, or geographically restricted vertebrate hosts - that may be co-endangered. Furthermore, Mihalca *et al*., (2011) have proposed a synopsis of the co-endangered status of ticks depending on the conservation status of their hosts as reported on the IUCN (International Union for Conservation of Nature) Red List of Threatened Species (https://www.iucnredlist.org). These include ticks of the African elephant (*Loxodonta africana*) (VU - vulnerable), Black Rhino (*Diceros bicornis*) (CR - critically endangered), Hippopotamus (*Hippopotamus amphibious*) (VU), giraffe

(*Giraffa camelopardalis*) (VU) and the king colobus (*Colobus polykomos*) (VU). It is therefore crucial to document and conserve ticks of these mammals, especially those that are host specific and are therefore at risk of coextinction with their hosts.

1.2 PROBLEM STATEMENT

Microscopy has traditionally been used to identify ticks based on their morphological characteristics. Aspects such as host and body site preference, morphological traits, geographical location, season, and ecological needs are critical in the identification and classification of tick species (Jongejan and Uilenberg, 1994). Although morphological characteristics of ticks are essential in tick identification and for describing new species, the lack of taxonomic expertise, combined with the inability to distinguish between closely related tick species, renders microscopy ineffective in identifying ticks (Nava *et al*., 2009).

Owing to recent changes in the use of land, human and animal movement, climate change, and illegal wildlife trade over time, the invasion of new hosts and habitats by alien tick species, and the introduction of novel pathogens in new habitats, host preference and geographical distribution of ticks may change. It is therefore essential to determine the effect of these anthropogenic factors on the distribution of ticks and tick-borne pathogens in order to predict hotspots for possible disease emergence or re-emergence.

DNA barcoding is a robust taxonomic technique that is used to distinguish species based on differences in short DNA sequences (barcodes) that occur within and between species (Hebert *et al*., 2003) and it therefore a useful tool to assist in identifying tick species on a molecular level and ultimately understanding their

geographic adaptation to new habitats and it in resolving taxonomic issues within and between species.

1.3 JUSTIFICATION OF THE STUDY

Although the occurrence and distribution of ticks of wildlife in southern Africa is well documented reviewed by Horak *et al.*, (2018a) this biodiversity has not been fully explored. According to the National Biodiversity Act (NBA) 2018 Synthesis report, most invertebrate groups are relatively poorly known and require taxonomic work. This study will provide additional foundational data with regards to the distribution of ticks of selected wildlife species in South Africa, as well as address ambiguities in the systematics of ticks. The tick collection at the SANBI Animal Biobank, and associated information in the Wildlife Disease Database, provides an opportunity to identify host-tick relationships in South Africa, conduct research on tick-borne infections associated with wild animals, and provide baseline data on the potential risk of zoonotic infections to livestock and humans, as well as the impact of tick-borne diseases on wildlife conservation.

It is crucial to identify ticks infesting wildlife because they form an economically significant group of vectors that may transmit disease to livestock, wildlife and humans (de la Fuente *et al*., 2008).

1.4 AIM AND OBJECTIVES

The study aims to determine the biodiversity of ticks of selected captive wildlife species in South Africa.

1.4.1 OBJECTIVES

The objectives of this study were to:

a. Mining of the Wildlife Disease database for metadata on ticks isolated from wild animals.
b. Morphological identification of ticks.
c. Molecular identification of ticks by DNA barcoding.
d. Determine the phylogenetic relationship of ticks from wildlife.

1.4.2 HYPOTHESIS

There is a wide range of tick species that infest wildlife in captivity.

CHAPTER 2: LITERATURE REVIEW

2.1 CLASSIFICATION OF TICKS (TAXONOMY)

Ticks (Acari: Ixodidae) are obligate ectoparasitic members of the phylum Arthropoda, class Arachnida, subclass Acari and order Ixodida (formerly known as the Metastigmata) (Barker and Murrell, 2004). Ticks have been classified into 905 species and three families Beati and Klompen, (2019). Moreover, an extinct fossil family, Deinocrotonidae has been described (Peñalver *et al*., 2017) and more evidence about the family has been added recently (Chitimia-Dobler *et al*., 2022). The three living families are *Nuttalliellidae,* which consists of a monotypic genus that has only one species, *Nuttalliella namaqua* (Barker and Murrell, 2004). The species is only found in Tanzania, Namibia, and South Africa and has morphological similarities to both argasids and ixodids (Latif *et al.*, 2012). The Ixodidae family, which has 702 species in 14 genera, is known as hard ticks because of their hard sclerotized dorsal scutum (Guglielmone *et al.*, 2010). The sclerotised plate covers the entire dorsal surface of the male, but only one-third of the female's dorsal surface. The Argasidae family is comprised of five genera with approximately 193 species (Guglielmone *et al.*, 2010).. They are referred to as soft ticks because of their leathery cuticle, which appears to be wrinkled and they lack the scutum that is present in the hard ticks The capitulum is positioned in the bottom of the body of the animal and is not clearly apparent whereas in hard ticks the capitulum extends from the body.

2.2 GENERAL MORPHOLOGY

The tick body is divided into two parts namely, the capitulum also referred to as the gnathosoma (Basu and Charles, 2017) as well as the idiosoma (body). It extends anteroventrally and contains the mouthparts as well as the bearing capitulum, a basal

chitinous section. According to Lindquist *et al*., (2017) the ring-like structure connects the capitulum to the body. Their mouthparts are divided into three types: toothed, elongated, and a basal chitinous piece called as the basis capitulum. Additionally, on the dorsal surface of the hypo-stoma are two chelicerae, one on each side of the mouth (Wall and Shearer, 2008). Each chelicera's free terminal forked (chelate) resulting in a dorsal, fixed toothed digitus externus and lateral moveable digitus internus.

The cheli-cerae function is a biting, tearing, and anchoring structure that opens the host's skin and allows entry of the whole capitulum, or at least the toothed hypostome (Sonenshine and Roe, 2013). A pair of palpi or pedipalp emerges from the basic capitulum's anteroventral sides. (Madder *et al*., 2014). While the tick is attached to the host, these structures function as counter-anchors. Mouthparts of different tick species, differ significantly. The body as a whole demonstrates distinctions between members of various genera. It does, however, have four pairs of legs, each of which is split into six parts known as coxa, trochanter, femur, patella tibia, and tarsus (Wall and Shearer, 2008 (Figure 2.1) In certain species, parts of these units are merged.

. The genital opening is placed on the mid-ventral border between the first and second pairs of legs. The anus is also ventral, midway between the level of the fourth pair of legs and the posterior edge of the body (Barker and Walker, 2014; Barker *et al*., 2014; Basu and Charles, 2017).

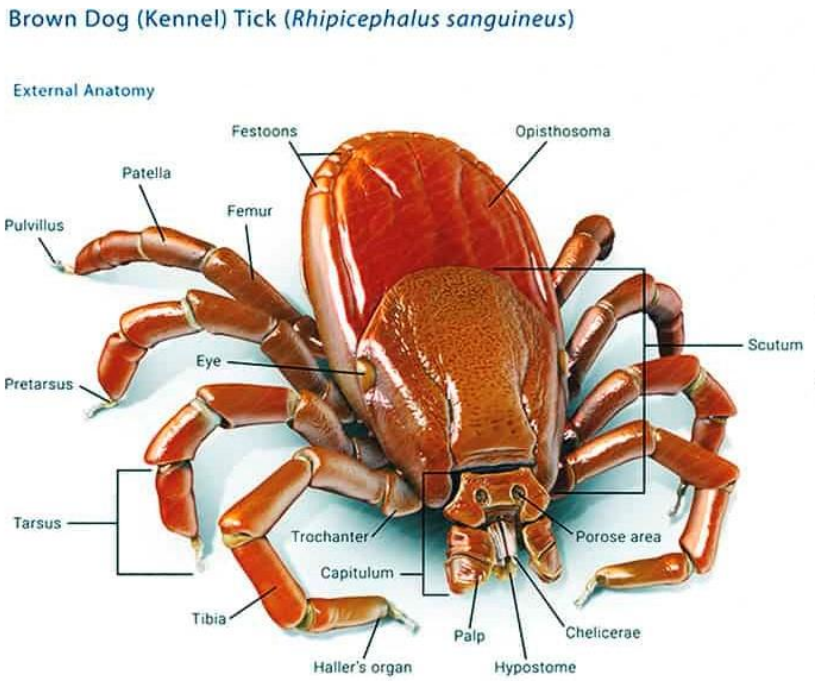

Figure 2.1: Morphological features of the genus Rhipicephalus, dorsal view. Picture adapted from: https://ticksafety.com/wp-content/uploads/Detailed-Tick-Anatomy-Graphic.jpg

2.3 TICK BIOLOGY

2.3.1 LIFE CYCLE

Ticks develop in four stages: embryonated egg, larva, nymph (one or many), and adult (Apanaskevich *et al.*, 2013), In most species, every active stage looks for a host, feeds, and reproduces in the wild (three-host life cycle) (Walker, 2003). Apart from members of the genus *Ixodes,* mating happens only on the host (e.g., during feeding). To begin the gonotrophic cycle, all ixodids other than species of *Ixodes* need a blood meal (Nicholson *et al.*, 2019). Ticks have four stages in their life cycle: eggs, larvae, nymphs, and adults (Gray *et al.*, 2013). According to Walker (2003), ticks of the Ixodid family undergo all three life cycles (one-host, two-hosts an the three host lifecycles) at a rapid rate, and they require a single host for each developmental stage (Dantas-Torres, 2010).

2.3.2 ONE- AND TWO-HOST LIFE CYCLES

In the one host – lifecycle, eggs hatch into the larvae form which climbs on a host to obtain a blood meal, one fully fed it moults into nymphs that also feed on the same host and develop into the adult stage ; Figure 2.2). At this stage, females obtain a full blood meal, detach and drop on the ground to lay a bunch of eggs and then dies.

The most intense variation of a one-host life cycle, for example, in the winter tick *Dermacentor albipictus* or the Asian blue tick (previously *Boophilus microplus*) and other *Boophilus* species. All life stages of these species remain on the host. Larvae and nymphs feed on the host and develop to the next developmental stage on the same host. Only the nourished, mated females descend to oviposit in the natural environment after moulting to the adult stage.

In a two-host lifecycle, eggs hatch into larvae finds a host, and attach to obtain a blood meal. Once fully engorged, the larvae moult on the host to nymphs. The nymphs attach and feed on the host, then detach and drop on the ground to moult (ovipost) which occurs in a safe environment such as (leaf litter, cracked walls, etc) After which the female dies.

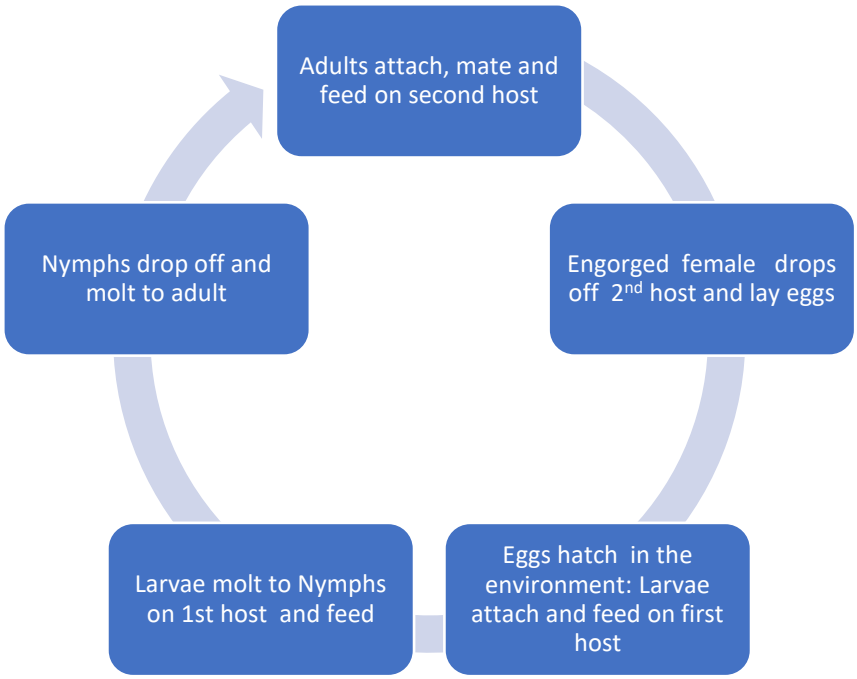

Figure 2.2: Two-host tick life cycle indicating the development from mating until the nymphs molt to adults. Information adapted from the CDC webpage- https://www.cdc.gov/dpdx/ticks/index.html

2.3.3 THREE-HOST LIFE CYCLES

Each active stage of a three-host life cycle replicates the sequence of host-seeking, feeding, and off-the-host moulting on the ground to nymphs, this stage is dependent on temperature.(Parola and Raoult, 2001). Under perfect conditions in the natural environment, the life cycle of such three-host ticks can be completed in less than a year, from the time the larvae hatch to the time the next generation hatches. However, according to (Parola and Raoult, 2001; Dantas-Torres, 2008) factors such as Photoperiod, temperature, and humidity may all affect the length of the life cycle, making it take anything from six months to six years.

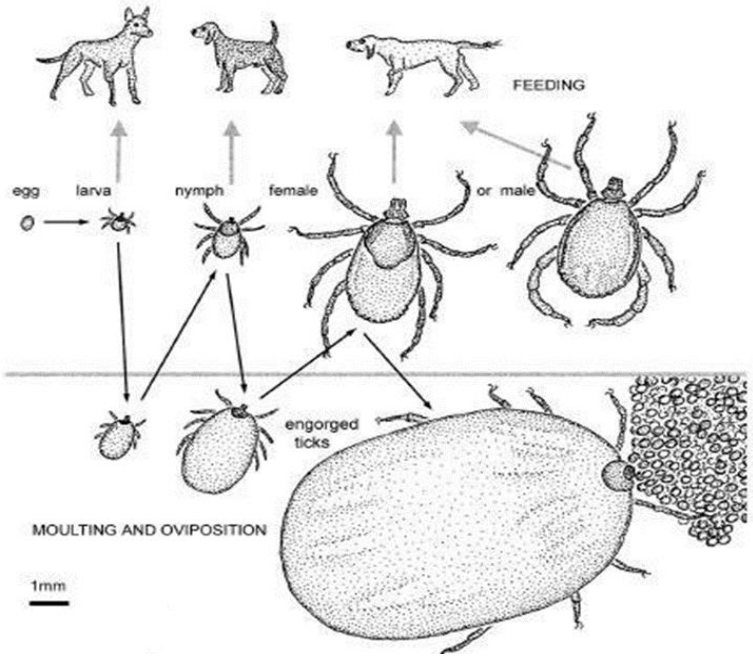

Figure 2.3: Life cycle of a three-host tick (Barker and Walker, 2014)

2.4 PARASITE-HOST RELATIONSHIP

Most tick species are host specialists , meaning they are host-specific and only feed on specific hosts; for example, the Asian blue tick tick *Rhipicephalus microplus* is a one-host tick, meaning it feeds and develops on the same host throughout its life cycle. Many ticks, on the other hand, are generalists who feed on anything they can find. *Amblyomma americanum*, for example, feeds on mammals, birds, and reptiles (Sonenshine and Roe, 2013), while *Ixodes ricinus* is reported to have a broad host range. Additionally, three-host ticks are the most relevant ticks for companion animals, with each growth stage developing on a new host. This diverse range of hosts serves as a reservoir for tick-borne illnesses, including the possibility of transmitting novel infections to hosts that have never been seen before. There are at least five factors that influence tick-host interaction. Seasonal and daily impacts (abiotic factors) such as day duration, temperature, and humidity are included. The rest consists of biotic

factors such as tick biological processes, host biological processes, and interactions between the two (Kovats *et al.*, 2001).

2.4.1 MECHANISMS USED TO FIND HOSTS (QUESTING)

Ticks that seek hosts identify a range of responses from potential hosts, which stimulates their host-finding activity. The most significant and well-studied stimuli are, scents They rely on wind direction to navigate along host-derived smells (Murlis *et al*., 2003). Carbon dioxide, found in the animal breath, and ammonia, found in urine and other animal wastes, are two of the most important host-derived substances (Randolph, 2013). They attract starving ticks into direct range to suitable hosts, which makes other, relatively short stimuli efficient.

Ticks have two methods for seeking hosts: endophilic ticks (nidicolous) live in burrows, nests, hollows, or holes near hosts' resting or breeding places (Gray *et al.*, 2013) whereas exophilic ticks (non-nidicolous) must look for their hosts. Endophilic and exophilic ticks are exposed to different environmental circumstances as a result of their divergent host-seeking activity. While endophilic ticks are more guarded and have more hosts accessibility, exophilic ticks are particularly vulnerable to environmental problems, and host availability is reliant on host ecology and population dynamics (Ruiz-Fons and Gilbert, 2010).

2.4.2 BLOOD FEEDING MECHANISM

Ticks feed by penetrating the host's skin tissue and attaching their mouthparts. Hard tick mouthparts are easily identifiable from above, whereas soft tick mouthparts are not. There are three obvious features in each of the two families: the two external joints are extremely dynamic palps, the chelicerae coupled among them protecting the central rod structure, the hypostome. Whilst the tick feeds on the side, the palps move and do not enter the host skin. The sharp hypostome contains numerous beak-like

hooks (Balashov, 1972; Sonenshine and Sonenshine, 1991). That is the component that ejects while biting into the skin of the host. The reverse extensions ensure that the attached tick is not easily removed. Furthermore, the salivary glands of most hard ticks release a cement-like material that practically binds the feasting tick in position on the host (Parola and Raoult, 2001; Dantas-Torres, 2010) the material dissolves when feeding is completed. The size of the particles injected, and the depth of attachment differ between species.

The duration of feeding differs between hard ticks and soft ticks. Hard ticks feed on hosts for longer periods – from several days to weeks – depending on the tick species, the developmental stage, and the kind of host (Parola and Raoult, 2001). The external area of the hard ticks or cuticle essentially accommodates the huge amount of consumed blood which, in mature ticks, is 200-600 times the unfed weight of the body (Parola and Raoult, 2001; Sonenshine and Sonenshine, 1991) Soft ticks feed on their hosts for short durations, ranging from a few minutes to several days, with specifics varied based on tick species, life stage, and host type. Many soft ticks might relate their feeding behavior to that of fleas or bedbugs, as they live on a host once they have found one.one Soft ticks feed numerous times throughout every stage of life and females deposit multiple tiny batches of eggs in their lifetimes between blood feeds. Soft ticks may gain body weight during a blood meal because their smooth outer epithelial tissue permits significant flexibility without further development to handle the volume of blood eaten, which can range from 5-10 times their unfed body mass (Sonenshine and Sonenshine, 1991).

2.5 DISTRIBUTION OF TICKS IN SOUTH AFRICA

Ticks are important parasites of wild and domestic animals and are also significant carriers of important diseases. Most ticks are thought to prefer domestic animals as

hosts; however, a significant proportion of ticks endemic to Sub-Saharan Africa are naturally parasites of wild animals and infect livestock and humans, especially at the wildlife-livestock-human interface. Nearly 90 tick species have been described in Southern Africa; 35 are linked with domestic animals and 15 are classified as economically important (Spickett *et al*., 2011). Several of these tick species are major carriers of tick-borne diseases of veterinary and medical importance. Tick species from the genera *Amblyomma*, *Hyalomma*, *Ixodes* and *Rhipicephalus* are reported to be indigenous to South Africa (Horak *et al*., 2018a). The authors have also shown that *A. hebraeum*, *I. rubicundus*, *R. appendiculatus* and *R.* (*Boophilus*) *decoloratus* are the most common species in most provinces of the country. However, Nyangiwe *et al*. (2013) has reported that the abundance of *R.* (*Boophilus*) *decoloratus* in most areas with high rainfall is decreasing and seem to be replaced by the invasive Asian tick, *R.* (*Boophilus*) *microplus*.

South Africa plays a huge role in the conservation of wildlife species, as a number of nature reserves, zoos, private game farms, and game parks are well established in the country. The huge diversity and distribution of wild animals kept in these facilities present potential hot spots for tick distribution as well as transmission of tickborne diseases (Cumming, 1998). Additionally, the increased animal movement in the country has also resulted in expansion of the geographical range of many tick species and the spread of tick-borne infections. Exotic tick species can find suitable habitats in a variety of environmental conditions; thus, their distribution is increasing. In addition, Jongejan and Uilenberg (2004) has shown that the survival of ticks in a specific habitat is determined by factors such temperature, rainfall, humidity as well as the presence of the preferred hosts. In a previous study, Hoogstraal and Aeschlimann

(1982) reported that most of the ixodid ticks have strict host preference, thereby limiting their geographical distribution.

2.6 TICKS OF WILDLIFE IN SOUTH AFRICA

Majority of ticks that are indigenous in sub-Saharan Africa are natural parasites of wild animals. *Amblyomma* species, especially *A. hebraeum* and *A. marmoreum* have been reported in wild animals such as African wild dog, lion, black-backed jackal, spotted hyena, civet cats, believed to be important carriers of diseases and other infections affecting humans and a wide range of wild bovids as well as scrub hares (Golezardy and Horak, 2007; Harris *et al*., 2018; Spickett *et al*., 1993; Viljoen *et al*., 2021). The unique climatic circumstances in South Africa support the vast diversity of tick species including *Amblyomma hebraeum*, which transmits *Erhlichia ruminantium*, which causes heartwater in domestic and wild ruminants, and is one of the four most common indigenous tick species that parasitize cattle and wildlife and cause major disease (Coetzer *et al*., 1994). In a recent review paper, Ledwaba *et al*. (2022) indicate that majority of *Rhipicephalus* species prefer a wide range of wild hosts; and that *R. warburtoni* and *R.* distinctus affect scrub hares as well as caracals and rock hyrax respectively. *Rhipicephalus microplus* and *R. decoloratus* transmit *Anaplasma* centrale and *A. marginale* (Potgieter 1987; Hove *et al*., 2018) as well as *Babesia bigemina*, the causal agent of African redwater disease.

Ixodes species associated with wild animals include *I*. *rubicundus* and *I.* pilosus. *Ixodes rubicundus* also referred to as the Karoo paralysis tick, is associated with wild bovids such as buffaloes, blesbok, common eland, gemsboks, red hartebeest, as well as in African wildcat, caracals, mice and rabbits. Horak *et al*. (2015) indicated that adults of this tick species infest ruminants dwelling or visiting mountainous, rocky areas whereas the nymphs and larva affect the elephant shrews and red rock rabbit.

The authors also reported that caracals are affected by all the life stages (larva, nymphs and adults) of this tick.

Haemaphysalis and *Hyalomma* species have also been reported in South African wild animals, for instance, Ha. elliptica is shown to be more prevalent in black-backed jackals, (Penzhorn *et al*., 2020). Moreover, *Ha. spinulosa* is reported in most carnivores (Cumming, 1998). Meanwhile, angulates are the preferred hosts of *Hyalomma* species and felids are only occasionally infected (Walker *et al*., 2003).

2.7 TICKBORNE DISEASES OF VETERINARY IMPORTANCE

Ticks are major carriers of bacteria, protozoans, and viruses that cause illness in both animals and humans (Barandika *et al*., 2008). These microbes have established themselves to benefit from the lifecycle of ticks in order to move from one host species to another (Ostfeld *et al*., 2006). Thus, pathogens get the opportunity to be transmitted during the tick's lifecycle which is done, firstly, when a tick gets the pathogens of an infected host organism during feeding (Randolph, 2008).

A study by Uilenberg, (1995) reported that four major tick-borne diseases affect livestock in South Africa namely: theileriosis, heartwater, African redwater, and gall sickness which are respectively caused by *Theileria parva, Ehrlichia ruminantium, Babesia bigemina, and Anaplasma marginale,* and they are believed to have spilled-over to wildlife hosts.

Tonetti *et al.* (2009) identified a number of wild herbivore species as significant reservoirs for tick-borne infections e.g. African buffalo as a reservoir for Corridor Disease and heartwater. Another study, undertaken by (Carmichael and Hobday, 1975; Peter *et al.*, 1998) indicated the transfer of pathogenic microorganisms from wild

herbivores to cattle. *Rickettsia africae*, which is the causal agent of African tick bite fever in humans have been reported from *A. sylvaticum* and *R. simus* that were collected from a leopard tortoise (Halajian *et al*., 2016)

Tick-borne pathogens that can infect humans and animals cause a variety of diseases such as spotted fever, human granulocytic anaplasmosis, Ehrlichiosis, African tick bite fever etc. Brites-Neto *et al.*, 2015). The distribution of endemic ticks is crucial in defining the overall epidemiology of tick-borne diseases.

Table 2.1: Ticks of veterinary importance and the pathogens they transmit

Tick Species	Diseases	Pathogens	Geographical distribution	References
Rhipicephalus spp.	Babesiosis Anaplasmosis	*Babesia bigemina* *Babesia bovis* *Anaplasma marginale*	Limpopo, Mpumalanga, Gauteng, North West, KwaZulu-Natal, Eastern and Northern Free state, and Eastern Cape	(Walker, 2000; Horak, Nyangiwe and De Matos, 2009; Spickett Arthur, 2013)
Hyalomma spp.	Anaplasmosis	*Anaplasma marginale*	Karoo, except Cape, South-western Southern Africa coastal Eastern KwaZulu-Natal, Eastern-Southern and South-western Cape	(Dreyer, Fourie and Kok, 1998; De Waal, 2000; Apanaskevich and Horak, 2008)
Rhipicephalus evertsi evertsi		*Anaplasma marginale*	Eastern half of Southern Africa,	(Walker, 2000; Spickett Arthur, 2013)
Rhipicephalus appendiculatus	East Coast Fever (ECF)	*Theilera parva*	Limpopo, Mpumalanga, KwaZulu-Natal, Western Cape	(Perry *et al.*, 1991; Lawrence, 1992; Walker, 2000)
Amblyomma hebraeum	Heartwater	*Ehrlichia ruminatium*	Limpopo, Mpumalanga, KwaZulu-Natal and Eastern and South-western Cape	(Yunker, 1996; Horak, Nyangiwe and De Matos, 2009)

2.6 IDENTIFICATION OF TICKS

2.6.1 MORPHOLOGICAL IDENTIFICATION

Traditionally identification of ticks is based on morphological characteristics (Mangold *et al*., 1998). Features such as the mouthparts, body size, anal grove, legs, and scutum, and the technique is still used broadly. However, it has its disadvantages such as the need to be done by an experienced taxonomist especially because morphological characteristics used for identification often have little variation (Dergousoff and Chilton, 2007). Other problems may arise because samples are sometimes damaged during sample collection or filled with blood in different stages making it difficult to do identification (Guglielmone *et al.*, 2006), thus requiring supplementation by other specific and sensitive methods.

2.6.2 POLYMERASE CHAIN REACTION (DNA BARCODING)

A polymerase chain reaction is a rapid and reliable technique and has been used successfully in many taxa as a tool for identification. It uses a standard fragment to identify species. The method is useful because it can identify previously unidentified provisional species that are often morphologically cryptic (Hebert *et al*., 2003). Its effectiveness has been assessed in several research studies and it has been effectively employed in the identification of a wide range of species. Since the degrees of divergence between individuals of the same species are often significantly lower than between closely related species and there is a barcoding gap between inter- and intraspecific divergences, this is considered to be more effective in identifying species. Standard molecular markers such as the internal transcribed space (ITS), *16S* rRNA, subunit I cytochrome oxidase (*COI*), *12S rRNA*, and *18S* rRNA, can be used with the application, however, in other cases, prior optimisation of primers may be required.

Mitochondrial *16S* rRNA has been frequently employed to investigate tick phylogeny and systematic evolution (Barker and Murrell, 2004; Beati and Keirans, 2001; Black and Piesman, 1994). All three authors described managed to use phylogeny to distinguish different tick species, particularly those that were grouped or clustered in wrong groups. The research also shows how phylogenetic trees may be used to better understand tick evolution and make informed modification to their taxonomy and nomenclature. Various research has evaluated the efficacy of DNA barcoding in the identification of tick species (Chao *et al*., 2011). DNA barcoding has been utilized effectively in a variety of species (Hebert *et al.,* 2004; Barrett and Hebert, 2005; Ward *et al.,* 2005; Smith *et al*., 2008). With the fast advancement of DNA barcoding in recent years, the number of DNA sequences in GenBank has exploded, while certain inconsistencies have accumulated (Mutanen *et al.*, 2016). These, inconsistencies negative impact on species identification and DNA barcoding.

CHAPTER 3: MATERIALS AND METHODS

3.1. ETHICAL APPROVAL

Approval of the study was provided by the SANBI Animal Research and Scientific Committee (RESC) [Project No: SANBI/RES/2020-12) Appendix A] and the University of Pretoria, Faculty of Veterinary Science, Research Ethics Committee (REC 167-20), as well as the Animal Ethics Committee (AEC) (Appendix B). 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) Appendix C].

3.2 MINING OF THE WILDLIFE DISEASE DATABASE

The Wildlife disease database is a depository of metadata of necropsies performed on different wildlife species from the SANBI animal collection and external wildlife owners from different locations in South Africa and neighbouring countries. The data includes information on the species, location, age, date of death, date of necropsy, samples collected from the carcasses, as well as the preliminary diagnosis. An example of the database is indicated in Appendix D. The database was mined for information on carcasses that were submitted for pathology and from which ticks were collected. Ticks at different development stages (nymph, adult, fully engorged) were manually pulled from the carcass using forceps and preserved in 70% ethanol, after which they were submitted to the SANBI Animal Biobank for storage.

3.3 HOST AND GEOGRAPHICAL DISTRIBUTION

A total of 30 hosts (12 species) from nine localities in South Africa, Namibia, and Botswana (Table 3.1; Figure 3.1) were investigated. Most of the hosts originated from localities in 6 provinces in South Africa. Two samples were from a leopard and two cheetahs from the Africat Foundation in Namibia and one sample originated from a

leopard from the Khutse game reserve in Botswana. Tick species from the genera *Rhipicephalus, Hyalomma, Amblyomma,* and *Haemaphysalis* were the most common.

Table 3.1: Geographical origin of hosts from the Wildlife Diseases database

Host		**Place of Origin**	
Species	No.	Locality	Province/Country
Cheetah (*Acinonyx jubatus)*	2	Africat Foundation	Namibia
Leopard (*Panthera pardus)*	1	Africat Foundation	Namibia
Cheetah (*Acinonyx jubatus)*	3	Ann Van Dyk Cheetah Centre	Northwest
Leopard (*Panthera pardus)*	1	Ann Van Dyk Cheetah Centre	Northwest
Tankwa feral goat (*Capra hircus*)	4	Carnavorn	Nothern Cape
African black-footed cat (*Felis nigripes)*	1	Cat Conservation Trust Karoo	Eastern Cape
Cheetah (*Acinonyx jubatus)*	1	Hoedspruit Enderngered species Centre	Mpumalanga
Leopard (*Panthera pardus)*	1	Khutse game reserve	Botswana
Brown snake eagle (*Circaetus cinereus)*	1	Mokopane Conservation Centre	Limpopo
Banded mongoose *(Mungos mungo)*	1	National Zoological Garden	Gauteng
White rhino (*Ceratotherium simum)*	1	National Zoological Garden	Gauteng
Leopard tortoise *(Stigmochelys pardalis)*	6	National Zoological Garden	Gauteng
Hog deer *(Axis porcinus)*	1	National Zoological Garden	Gauteng
Cheetah (*Acinonyx jubatus)*	1	National Zoological Garden	Gauteng
Porcupine (*Erethizon dorsatum)*	1	National Zoological Garden	Gauteng
Bosc's monitor lizard (*Varanus exanthematicus*)	1	National Zoological Garden	Gauteng
Roan antelope (*Hippotragus equinus)*	1	National Zoological Garden	Gauteng
Vervet monkey (*Chlorocebus pygerythrus)*	1	National Zoological Garden	Gauteng
Cheetah (*Acinonyx jubatus)*	1	Rietvlei Nature Reserve	Gauteng
Total	**30**	**9**	**8**

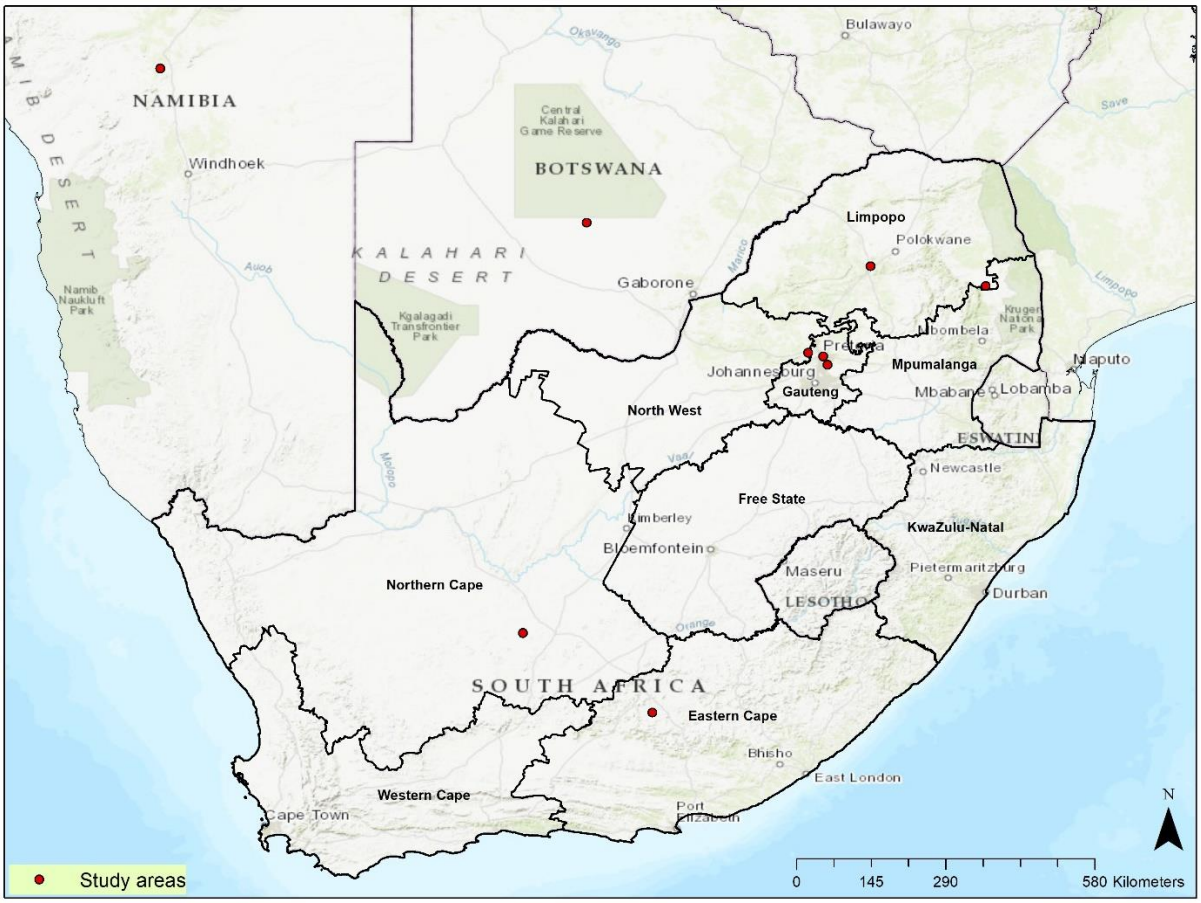

Figure 3.1: Map of South Africa showing origin of ticks (source: created using the ESRI 2020. ArcGIS Desktop: ArcMap 10.8.1 software)

3.4 MORPHOLOGICAL IDENTIFICATION OF TICKS

A total of 48 ticks were identified morphologically by an expert taxonomist (Dr. Deon Bakkes, Gertrud Theiler Tick Museum, ARC-OVI) using a Zeiss Discovery.V20 Stereomicroscope (Zeiss Research Microscopy Solutions, Zeiss Group). Identification was done to species level (where possible) using established taxonomic characters previously described (Banks, 1915; Horak *et al*., 2018b; Walker *et al*., 2005). Additional information such as the sex, life stage, and the number of tick samples was also noted. Upon completion of tick identification, ticks were stored in 70% ethanol and submitted back to SANBI-NZG for further identification using molecular techniques (DNA barcoding).

3.5 DNA BARCODING

Molecular identification targeting specific mitochondrial genes (*16S* rRNA & *COI*) was done to confirm the morphological identification of ticks.

3.5.1 Preparation of buffers and surface sterilization of tick legs

To prepare the wash buffer solution, a tablet of phosphate buffer saline (PBS) was dissolved in 200 ml of distilled water to make 1X PBS. Half of the prepared PBS was then mixed with 50 µl of Tween-20 to generate 0.05% PBS-T. To remove surface contamination, the ticks were washed with PBS-T for 1 hour, rinsed twice with 70% ethanol, then rinsed three times with double distilled water, vortexing for 1 minute each time (Van Wyk, 2019).

3.5.2 DNA EXTRACTION FROM TICK LEGS

Before DNA extraction, tick legs were crushed using sterile blades to enhance the breaking down of the cell membrane. Subsequently, DNA was extracted using the

ZymoBIOMICS™ DNA Miniprep kit according to the manufacturer's instructions (Zymo Research, Freiburg im Bresgau, Germany). Briefly, samples were added to ZR Bashingbead™ Lysis tubes, which were then vortexed in 750µl ZymoBIOMICS™ Lysis solution. Samples were incubated for 24 hours at 55 °C on a heating block for complete lysis. After the incubation period, the mixture was centrifuged at 12,000 rpm for 1 minute and 400 µl of the supernatant was transferred to a collection tube on a Zymo-Spin™ III-F filter and centrifuged at 12,000 rpm for 1 minute, after which the collection tube was removed disposed of. Then 1,200 µl of ZymoBIOMICS DNA Binding Buffer was added and mixed well. Thereafter, 800 µl of the mixture was transferred to a Zymo-Spin™ IICR column in a collection tube and centrifuged at 10,000 x g for 1 minute before discarding the flow-through and repeating the previous procedure. This was followed by the addition of 400 µl of ZymoBIOMICS™ DNA Wash Buffer 1 to the column in a new collection tube and centrifuged for 1 minute and discarded the collection tube.

Following that, 700 µl of DNA wash buffer 2 was added, centrifuged for 1 minute, and the collection tube was emptied. An additional 200 µl of DNA wash buffer 2 was added and the sample was centrifuged for 1 minute and the collection tube with the run-through was discarded. The Zymo-Spin™ IICR Column mixture was then transferred to a clean 1.5 ml microcentrifuge tube and 100 µl Symbiotic™ DNase/RNase Free Water was added directly to the column matrix and incubated for 1 minute. Then centrifuged at 10,000 x g for 1 minute to elute DNA. ZymoBIOMICS™ HRC Prep Solution (600 µl) was added and the sample was centrifuged at 12 000 rpm for 3 minutes. The eluted DNA was transferred to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuged at exactly 16,000 x g for 3 minutes. The DNA was stored at -20 °C until further use. DNA quality and quantity were

confirmed using gel electrophoresis and Nanodrop (ND-1000 Spectrophotometer, Thermo Fisher Scientific Nanodrop Technologies).

3.5.3 SURFACE STERILIZATION OF WHOLE TICKS

The ticks were individually washed three times with phosphate buffer saline (PBS) and allowed to dry. A pestle and mortar (both lined with foil to prevent cross-contamination) were used to crush the ticks. Subsequently, DNA was extracted using the Zymo Quick-DNA Miniprep plus kit (as outlined by the manufacturer (Zymo Research, Freiburg im Bresgau, Germany). Briefly, 95 µl of water, 95 µl of solid tissue buffer, and 10µl of proteinase K were mixed by vortexing. The samples were incubated for 48 hours at 55°C on a heating block for complete lysis. After incubation, the mixture was centrifuged at 12,000 rpm for 1 minute and the supernatant was transferred to a clean tube. Afterward, 400 µl of genomic binding buffer was added to the supernatant and vortexed for 10 seconds, the mixture was then transferred to a collection tube on a Zymospin IIC-XLR column and centrifuged at 12,000 rpm for 1 minute, after which the collection tube was discarded. This was followed by the addition of 400µl of DNA Pre-Wash Buffer to the column in a new collection tube and centrifuged for 1 minute, the collection tube was then emptied. Subsequently, 700µl of g-DNA wash buffer was added, centrifuged for 1 minute and the collection tube was emptied. Another 200 µl of g-DNA wash buffer was added and centrifuged for 1 minute, and the collection tube with the flow-through was discarded.

DNA was then eluted by adding 50 µl of DNA elution buffer and incubated for 5 minutes before being centrifuged for 1 minute at 12 000 rpm. The DNA was stored at -20° C until further use. DNA quality and quantity were confirmed using gel electrophoresis and Nanodrop (ND-1000 Spectrophotometer, Thermo Fisher Scientific Nanodrop Technologies).

3.4.4 AMPLIFICATION OF COI AND 16S RRNA GENES USING DNA FROM WHOLE TICKS

The concentration of the DNA extracted from the tick legs was low and there was no amplification of the DNA, and therefore PCR was performed using DNA extracted from whole ticks.

The methods described by (Li *et al.*, 2018; Damian *et al.*, 2021) were ere optimized to perform selective amplification of desired gene regions. 23 Two primer sets (Table 3.2) and touch down PCR conditions were optimized to perform amplification of the desired gene regions (Table 3.3) obtained as indicated in the literature (Black and Piesman, 1994; Folmer *et al*., 1994; Ondrejicka *et al*., 2017). PCR primers were individually dissolved in sterile nuclease-free water and mixed well to prepare 100 µM stock solutions. Once dissolved the sterile stock primer sets were stored at -20° C to increase the stability and storage time. A 10 µM working dilution of the different primer sets was used to perform PCR. Each PCR reaction was made up to a total volume of 25 µl, compromising 12.5 µl master mix (Ampliqon Taq DNA Polymerase RED; LASEC, Denmark), 1 µl of 10 µM of each primer, 8.5 µl nuclease-free water, and 2 µl of DNA template. DNA isolated from fresh ticks obtained from a tiger and a cheetah from SANBI Mokopane Conservation Centre were used as positive controls, and double-distilled water (ddH_20) was used as a negative control. The PCR was carried out using the SimpliAmp^{TM} thermal cycler (Thermo Fisher Scientific).

3.4.5 VISUALIZATION OF THE AMPLICON WITH AGAROSE GEL ELECTROPHORESIS

Following PCR, amplification was confirmed by gel electrophoresis, using a 2% agarose gel by dissolving 4g of Seakem ® LE agarose in 200 ml of 1x Tris -acetate-

EDTA (TAE) buffer (Thermo Fisher Scientific).Three (3) µl of SYBR safe stain (Thermo Fisher Scientific) was added to the gel to enhance visualization. The gel was poured into a gel tray ray and left to solidify before loading the PCR products. Two µl of PCR product was loaded and 3 µl of 1kb plus DNA Ladder (Thermo Fisher Scientific) was used for fragment size determination. The samples were run at 100 V for 45 minutes and visualized using the gel doc system (Bio-Rad Gel Doc TM EZ Imager).

Table 3.2: Summary of primer sets analyzed for amplification and protocol references used for the optimization of PCR

Primer Pairs	**Primer Name**	**Primer Sequence**	**Length of expected amplicon**	**References**
COI	HCO2198 LCO1490	Forward: 5’ -TAA ACT TCA GGG TGA CCA AAA AAT CA- 3’ Reverse: 5’ -GGT CAA CAA ATC ATA AAG ATA TTG G-3	675-820	(Folmer *et al.*, 1994)
16S	16S+1 16S-1	Forward: 5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3' Reverse: 5’-CCG GTC TGA ACT CAG ATC AAG T-3’	454	(Black and Piesman, 1994)

Table 3.3: Cycling protocols that were analyzed for amplification using three primer sets and annealing temperatures that were modified

Primer sets	***COI (Ondrejicka et al., 2017)***	***16S* rRNA (Black and Piesman, 1994)**
Touch Down Protocol	Initial denaturation at 94°C for 1 min — 1 Cycle Denaturation at 94°C for 5 min Annealing at 45°C for 1.5 min Extension at 72°C for 1 min — 5 Cycles Denaturation at 94°C for 1 min Annealing at 51°C for 40 sec Extension at 72°C for 1 min — 35 Cycles Final extension at 72 °C for 5 min Hold at 4°C	Initial denaturation at 95°C for 5 min — 1 Cycle Denaturation at 92°C for 1 min Annealing at 48°C for 1 min Extension at 72°C for 30 Sec — 10 Cycles Denaturation at 92°C for 1 min Annealing at 54°C for 35 sec Extension at 72°C for 30 sec — 32 Cycles Final extension at 72 °C for 7 min Hold at 4°C

3.5 SEQUENCING AND SEQUENCE ANALYSIS

PCR products of the *16S* rRNA and *COI* genes from selected samples were submitted to Inqaba Biotechnology (South Africa) for bi-directional sequencing using the PCR primers. Forward and reverse reads (sequences) were assembled and aligned using CLC main work Bench workbench (https://digitalinsights.qiagen.com/). Consensus sequences were compared to published sequences from GenBank using BLASTN (Baulcombe *et al.*, 1998), in order to identify homologous sequences. *COI* and *16S* rRNA sequences from the GenBank that showed 95 - 100% similarity to the query sequence were included in the alignment.

Neighbour-Joining (NJ) and Maximum Likelihood (ML) phylogenetic trees were constructed using MEGA7 (Kumar *et al*., 2016). The best-fitting model was TN93+G for *COI* and HKY for the *16S* rRNA gene sequences. For both trees, the default settings in each programme was used to draw the tree, with a 1000 bootstrap analysis (Felsenstein, 1985). Evolutionary divergence between the *COI* and *16S* sequences was estimated by determining the number of base-pair differences between the new sequences and the reference sequences from GenBank.

CHAPTER 4: RESULTS

4.1 MORPHOLOGICAL IDENTIFICATION OF TICKS

Following previously described morphological keys (Banks, 1915; Walker, 2000; Horak *et al.*, 2018b), ticks were successfully identified to species level, with just one (*Ixodes* spp.) identified only to genus level.

A total of 48 ticks (adults, larvae, nymphs), from 13 host species and nine localities in South Africa, Namibia, and Botswana (Table 4.1) were identified in this study. The results indicated that the ticks belonged to seven genera; namely, *Amblyomma, Hyalomma, Rhipicephalus, Haemaphysalis, Ixodes, Rhipicentor,* and *Otobius.* Out of the seven genera, a total of 15 ixodid tick species and one soft tick were identified. The ixodid tick species identified included*Amblyomma hebraeum* Koch, 1844 (plate 4.1 - 4.3); *Amblyomma marmoreum* Koch, 1844 (plate 4.4 - 4.7); *Amblyomma nuttalli* Dönitz, 1910 (plate 4.8 - 4.9); *Amblyomma (Aponomma) exornatum* Koch, 1844 (plate 4.10)*; Hyalomma truncatum* Koch, 1844 (plate 4.11 - 4.12); *Hyalomma rufipes*, Koch 1844 (plate 4.13)*; Rhipicephalus evertsi evertsi* Neumann, 1897 (plate 4.14 - 4.15); *Rhipicephalus simus* Koch, 1844 (plate 4.16); *Rhipicephalus follis* Dönitz, 1910 (plate 4.17- 4.18); *Rhipicephalus evertsi mimeticus* Dönitz, 1910 (plate 4.19 - 4.20)*; Rhipicephalus decoloratus*, Koch, 1844 (plate 4.21 - 4.22); *Rhipicephalus pravus* Dönitz, 1910 (plate 4.23)*; Haemaphysalis elliptica* Koch, 1844 (plate 4.24); *Rhipicentor nuttalli* Cooper and Robinson, 1908 (plate 4.25); and *Ixodes* spp. (plate 4.27),), while the Argasidae identified included *Otobius megnini* Dugès, 1884 (plate 4.26).

Amblyomma species were present in Gauteng, North West, Limpopo, and in the Northern Cape, while the *Hyalomma* species were present in the North West, Gauteng, and Northern Cape Provinces, respectively. The *Rhipicephalus* species

mainly were present in the North West Province, Gauteng, and the Northern Cape. Both the *Haemaphysalis* and *Rhipicentor* were present in the North West Province while *Otobius* and the *Ixodes* species were present in Gauteng.

Amblyomma species

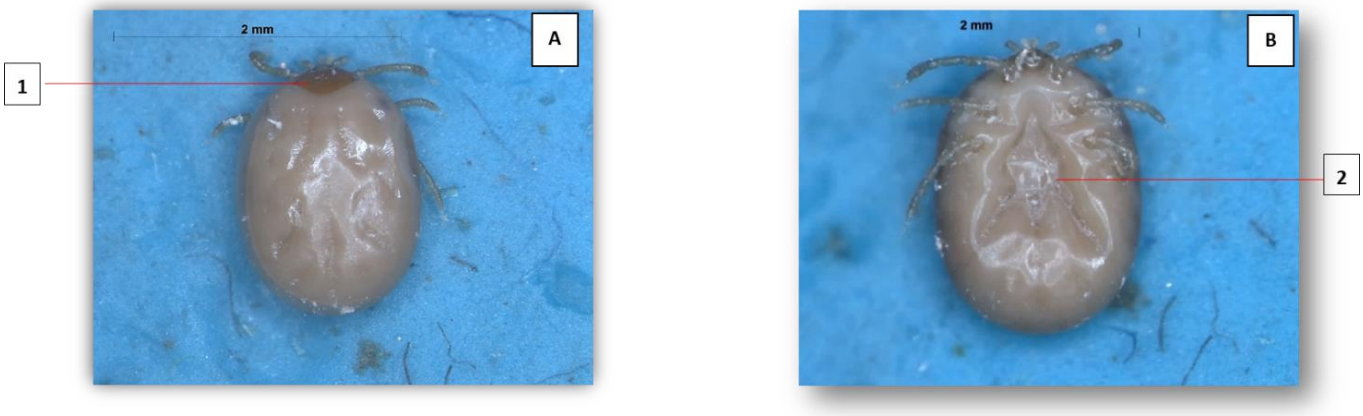

Plate 4.1: *A. hebraeum* larvae; **A** Dorsal habitus**;(1)** Scutum; **B** Ventral habitus; **(2)** Genital groove.

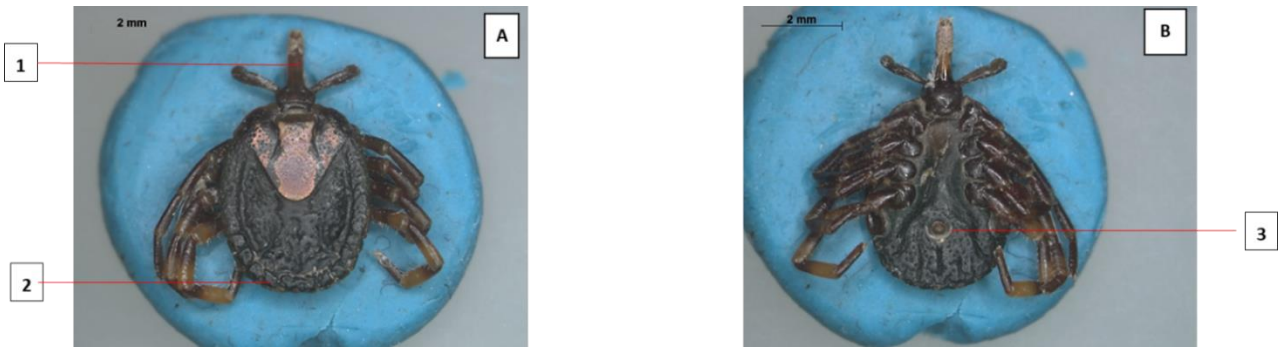

Plate 4.2: *A. hebraeum* (female); **A** Dorsal habitus;(**1**) Palps; **B** Ventral habitus; **(3)** genital aperture.

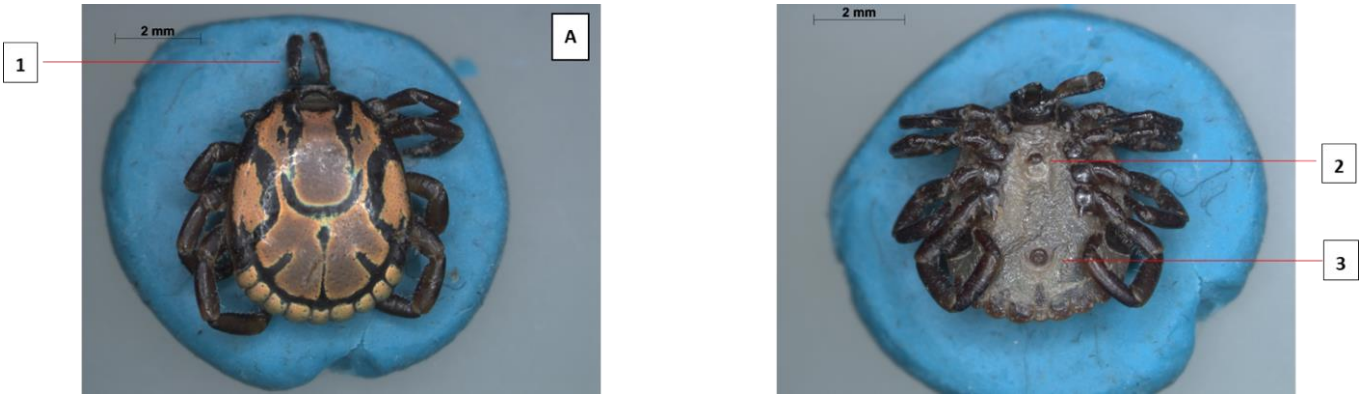

Plate 4.3: *A. hebraeum* (male); **A** Dorsal habitus; (**1**) Palps; **B** Ventral habitus; **(2)** Genital aperture **(3)** Genital aperture.

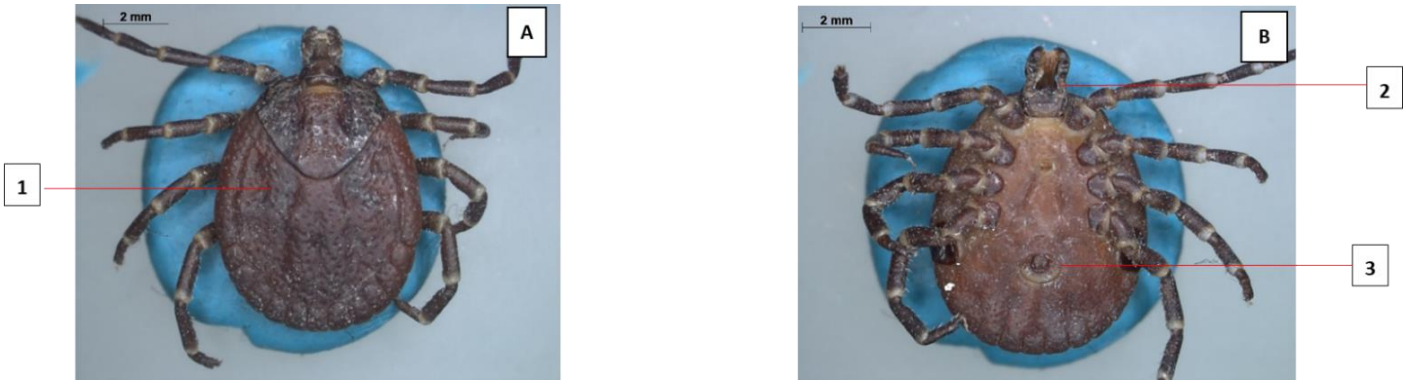

Plate 4.4: *A. marmoreum* (female); **A** Dorsal habitus; **(1)** Scutum; **B** Ventral habitus; **(2)** Mouth parts; **(3)** Anus.

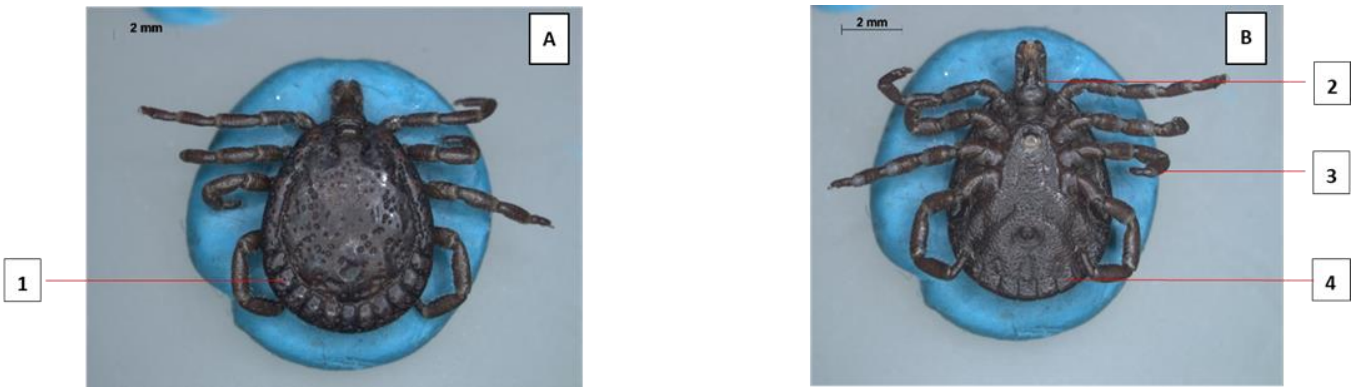

Plate 4.5: *A. marmoreum* (male); **A** Dorsal habitus; **(1)** Festoons; **B** Ventral habitus; **(2)** Mouth parts; **(3)** Legs; **(4)** Festoons ventral side.

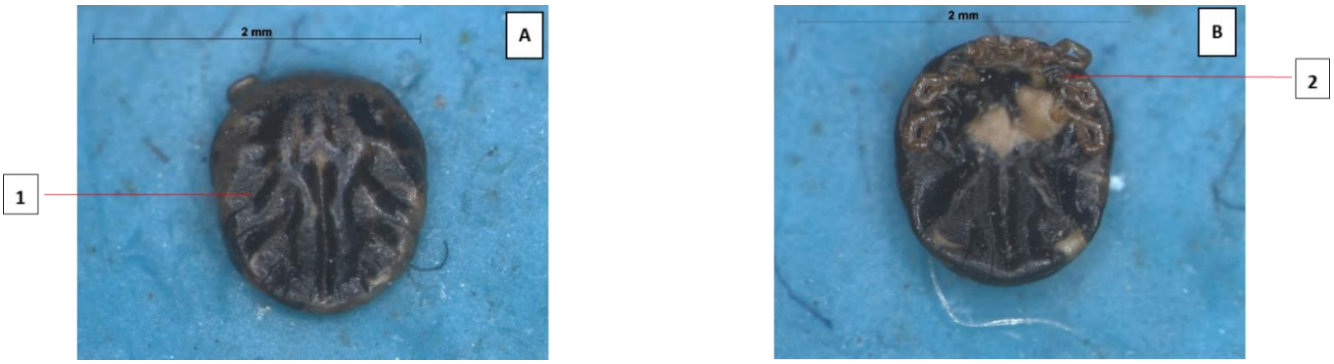

Plate 4.6: *A. marmoreum* larvae; **A** Dorsal habitus; **(1)** Scutum; **B** Ventral habitus; **(2)** Legs.

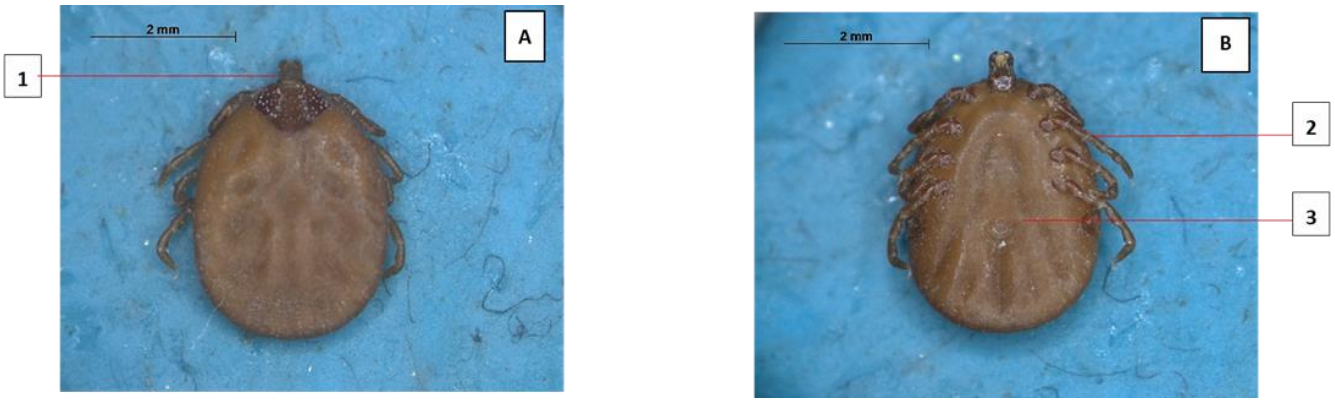

Plate 4.7: *A. marmoreum* nymph; **A** Dorsal habitus; **(1)** Scutum; **B** Ventral habitus; **(2)** Legs; **(3)** Anus.

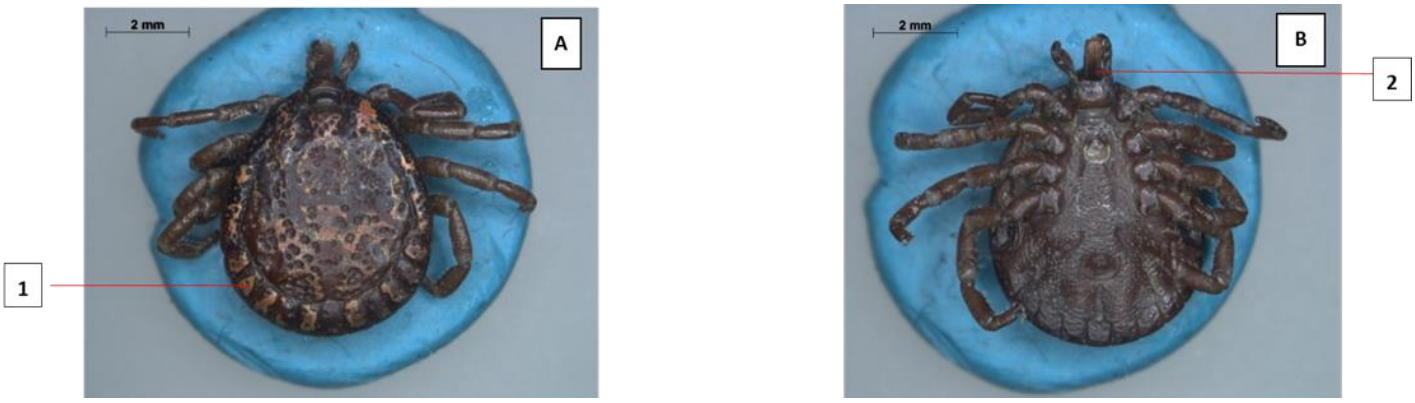

Plate 4.8: *A. nuttalli* (male); **A** Dorsal habitus; **(1)** Festoons; **B** Ventral habitus; **(2)** Mouth parts.

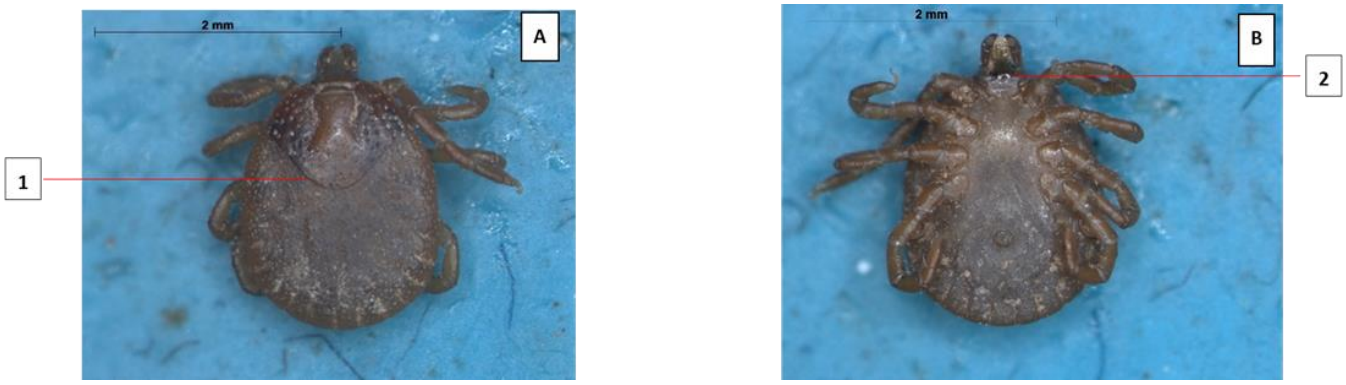

Plate 4.9: *A. nuttalli* nymph; **A** Dorsal habitus; **(1)** Scutum; **B** Ventral habitus; **(2)** Mouth parts.

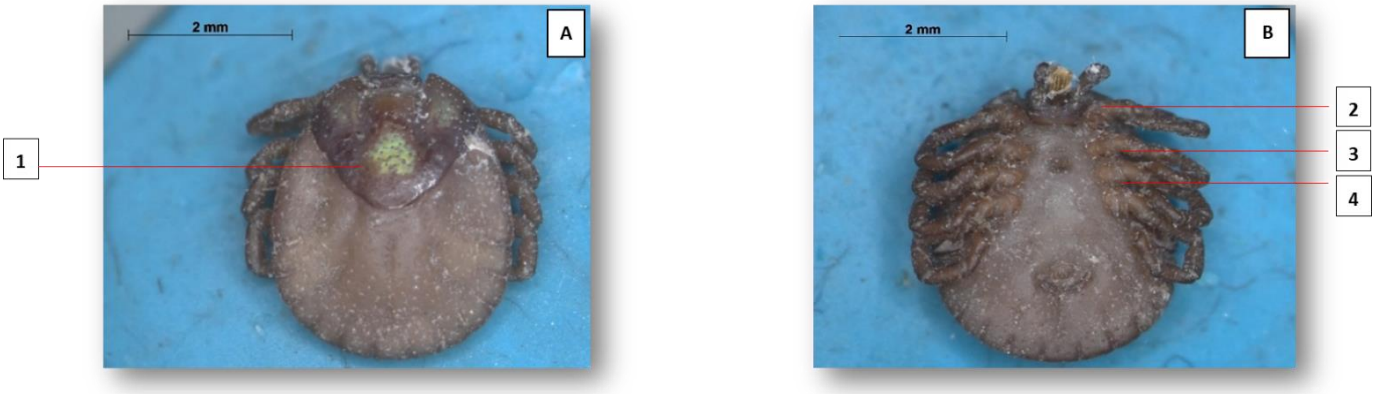

Plate 4.10: *A. exornatum* (female); **A** Dorsal habitus; **(1)** Scutum; **B** ventral habitus; **(2)** Coxa ; **(3)** Coxa II; Coxa III.

***Hyalomma* Species**

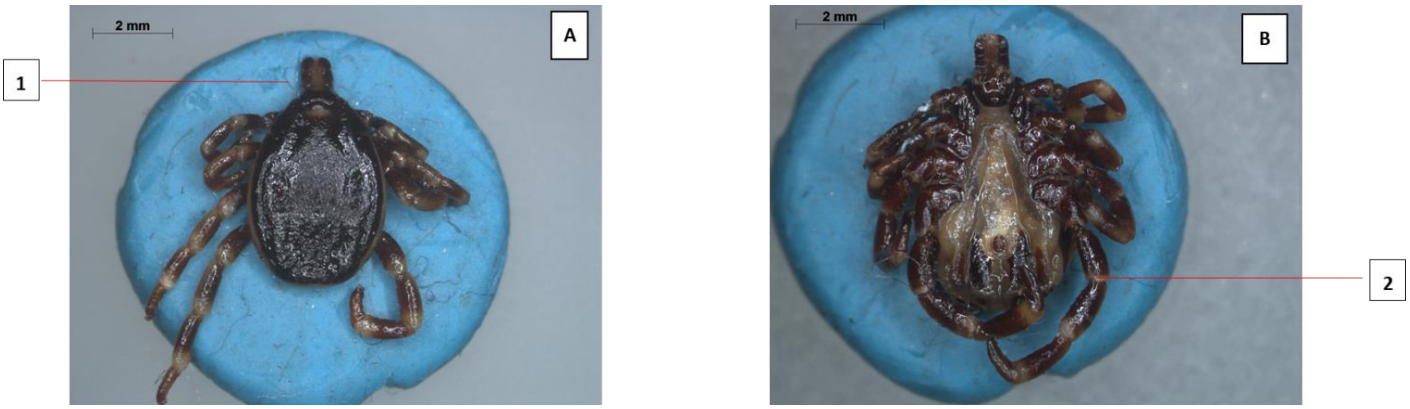

Plate 4.11: *H. truncatum* (female), **A** Dorsal habitus; **(1)** Mouthparts; **B** Ventral habitus; **(2)** Legs.

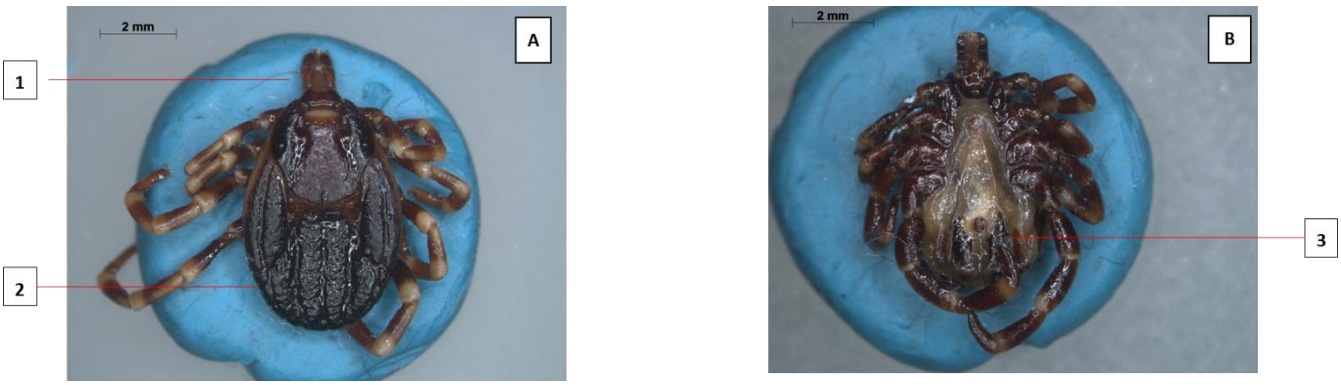

Plate 4.12: *H. truncatum* (male), **A** Dorsal habitus; **(1)** Mouthparts; **B** Ventral habitus; **(2)** Legs.

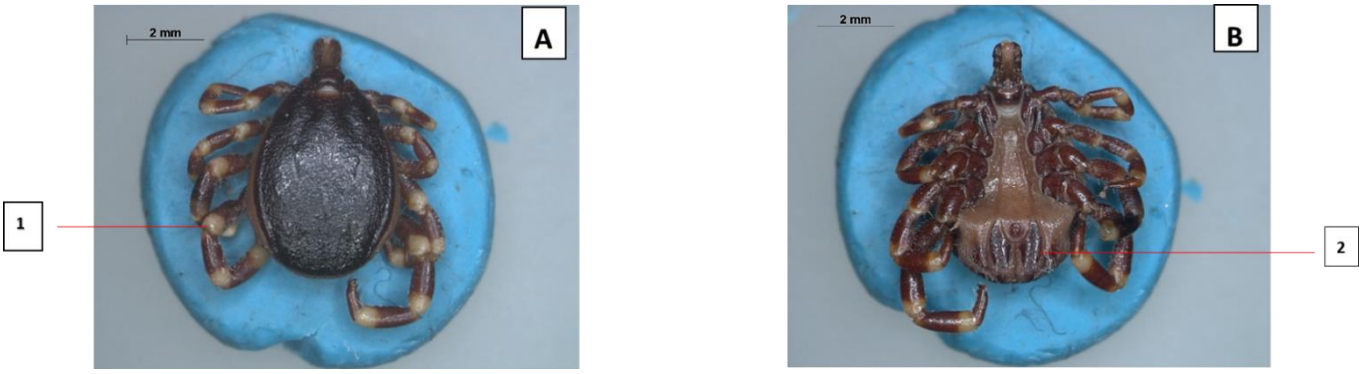

Plate 4.13: *H. rufipes* (male); **A** Dorsal habitus; **(1)** Legs; **B** Ventral habitus; **(2)** Sclerotized plates aligned with anus.

Rhipicephalus species

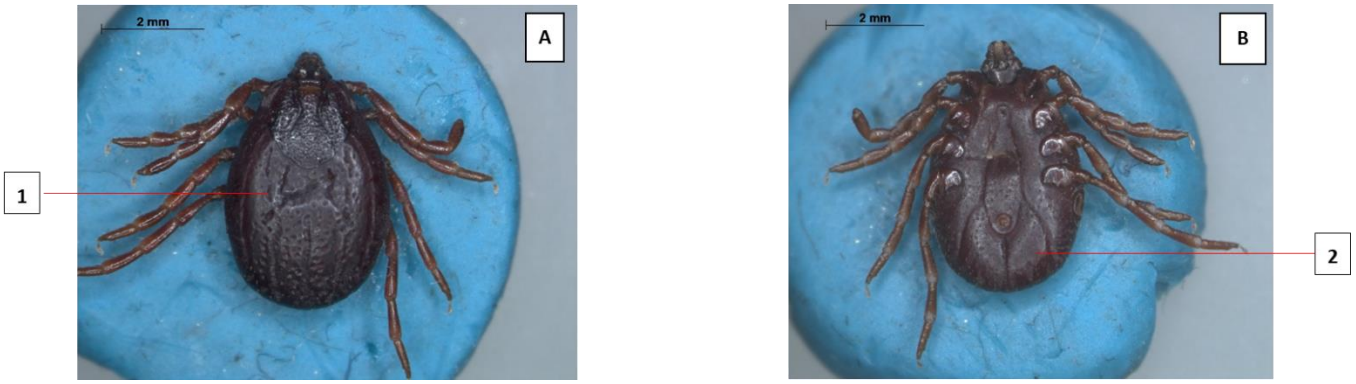

Plate 4.14: *R. evertsi evertsi* (female) ; **A** Dorsal habitus ; **(1)** Scutum; **B** Ventral habitus; **(2)** Ventral plates.

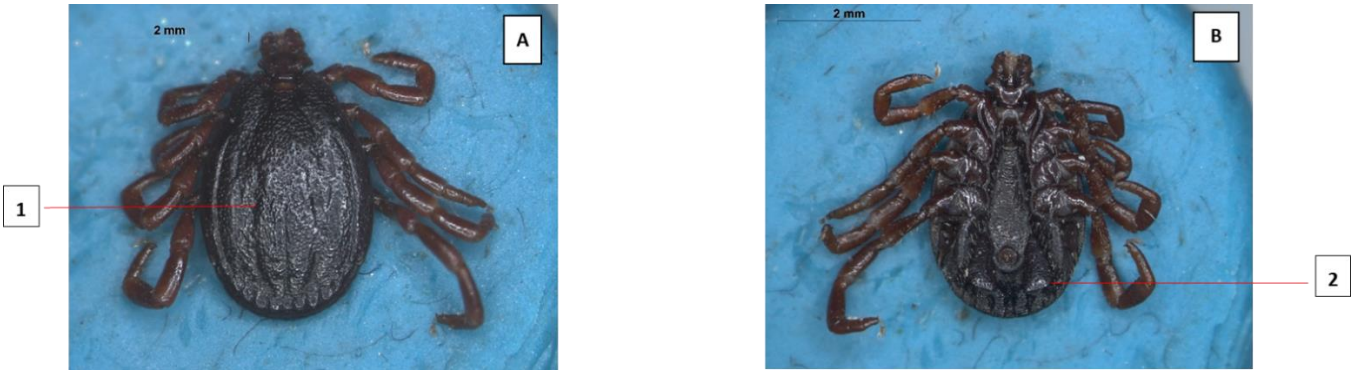

Plate 4.15: *R. evertsi evertsi* (male); **A** Dorsal habitus; **(1)** Alloscutum; **B** Ventral habitus; **(2) A**ccessory plate

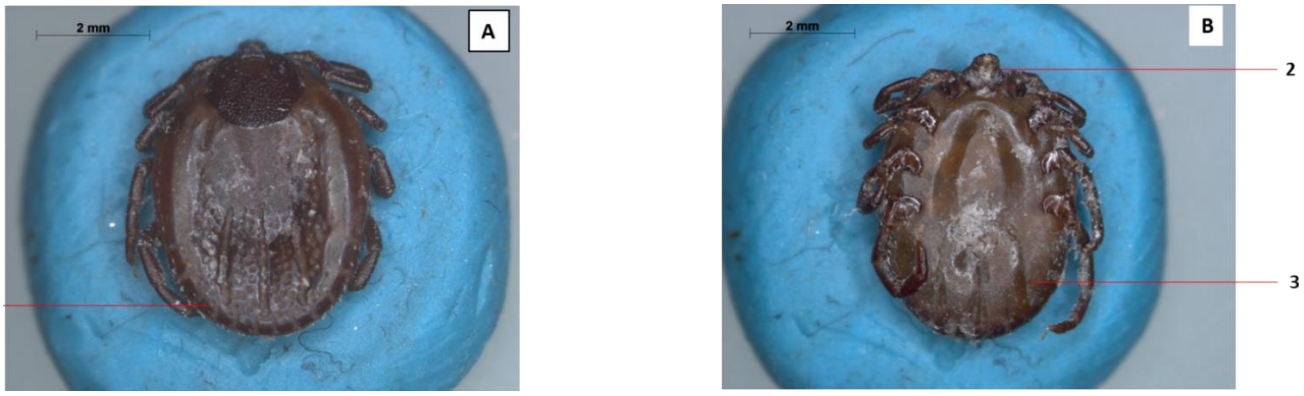

Plate 4.16: *R. simus* (male); **A** Dorsal habitus; **(1)** Festoons; **B** Ventral habitus; **(2)** Mouthparts; **(3)** Genital groove.

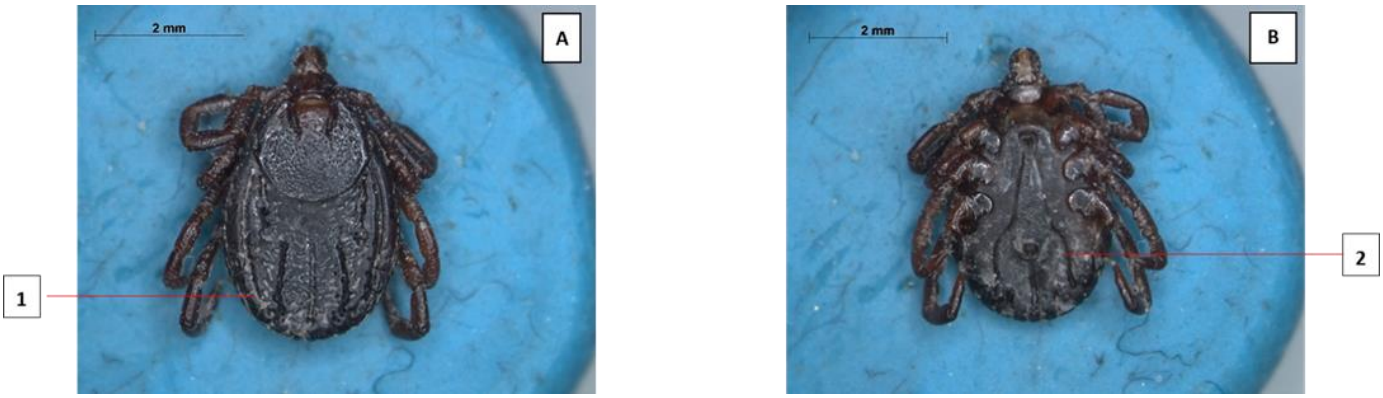

Plate 4.17: *R. follis* female; **A** Dorsal habitus; **(1)** Scutum; **B** Ventral habitus; **(2)** Accessory plate.

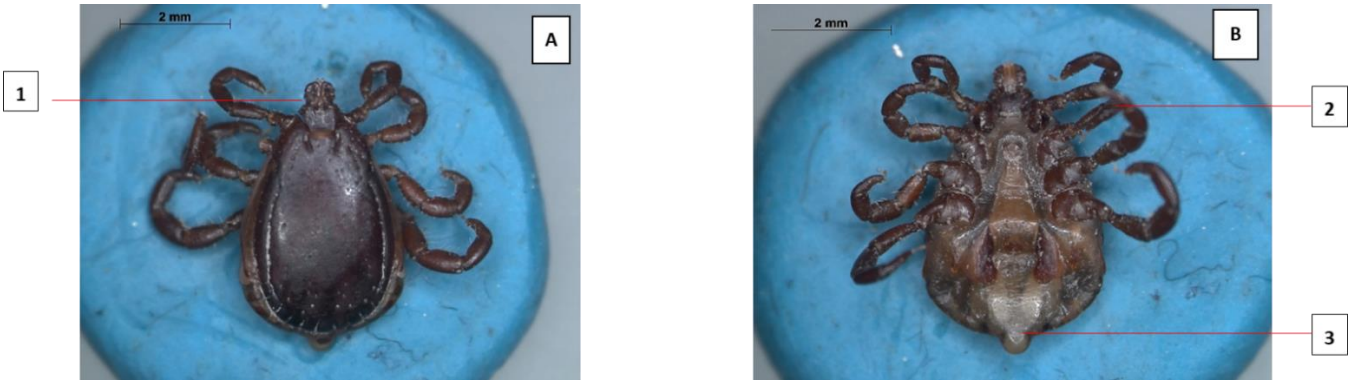

Plate 4.18: *R. follis* (male); **A** Dorsal habitus; **(1)** Mouth parts; **(2)** Legs; **B** Ventral habitus; **(2)** Legs; **(3)** Caudal appendage.

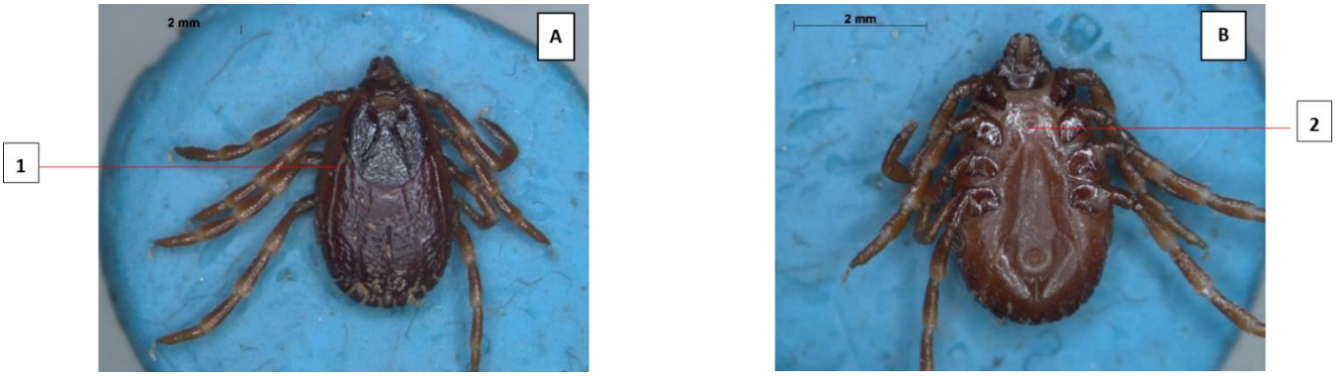

Plate 4.19: *R. evertsi mimeticus* (female); **A** Dorsal habitus; **(1)** Scutum; **B** Ventral habitus; **(2)** Genital aperture.

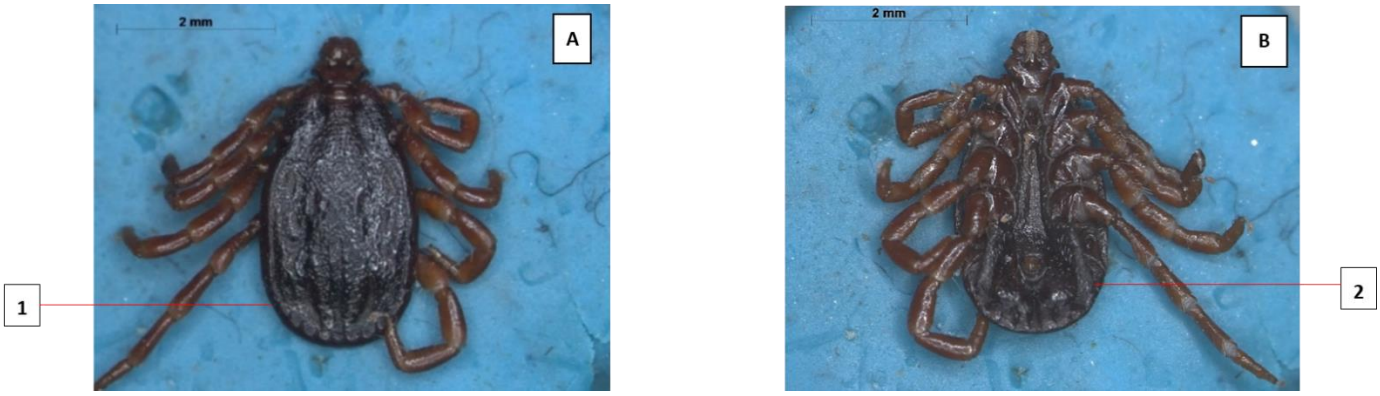

Plate 4.20: *R. evertsi mimeticus* (male); **A** Dorsal habitus; **(1)** Scutum; **B** Ventral habitus; **(2)** Genital aperture.

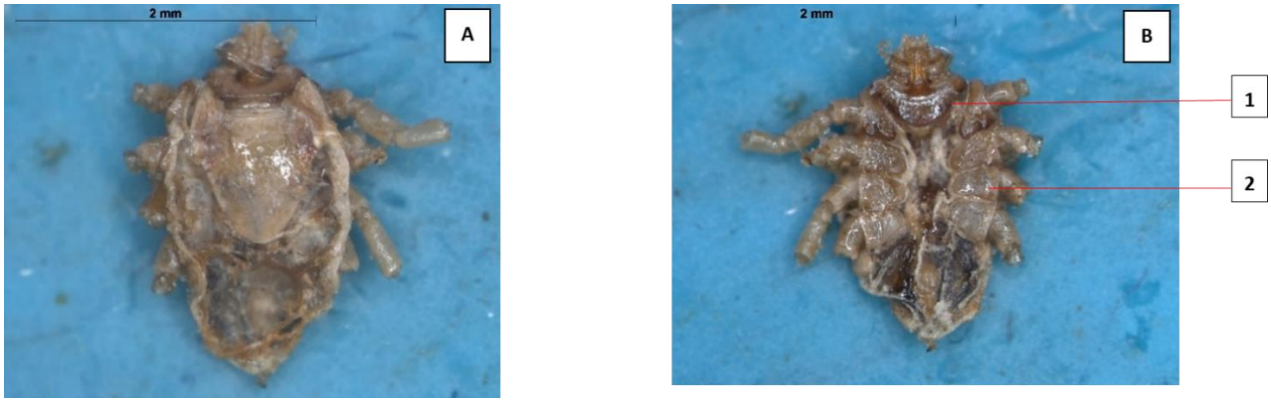

Plate 4.21: *R. decoloratus* (female); **A** Dorsal habitus (damaged); **B** Ventral habitus; **(1)** Basis capituli; **(2)** Coxa.

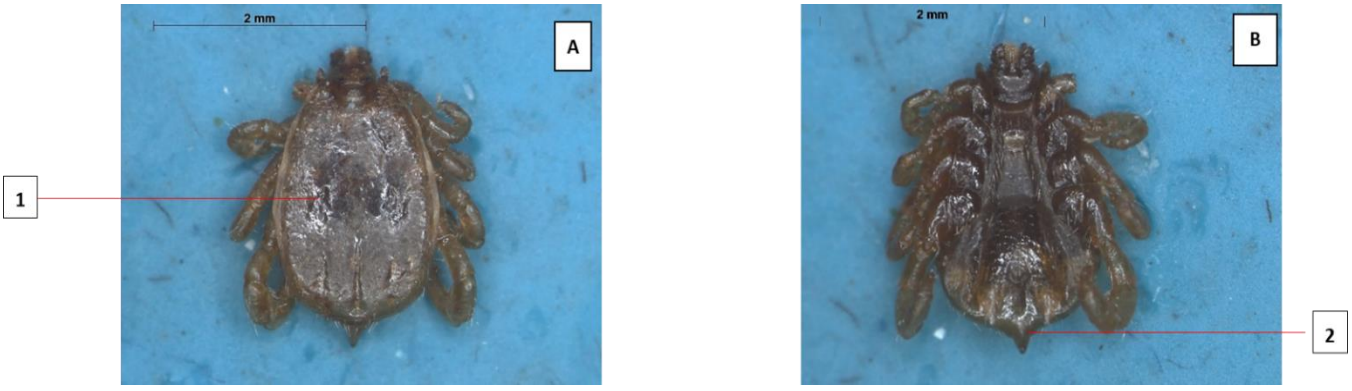

Plate 4.22: *R. decoloratus* (male); **A** Dorsal habitus; **(1)** Alloscotum; **B** Ventral habitus **(2)** Caudal appendage.

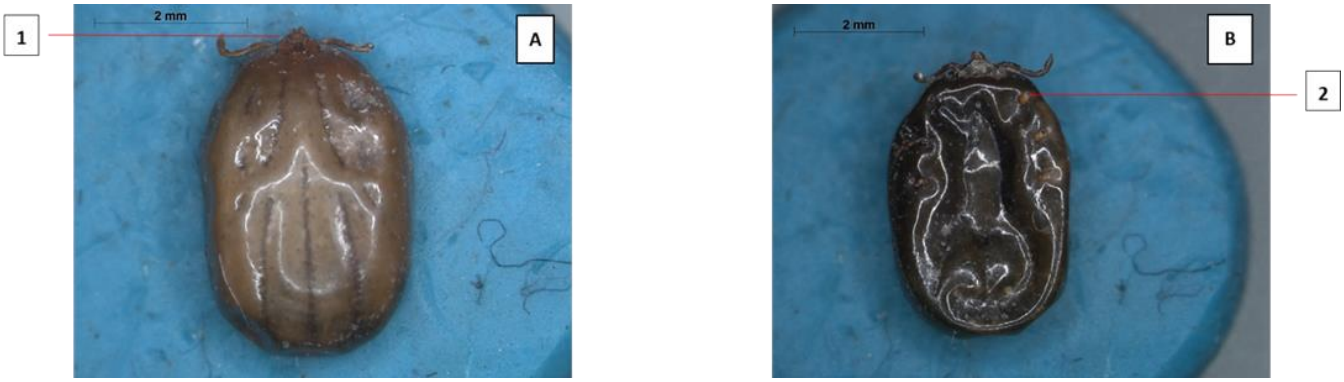

Plate 4.23: *Rhipicephalus* spp. cf. sp nr *pravus*, **A** Dorsal habitus; **(1)** Mouthparts; **B** Ventral habitus; **(2)** Legs.

***Haemaphysalis* species**

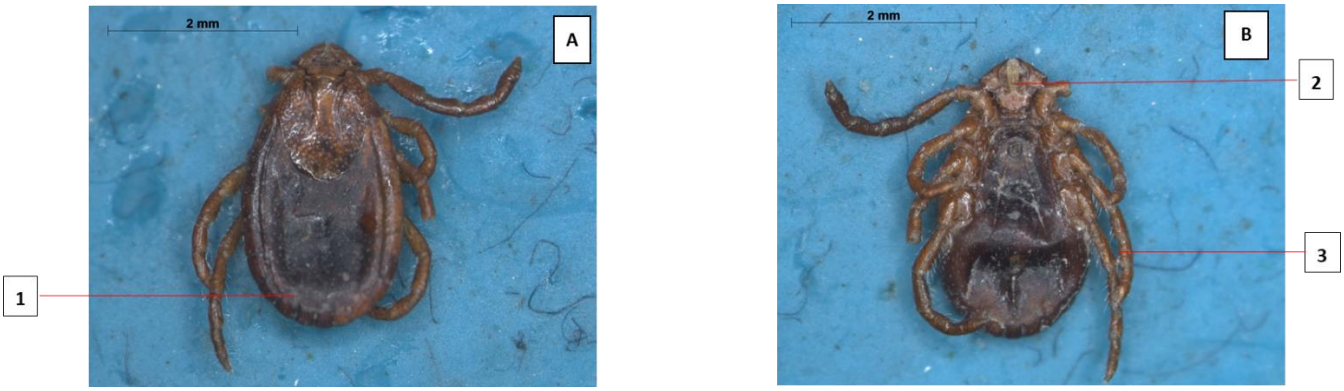

Plate 4.24: *H. elliptica* **; A** Dorsal habitus; **(1)** Festoons **(2)** Mouth parts; **B** Ventral habitus; **(2)** Legs.

***Rhipicentor* species:**

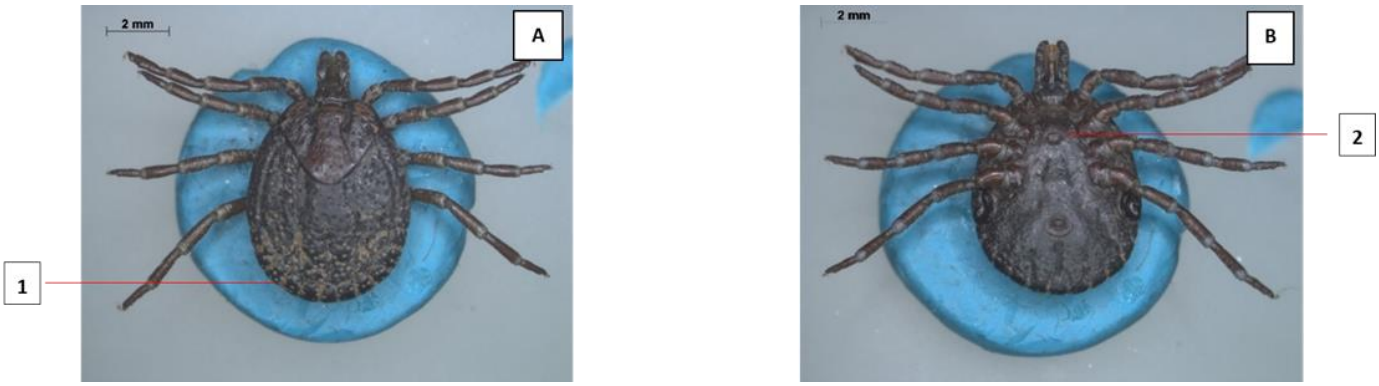

Plate 4.25: *R. nuttalli* (female) **A** Dorsal habitus; **(1)** Festoons; **B** Ventral habitus; **(2)** Legs.

***Otobius* species**

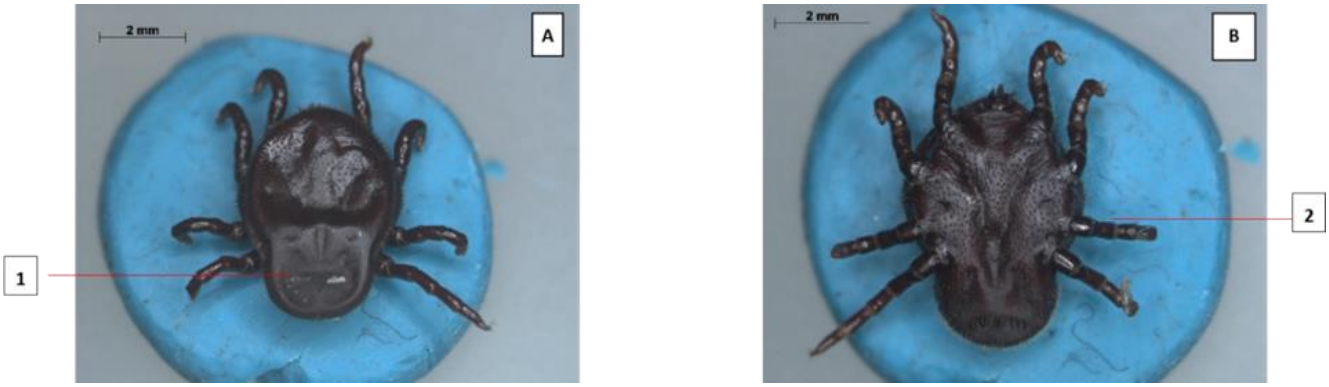

Plate 4.26: *O. megnini* nymph; **A** Dorsal habitus; **(1)** Mouthparts; **B** Ventral habitus; **(2)** Legs.

***Ixodes* species**

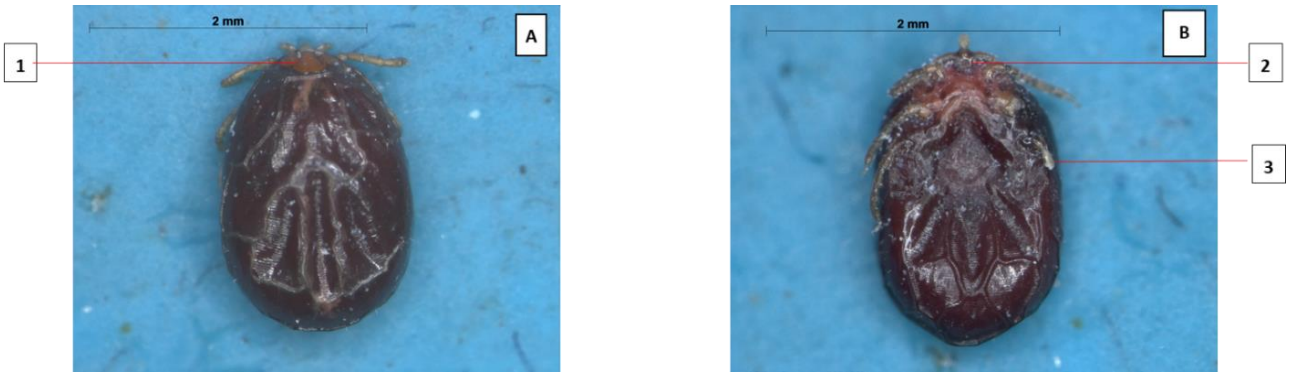

Plate 4.27: *Ixodes* spp. ; **A** Dorsal habitus; **(1)** Scutum; **B** Ventral habitus;**(2)** Mouth parts; **(3)** Legs.

4.1.1 HOST, AGE, AND GEOGRAPHICAL DISTRIBUTION

Amblyomma marmoreum (22.9%) was and collected from three hosts originating from Gauteng, Eastern Cape, and Limpopo provinces. This tick species was collected from leopard tortoise, brown snake eagle, and leopard, with most ticks originating from the leopard tortoise. Ticks collected from a brown snake eagle, African black-footed cat, cheetah as well as roan antelope from four provinces in South Africa(Gauteng, North West, Mpumalanga, and the Eastern Cape) were identified as *Amblyomma hebraeum* (10.4%). *Amblyomma nuttalli* (8.3%) and *A. (Aponomma) exornatum* (2%) were collected from a leopard tortoise and Bosc's monitor lizard that originated from the NZG, Gauteng.

Haemaphysalis elliptica (10.4%), was collected from cheetah, originating from Gauteng, North West, and Mpumalanga provinces. *Hyalomma truncatum* (12.5%) was collected from a feral Tankwa goat from the Northern Cape province and a leopard tortoise from Gauteng.

Rhipicephalus evertsi evertsi (6.3%) was collected from leopard and cheetah that originated from the Africat Foundation in Namibia, as well as from a roan antelope from the NZG. *Rhipicephalus simus* (4.2%) was the second most identified tick species in the genus *Rhipicephalus* and was collected from cheetah, feral Tankwa goat, and porcupine at the NZG, Rietvlei nature reserve in Gauteng as well as from the Hoedspruit Endangered species Centre in Mpumalanga. On the other hand, *Rhipicephalus follis* (6.3%) was collected from feral Tankwa goats from the Northern Cape and *R. spp cf. sp. nr pravus* (2%) and *R. decoloratus* (2%) were collected from a roan antelope from NZG in the Gauteng. Lastly, *R evertsi mimeticus* (2%) were collected from a leopard that came from Namibia.

Rhipicentor nuttalli (2%) was the only species from the genus *Rhipicentor* that was identified in this study. It was collected from a leopard originating from the Ann Van Dyk Cheetah Centre in North West Province. A single species, *Ixodes* (2%) collected from a banded mongoose from Gauteng was identified to genus level. One soft tick, *O. megnini* nymph (2%) was identified from a hog deer also from the Gauteng province.

When determining the age distribution, *Hyalomma* and *Rhipicephalus* samples collected from the Northern Cape were adults ticks collected from Feral Tankwa goats. The species was the most dominant (Table 4.1). From North West Province, adult ticks belonging to the genera *Haemaphysalis*, *Rhipicentor,* and *Amblyomma* were collected from cheetahs and a leopard from the Ann Van Dyk Cheetah Centre. In addition, *H. elliptica* larvae was also collected from a cheetah from the Ann Van Dyk Cheetah Centre.

The majority of ticks collected from various hosts in the Gauteng province were adults (n=13) belonging to the genus *Hyalomma, Amblyomma*, *Haemaphysalis, and*

*Rhipicephal*us (Table 4.1), while larvae (n=5) and nymphs (n=6) belonged to the genus *Ixodes* and *Otobius*, respectively (Table 4.1). *Amblyomma marmoreum* larvae and nymphs were collected from a brown snake eagle from Limpopo province (Table 4.1). Moreover, *A. marmoreum* nymphs, *A. hebraeum* larvae as well as fleas were collected from an African black-footed cat from the Eastern Cape. In Mpumalanga, two adult ticks belonging to *Amblyomma* and *Rhipicephalus* genera as well as a nymph of *Haemaphysalis* were collected from a cheetah.

Additionally, adult ticks collected from a leopard in Botswana was identified as *H. truncatum.* In Namibia, ticks were collected from both cheetah and leopard. The ticks identified on cheetah included *R. evertsi evertsi* and *H rufipes* while *R evertsi mimeticus* was collected from leopard.

Table 4.1: Geographical origin of hosts and tick species identified in the study

Hosts			Ticks		
Species	Locality	Province/Country	Sample ID	Life cycle stage	Morphology (Species)
Banded Mongoose *(Mungos mungo)*	National Zoological Garden	Gauteng	TIC 2	larvae	*Ixodes* spp.
White Rhino (*Ceratotherium simum)*	National Zoological Garden	Gauteng	TIC 3 (364)	adults (female and male)	*Hyalomma truncatum*
Leopard Tortoise *(Stigmochelys pardalis)*	National Zoological Garden	Gauteng	TIC 4	female	*Amblyomma marmoreum*
Hog Deer *(Axis porcinus)*	National Zoological Garden	Gauteng	TIC 7	nymph	*Otobius megnini*
Cheetah (*Acinonyx jubatus)*	National Zoological Garden	Gauteng	TIC 8	adults (female and male)	*Haemaphysalis elliptica*
Porcupine (*Erethizon dorsatum)*	National Zoological Garden	Gauteng	TIC 12	female	*Rhipicephalus simus*
Bosc's monitor lizard (*Varanus exanthematicus*)	National Zoological Garden	Gauteng	TIC 13	adults (males and females)	*Amblyomma (Aponomma) exornatum*
			TIC 14 (a)	adults (males and females)	*Haemaphysalis elliptica*
Cheetah (*Acinonyx jubatus)*	Rietvlei Nature Reserve	Gauteng	TIC 14 (b)	female	*Rhipicephalus simus*
Leopard Tortoise *(Stigmochelys pardalis)*	National Zoological Garden	Gauteng	TIC 16	male	*Amblyomma marmoreum*
			TIC 22 (a)	adults (males and females)	*Amblyomma marmoreum*
Leopard Tortoise *(Stigmochelys pardalis)*	National Zoological Garden	Gauteng	TIC 22 (b)	nymph	*Amblyomma nuttalli*
			TIC 23 (a)	larvae	*Amblyomma marmoreum*
			TIC 23 (b)	adults (male and female)	*Amblyomma marmoreum*
			TIC 23 (c)	nymph	*Amblyomma nuttalli*
Leopard Tortoise *(Stigmochelys pardalis)*	National Zoological Garden	Gauteng	TIC 23 (d)	nymph	*Amblyomma marmoreum*
			TIC 25 (a)	adults (males and females)	*Amblyomma hebraeum*
			TIC 25 (b)	nymph	*Rhipicephalus* spp *cf.* sp nr *pravus*
			TIC 25 (c)	larvae	*Rhipicephalus evertsi evertsi*
Roan Antelope (*Hippotragus equinus)*	National Zoological Garden	Gauteng	TIC 25 (d)	male	*Rhipicephalus (Boophilus) decoloratus*
			TIC 26 (a)	adults (males and females)	*Amblyomma nuttalli*
			TIC 26 (b)	males	*Amblyomma marmoreum*
			TIC 26 ©	nymph	*Amblyomma marmoreum*
Leopard Tortoise *(Stigmochelys pardalis)*	National Zoological Garden	Gauteng	TIC 26 (d)	adults (male and female)	*Amblyomma marmoreum*
Leopard Tortoise *(Stigmochelys pardalis)*	National Zoological Garden	Gauteng	TIC 27	larvae	*Amblyomma marmoreum*
Vervet Monkey (*Chlorocebus pygerythrus)*	National Zoological Garden	Gauteng	TIC 28	larvae	*Amblyomma hebraeum*
			TIC 5 (a)	adult	*Hyalomma truncatum*
Tankwa feral Goat (*Capra hircus*)	Carnavorn	Nothern Cape	TIC 5 (b)	male	*Rhipicephalus follis*
			TIC 17 (a)	adults (male and female)	*Hyalomma truncatum*
			TIC 17 (b)	adults (male and female)	*Rhipicephalus follis*
Tankwa feral Goat (*Capra hircus*)	Carnavorn	Northen Cape	TIC 17 (c)	adults (male and female)	*Rhipicephalus simus*
Tankwa feral Goat (*Capra hircus*)	Carnavorn	Nothern Cape	TIC 11	adults (males)	*Hyalomma truncatum*
Tankwa feral Goat (*Capra hircus*)	Carnavorn	Northen Cape	TIC 19	male	*Hyalomma truncatum*
Leopard (*Panthera pardus)*	Ann Van Dyk Cheetah Centre	Northwest	TIC 9	female	*Rhipicentor nuttalli*
Cheetah (*Acinonyx jubatus)*	Ann Van Dyk Cheetah Centre	North West	TIC 20	adults (males and females)	*Amblyomma hebraeum*
Cheetah (*Acinonyx jubatus)*	Ann Van Dyk Cheetah Centre	North West	TIC 24	larvae	*Haemaphysalis elliptica*
			TIC 21 (a)	adults (males and females)	*Amblyomma hebraeum*
			TIC 21 (b)	nymph	*Haemaphysalis elliptica*
Cheetah (*Acinonyx jubatus)*	Hoedspruit Enderngered species Centre	Mpumalanga	TIC 21 (c)	adults (males and females)	*Rhipicephalus simus*
Cheetah (*Acinonyx jubatus)*	Africat	Namibia	TIC 6	male	*Hyalomma rufipes*
			TIC 18 (a)	adults (male and female)	*Rhipicephalus evertsi evertsi*
Leopard (*Panthera pardus)*	Africat	Namibia	TIC 18 (b) (466)	adults (male and female)	*Rhipicephalus evertsi mimeticus*
Cheetah (*Acinonyx jubatus)*	Africat	Namibia	TIC 10	female	*Rhipicephalus evertsi evertsi*
Leopard (*Panthera pardus)*	Khutse game reserve	Botswana	TIC 15	male	*Hyalomma truncatum*

4.3 IDENTIFICATION OF TICKS USING DNA BARCODING

4.3.1 PCR AMPLIFICATION, SEQUENCING, AND PHYLOGENETIC ANALYSES OF THE *COI* AND *16S* RRNA GENES

Of the 49 ticks that were morphologically identified, seven were submitted to the biobank as reference samples*: Rhipicentor nuttalli from a leopard and feral goat, Haemaphysalis elliptica from a cheetah, Hyalomma truncatum from a leopard,* as well as *Amblyomma marmoreum* from a leopard tortoise and African black-footed cat as they were represented by only one tick specie. The remaining 42 samples were subjected to amplification using the optimized cycling conditions for both the *COI* and *16S* rRNA genes, only 16 and 18 samples yielded amplicons of the expected sizes 645 bp and 454 bp respectively (Fig. 4.1 and 4.2). A total of 21 samples either did not amplify or they showed faint bands and therefore excluded from further analysis.

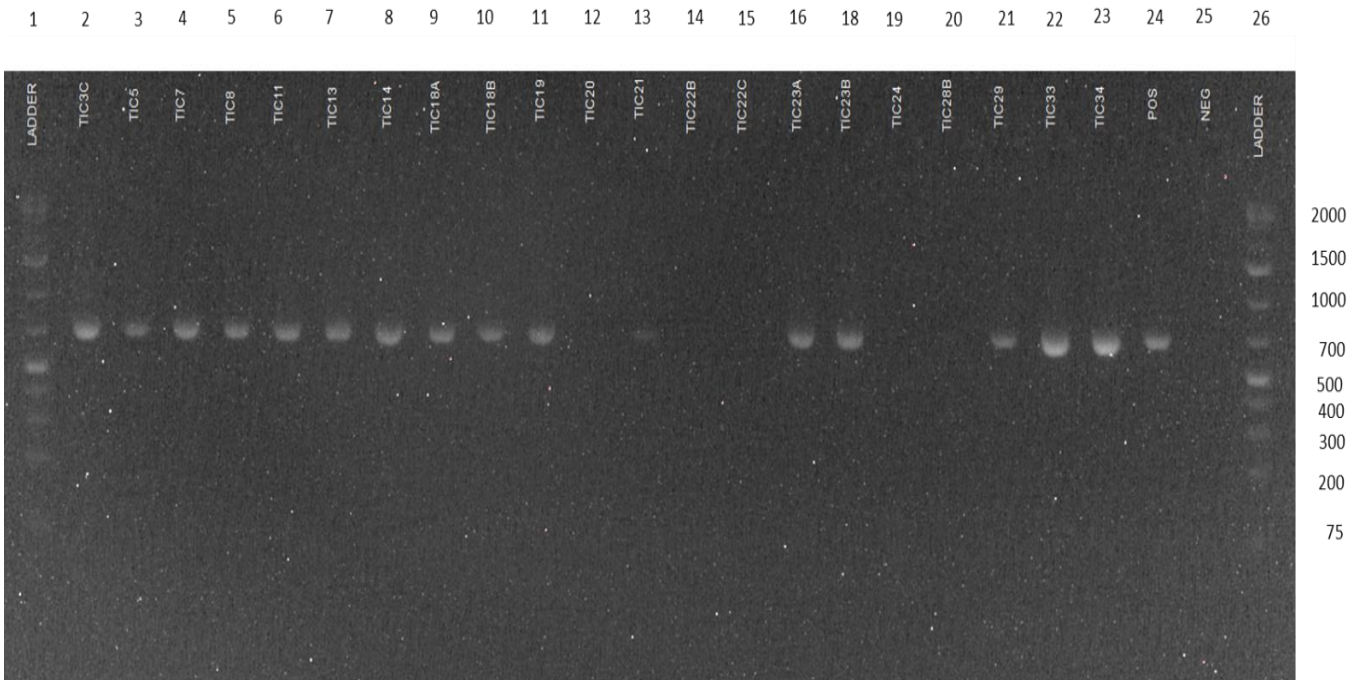

Figure 4.1: Agarose gel electrophoresis of the *COI* gene with the expected product size of 675 bp. Lane 1 and 26: (1 Kb plus molecular ladder). Lanes 2- 23: tick DNA samples, Lane 24: positive control, Lane 25: negative control.

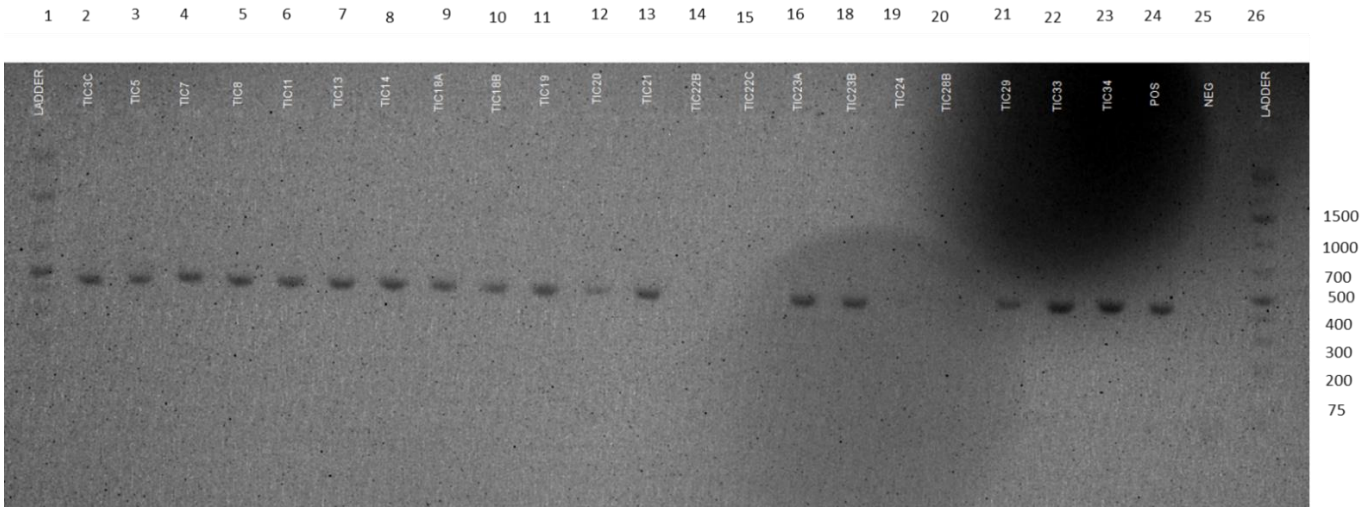

Figure 4.2: Agarose gel electrophoresis of the *16S* rRNA gene with the expected product size of 454 bp. Lane 1: and 26 (1 Kb plus Ladder). Lane 2-23 tested DNA tick samples, Lane 24: positive control, lane 25: negative control.

Good quality sequences were obtained from 15 tick samples for the *16S* rRNA gene and nine sequences were obtained for the *COI* gene (Table 4.2). The forward and reverse reads were assembled, edited, and aligned with similar sequences from GenBank. The final alignments comprised of 36 and 20 sequences, respectively, for the *16S* rRNA and *COI* genes. Blastn homology search indicated that the new sequences from ticks collected from wild animals were 95 -100% identical to published *COI* and *16S* rRNA gene sequences of tick species from various hosts or vegetation in South Africa or elsewhere (Table 4.2; Appendix E). The sequencing results were similar to the morphological results, with the exception of two samples (TIC 5 and TIC 19) which were morphologically identified as *Hyalomma truncatum,* however, sequencing results indicated that they are closely similar to published *Hyalomma glabrum* sequences (Table 4.2).

The following species were identified based on the analysis of *COI* sequences: (*Otobius megnini*, *Amblyomma (Aponomma) exornatum, A. marmoreum, Hyalomma glabrum*, *H.*

truncatum, *Haemaphysalis elliptica*). Only three sequences were identical to published *COI* sequences of *Haemaphysalis elliptica* (TIC 8) and *A. marmoreum* (TIC 23A and TIC 23B). Three more species (*Amblyomma hebraeum, Rhipicephalus evertsi mimeticus,* and *R. simus/R. gertrudae*) were identified based on the *16S* rRNA gene sequences (Table 4.2). Sequence analysis indicated that TIC 18A, which was morphologically identified as *R. evertsi evertsi* was closely related to *R. evertsi mimeticus.* Evolutionary divergence between the new *COI* and 16S rRNA gene sequences and similar sequences from GenBank was estimated by determining the number of base-pair differences. Intraspecific differences ranged from 0 – 29 bp for *COI* (Table 4.3) and 0 – 19 bp for *16S* rRNA (Tables 4.4)

Table 4.2: Sequencing results of the *COI* and *16S* rRNA genes

Morphological results	**Sequencing results**				
	Sequence ID	***COI* gene**	**BLASTn**	***16S* rRNA**	**BLASTn**
			(% identity)		(% identity)
Hyalomma truncatum	TIC 5	*Hyalomma glabrum* KU130596	99.10%	*Hyalomma glabrum* KU130432	100%
Otobius megnini	TIC 7	*Otobius megnini* MG582606	100%	*Otobius megnini* L34325	95.87%
Haemaphysalis elliptica	TIC 8	*Haemaphysalis elliptica* MZ351133	100%	*Haemaphysalis elliptica* HM068961	98.99%
Hyalomma truncatum	TIC 11	*Hyalomma truncatum* OK481109	99.64%	*Hyalomma truncatum* KU130478	98.99%
Amblyomma (Aponomma) exornatum	TIC 13	*Amblyomma (Aponomma) exornatum* MN150167	95.12%	*Amblyomma (Aponomma) exornatum* MN 150173	96.17%
Haemaphysalis elliptica	TIC 14	*Haemaphysalis elliptica* MZ351133	97.65%	*Haemaphysalis elliptica* HM068961	100%
Rhipicephalus evertsi evertsi	TIC 18A			*Rhipicephalus evertsi mimeticus* OK481095	99.75%
Rhipicephalus evertsi mimeticus	TIC 18B			*Rhipicephalus evertsi mimeticus* OK481095	100%
Hyalomma truncatum	TIC 19	*Hyalomma glabrum* KU130596	99.82%	*Hyalomma glabrum* KU130432	99.75%
Amblyomma hebraeum	TIC 20			*Amblyomma hebraeum* L34316	100.00%
Rhipicephalus simus	TIC 21			*Rhipicephalus gertrudae* MW080139	99.73%
				Rhipicephalus simus LC634554	99.46%
				Rhipicephalus sp. KT382478	99.00%
Amblyomma marmoreum	TIC 23A	*Amblyomma marmoreum KY457515*	100%	*Amblyomma marmoreum* MW290508	97.71%
Amblyomma marmoreum	TIC 23B	*Amblyomma marmoreum KY457515*	100%	*Amblyomma marmoreum* MW290508	97.71%
Amblyomma marmoreum	TIC 23D			*Amblyomma marmoreum* MW290508	97.71%
Amblyomma hebraeum	TIC 28			*Amblyomma hebraeum* L34316	100%

* No *CO1* sequence

The observed relationships between the new *COI* and *16S* rRNA gene sequences of ticks from South African wildlife and published sequences were confirmed by Neighbor-Joining (NJ) and Maximum Likelihood (ML) analyses. Representative trees are indicated in Figures 4.3 and 4.4. Five and seven distinct clades were obtained from the *COI* and *16S* trees respectively. Clade A is composed of two sub-clades, namely, *Hyalomma glabrum* and *Hyalomma truncatum* groups. Clade B is the *Haemaphysalis elliptica* group, clade C is the *Amblyomma marmoreum* group, clade D is the *A. exornatum* group and clade E is composed of the *Otobius megnini* sequences. Two additional clades, F (*Rhipicephalus simus* and *R. evertsi mimeticus*) and (*Amblyomma hebraeum*) are indicated in the *16S* rRNA gene tree (Fig. 4.4). The topologies of the NJ and ML trees were similar.

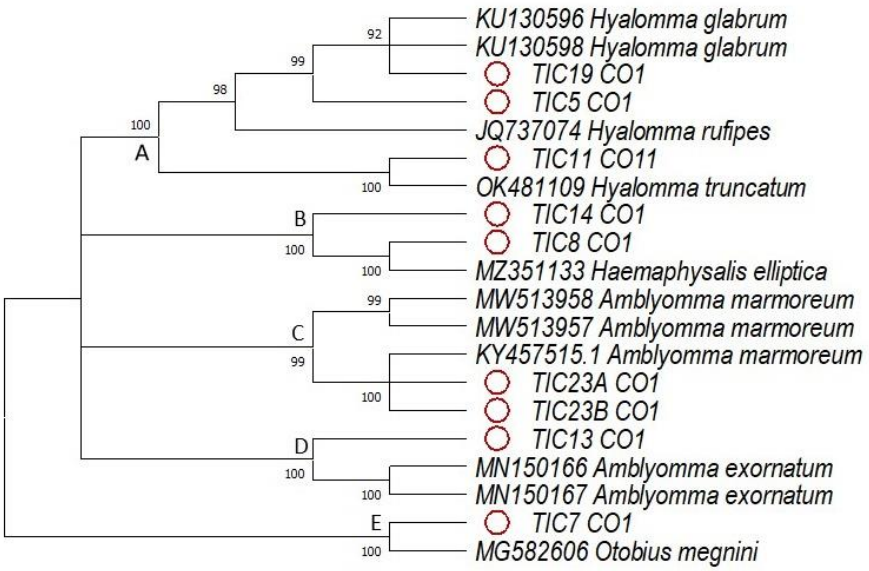

Figure 4.3: Phylogenetic tree of *COI* gene inferred using Maximum Likelihood method (ML) based on the Tamura-Nei model (Tamura and Nei, 1993) The analysis involved 20 nucleotide sequences. The percentage of replicate trees in which the associated

taxa clustered together in the bootstrap test (1000 replicates) (Felsenstein, 1985) are shown next to the branches.

All positions containing gaps and missing data were eliminated. There was a total of 553 positions in the final dataset. Evolutionary analyses were conducted in MEGAX (Kumar *et al*., 2018) . Clades (A – E) are indicated at the nodes.

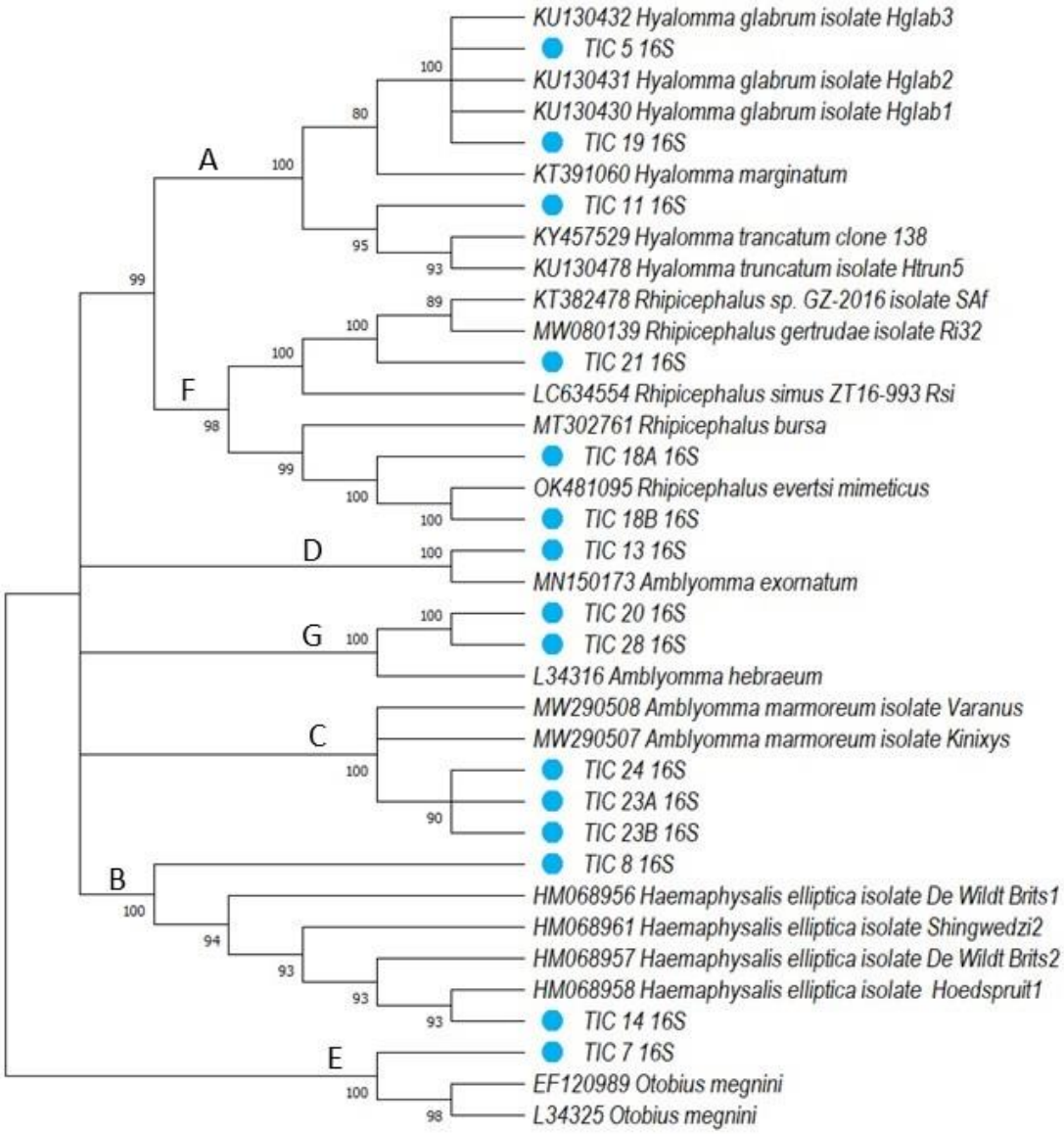

Figure 4.4: Phylogenetic tree of *16S* rRNA gene inferred using Neighbor-Joining (NJ) method based on the Tamura-Nei model (Tamura and Nei, 1993). The percentage of

trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 36 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 553 positions in the final dataset. Evolutionary analyses were conducted in MEGAX (Kumar *et al*., 2016). Clades (A – G) are indicated at the nodes.

CHAPTER 5: DISCUSSION

Several surveys have previously been carried out on ticks of captive and free-living species of wild animals in South Africa (Fourie *et al.*, 2002; Horak *et al.*, 2006). The current study identified tick specimens of various life stages (larvae, nymph, adult males, and females) collected opportunistically from carcasses of captive animals that were submitted for necropsy at the SANBI-NZG and stored in the biobank.

The ticks were identified morphologically and confirmed by PCR amplification targeting two mitochondrial markers, *COI* and *16S* rRNA. The majority of the adult specimens were identified morphologically however, immature life stages of some of the specimens hindered accurate morphological identification; hence, the use of molecular analysis to assist with resolving the identification of those immature life stage specimens. A study by (Couper and Swei, 2018), has indicated that morphologically similar taxa, damaged specimen, and immature tick stages can impair morphological identification.

Furthermore, sequence and phylogenetic analyses, was carried out to confirm the results of the morphological identification. Phylogenetic analysis of the sequences generated in this study showed that they clustered with related species from the NCBI database (Available from: https://www.ncbi.nlm.nih.gov) with high bootstrap support values ranging from 95 – 100%. These findings are in support with observations made by (Silatsa *et al.*, 2019) on ticks from Cote d'Ivoire, (Muhanguzi *et al.*, 2020) in Uganda, and (Alghamdi *et al.*, 2021) in Saudi Arabia. The current molecular approach (DNA barcoding) has become a significant trend in taxonomic and phylogenetic studies of tick species to resolve the difficulty of identification problems especially of tick related species or cryptic species complexes.

The targeted markers (*COI* and 16S rRNA) have been used in other studies (Zhang and Zhang, 2014; Abdullah *et al.*, 2018; Roth *et al.*, 2019) and have provided more knowledge of the taxonomy and systematics of ticks. Furthermore, a previous study conducted by (Lv *et al.*, 2014) demonstrated that the *16S* rRNA and *COI* genes are suitable markers to be used for tick identification as compared to 12S and ITS2 gene markers.

The current study indicated that the samples identified belong to the genera *Amblyomma*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Otobius*, *Rhipicentor,* and *Rhipicephalus*. (Horak *et al.*, 2018a) have also indicated that some of the tick species from the genera *Amblyomma*, *Rhipicephalus*, *Ixodes,* and *Hyalomma* are indigenous to South Africa and affect both the domestic and wild animals. The authors further reported that *A. hebraeum*, *I. rubicundus*, *R. appendiculatus,* and *R.* (*Boophilus*) *decoloratus* are the most common species in most provinces of the country.

5.1 IDENTIFIED TICK SPECIES

Amblyomma spp.

Species from this genus were the most commonly identified. Similarly, a previous study investigating ticks collected from goats in Mpumalanga also indicated that the species from the genus *Amblyomma* as the most identified in goats (Jongejan *et al.*, 2020). Furthermore, *Amblyomma* spp. displayed three distinct lineages from the *16S* rRNA tree topology; thus, supporting polyphyly in this genus . These results are supported by a study looking at phylogenies from mitochondrial genomes of 120 species of ticks that was conducted by (Kelava *et al.*, 2021). The findings showed that *Amblyomma* spp. are polyphyletic, and monophyly can only be supported within the genus when *Amblyomma transversale* and *A. fimbriantum* are excluded from the analysis. The above-mentioned authors also emphasized that it is however essential

to note that phylogenetic trees from a single gene may not be representative of the evolution of *Amblyomma* species (Kelava *et al.*, 2021). Additional markers are therefore required to confirm the evolution of *Amblyomma* species.

Amblyomma (Aponomma) exornatum is commonly known as the tick of varanid lizards (Horak *et al.*, 2006). In Africa, this tick species may be found in savannah, woodland savannah, and rainforest regions (Elbl and Anastos, 1966). The species has been reported from collections from mammalian and avian hosts (Elbl and Anastos, 1966). In addition, (Horak *et al.*, 2006; Hornok *et al.*, 2020; Mofokeng, 2021) also indicated that the species prefers monitor lizard. In the current study, it was collected from a Boc's monitor lizard from the Gauteng province, which reflects what has been reported in previous studies (Horak *et al.*, 2018b). Moreover, (Hornok *et al.*, 2020) has shown that *A. exornatum* groups separately from the other *Amblyomma* species. The findings in my study are also similar to what has been described in previous studies, as the species has grouped separately on the *16S* tree.

In southern Africa, *A. hebraeum,* also known as the South African Bont tick, is predominantly found along the coasts of the Eastern Cape and KwaZulu-Natal provinces; however, it's distribution extends inland through, Mpumalanga, Limpopo, North West provinces as well as in Swaziland and southern Mozambique (Horak *et al*., 2009) According to (Spickett, 2013) this tick species is native to southern Africa and is sexually dimorphic. The nymph and adult stages of this tick species are vectors of *Ehrlichia ruminantium,* which causes heartwater disease and affects various species of domesticated ruminants, while wild ruminants remain susceptible to the disease (Allsopp *et al*., 1999). In this study, *A. hebraeum* was identified in hosts from four provinces; namely, Mpumalanga, Gauteng, Eastern Cape, and North West, which are known to be warm and receive acceptable rainfall (Theiler and Salisbury, 1959).

This finding supports those of (Uilenberg, 2018), which indicated that this species prefers warm and moist regions. In South Africa, it commonly infests cattle, sheep, and goats, as well as a variety of wildlife species (Horak *et al.*, 2018b, 2018a; Jongejan *et al.*, 2020). In the current study, it was collected from cheetah, vervet monkey, an African black-footed cat, and roan antelope. Sequence analysis confirmed that *A. hebraeum* is similar or closely related to the *16S* rRNA gene sequences of *A hebraeum* (L34316) deposited on GenBank.

Amblyomma marmoreum was the most common tick species identified in this study, and was identified from host in three provinces. It is commonly referred to as the South African tortoise tick or the tortoise tick as leopard tortoise is known as its preferred host. (Horak *et al.*, 2006) has shown that this tick is widely distributed in South Africa as well as other countries such as Namibia, Swaziland, Botswana, and Mozambique (Theiler and Salisbury, 1959). The larvae and nymph stages of this tick species are known to parasitize reptiles, birds, and mammals whilst, adults are found largely on tortoises and snakes (Norval, 1975). In the current study, adult ticks were found on the Leopard tortoise while nymphs were collected from a Brown snake eagle and an African black-footed cat. Additionally, it has been previously isolated from humans, as previously reported by Horak *et al*. (2000). This tick species transmits pathogens such as *Ehrlichia ruminantium* to susceptible hosts. Sequence analysis confirmed that *A. marmoreum* obtained from this study is similar (97.71%) or closely related to published sequences deposited in GenBank.

Amblyomma nuttalli also known as the Pan-African tortoise tick, is often difficult to distinguish from other species of the *A. marmoreum* group (Horak *et al.*, 2006). Collections of this species have been made in South Africa and Maputo province, Mozambique, mostly from savanna and forest biomes. The immature stages of this

tick species have been reported on reptiles, birds, and sometimes mammals, while adult ticks have been reported from reptiles such as the Bell's hinged tortoise, monitor lizards as well as snakes (Horak *et al.*, 2006). In a recent study Nardi *et al.* (2021) reported on the occurrence of a *Coxiella* endosymbiont in *A. nuttalli*. However, this tick bacterial species was reported to habour *Coxiella endosymbiont which* are related but not identical to *Coxiella burnetii*, the causative agent of Q fever, Sequencing of the ticks could not be done as there were very few samples available, and these were stored as reference samples at the SANBI-Biobank.

***Haemaphysalis* spp.**

Species from this genus were shown to be monophyletic; thus, in agreement with the findings reported by (Kelava *et al.*, 2021). *Haemaphysalis elliptica* commonly called the southern Africa yellow dog tick and was the only species from this genus identified in the current study. It is widespread in southern Africa, in provinces such as Kwa-Zulu Natal, Eastern Cape, Gauteng, Northwest, Mpumalanga, Limpopo, and Free state and feeds on various wild and domestic carnivores including domestic dogs and cats as well as lions (*Panthera leo*), leopards (*Panthera pardus*) and cheetahs (*Acinonyx jubatus*) (Horak *et al*., 2010; Horak *et al.*, 2018b). Apanaskevich *et al*. (2007) indicated *H. elliptica* has been incorrectly identified as *H. leachi* in most studies in South Africa, and the latter only occurs in the Northern regions of Africa. On the other hand, murid rodent species are the preferred hosts of its immature stages (Hoogstraal, 1956; Penzhorn *et al*., 2020). In the current study, *H. elliptica* was collected from a cheetah which is in agreement with previous reports (Horak *et al*., 2010), which reported *H. elliptica* as the most dominant species infesting domestic cats and wild felids in South Africa. The total collection from this study had more male ticks collected than female ticks. The high number of male ticks recorded

in this study compared with the females is unsurprising since the females are known to detach from their hosts a few days after feeding to oviposit while the males stay longer before dropping (Hoogstraal, 1956).This species is known to transmit *Babesia rossi* which causes canine babesiosis in domestic dogs (Penzhorn *et al*., 2020). Sequence analyses revealed that *H. elliptica* is comparable to or closely related to the *COI* and *16S* rRNA gene sequences submitted in GenBank

***Hyalomma* spp.**

Members of this genus are reported to parasitize a wide range of hosts such as large domestic and wild herbivores, which are preferred by adult ticks while the larva and nymphs feeds on rodents, gerbils, scrub hares and ground frequenting birds (Walker, 2003). Previous studies (Murrell *et al*., 2000) have indicated that *Hyalomma spp* are monophyletic and the phylogenetic results from our study confirms the same findings. *Hyalomma rufipes* also known as the coarse Bont-legged tick is a- two-host tick species and commonly distributed tick of this genus in Africa (Hornok and Horváth, 2012). South Africa, Botswana, Zimbabwe, Northern Namibia, Kenya, Ethiopia, and Somalia, as well as the southern regions of West Africa, are all home to this tick species (Apanaskevich and Horak, 2006a, 2006b, 2008). In the current study the tick species was identified from a cheetah that came from Namibia, which supports the report by Hornok and Horváth, (2012), which indicated that both the larval and nymphal stages of this species prefer small mammals and birds as host, while adults are mainly found on cattle, sheep, goats, and wild ungulates as well as horses (Chitimia-Dobler *et al.*, 2019).

Hyalomma rufipes is a well-known vector of Crimean Congo hemorrhagic fever (CCHF) virus in humans (Gargili *et al*., 2017) as well as and other viruses such as *Rickettsia aeschlimannii*, *Anaplasma marginale* and *Babesia occultans* (Papa *et al.*,

2015; Chitimia-Dobler *et al.*, 2019). In humans, CCHF virus is transmitted either through a tick bite or contact with infected animal blood or tissues and has a fatality rate of up-to 40%. No sequence data was obtained from this tick species.

Hyalomma truncatum is generally known as the shiny *Hyalomma* or small smooth Bont-legged tick. This species is said to be heat and drought tolerant and is generally found in regions with these climatic conditions allow. In the current study *H. truncatum* was collected from feral Tankwa goat from the Northern Cape province, which is known for its high temperatures and dryness, thus confirms what has been previously stated in literature. It also transmits diseases of human and animals, including the CCHF virus and *Rickettsia conorri* (*R. conori)*, as well as anaplasmosis, babesiosis, and sweating sickness in livestock (Hoogstraal, 1956). Depending on the host species involved, this tick species has both two- and three-host life cycles. It prefers domestic and wild herbivores whilst, hares and rodents are preferred for immature stages (Magano *et al*., 2000). Adults may be spotted in huge numbers after the rains in late summer, and juvenile stages can be found in the dry fall and spring months (Magano *et al*., 2000). Although ticks TIC5 and TIC19 were morphologically identified as *H. truncatum*, sequence analysis indicate that these are identical or closely similar to *Hyalomma glabrum* sequences identified from different livestock and wildlife species in South Africa (Sands *et al.*, 2017).

Previously, *H. glabrum* was referred to as *H. marginatum turanicum* but has been redescribed as a valid species by (Apanaskevich and Horak, 2006b). It's distribution is restricted to Southern regions of Africa, predominantly South Africa, extending to Namibia, Botswana, and Swaziland (Apanaskevich and Horak, 2006a, 2008).

***Ixodes* spp.**

Ixodes spp., the largest genus in the Ixodidae family, includes over 245 species and is highly sophisticated morphologically and physiologically. It is found all over the world and is known to infect various species, including mammals, and birds, and is sometimes found on reptiles (Ash *et al.*, 2017). It was collected from a banded mongoose in Gauteng from the current investigation. Due to the small number of samples in the biobank collection, this tick was not sequenced.

***Otobius* spp.**

Otobius megnini, also known as the spinose ear tick, was the only member of this genus identified in this study. It is a soft-bodied, single-host tick that is only parasitic in the larval and nymphal stages (Walker, 2003). This tick species is primarily found in arid areas of North and South America, India, and southern Africa. The tick spreads easily because of its ability to infest and hide for a long time in the host's ears; then carried along during translocation of animals (Walker, 2003). Adult ticks aren't parasitic, but their larvae and nymphs infest animals' external ear canals. In dogs, these ticks are infrequent, and in cats, they are relatively rare (Miller, 2020). It is known to induce paralysis in horses (Miller, 2020). There are currently no known pathogens transmitted by the tick species (Barker and Walker, 2014), though reports of the presence of infectious agents such as *Coxiella burnetii*, the agent of Q fever, spotted fever rickettsia, *Ehrlichia canis*, *Borrelia burgdorferi*, and *Babesia* have been reported to be present in *O. megnini*, though its role in transmitting these pathogens has not been confirmed. Because it is easily transported by hosts, occurrence of 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. Infected animals

exhibit intermittent painful non-exertional muscle contraction and mild to moderate rhabdomyolysis (Miller, 2020).

For both markers, sequence analysis showed that *O. megnini* (TIC 7) collected from a hog deer from the NZG in Gauteng is identical (16S rRNA gene) or closely similar (*COI* gene) to *O. megnini* that were collected from cattle from Iran and Argentina (Black and Piesman, 1994; Nava *et al*., 2009).

***Rhipicentor* spp.**

The genus *Rhipicentor* comprises of only two species, *R. bicornis* and *R. nuttalli* both of which are confined to the African continent (Fourie *et al.*, 2002). In South Africa, *R. nuttalli* has been recorded in the Eastern and Western Cape, Free State, Gauteng, Limpopo, and North-West provinces (Fourie *et al.*, 2002). A study conducted by (Fourie *et al.*, 2002) reported that this tick species has been found to cause paralysis in dogs. However, in the current study, this tick species was reported from a cheetah. Samples of this tick species did not amplify thus no sequence data was obtained.

***Rhipicephalus* spp.**

Previous studies (Dreyer *et al*., 1998; Nyangiwe *et al.*, 2017) conducted in other provinces of South Africa have reported that *Rhipicephalus* species is the most dominant tick species infesting cattle in central-western regions of South Africa, excluding the western Free State, the Karoo, and the Northern Cape Province. Members of this genus were not included in the construction of the *COI* tree as a result of poor sequences generated. Despite the lack of some species in the *COI* tree topology, there is substantial support of monophyly of ticks in the *16S* rRNA tree topology. These findings are in accordance to those of (Kelava *et al.*, 2021), where they assessed the phylogeny of 120 tick species using mitochondrial genome sequences.

On the contrary, (Murrell *et al*., 2000) reported that the genus *Rhipicephalus* is paraphyletic and can only be monophyletic if members of the genus *Boophilus* are incorporated into the phylogenetic analysis. In the current study, *Boophilus* spp. were not included in both tree topologies. *Rhipicephalus* spp. from the *16S* rRNA tree topology is polyphyletic.

Rhipicephalus decoloratus is endemic to the eastern grasslands of the Free State Province and widespread across South Africa with the exceptions of the western Free State, the Karoo, and the Northern Cape Province (Walker, 2000). This tick is a one-host species and transmits *Babesia bigemina*, the causative organism of babesiosis in cattle while infected wild animals act as reservoir hosts (Booysen *et al.*, 2017). It was collected from a roan antelope, this finding indicates its host preference for large ungulates (Mason and Norval, 1980). Due to the poor sequences generated, no sequence analysis was done.

Rhipicephalus evertsi evertsi, also known as the red-legged tick is the most common tick species throughout sub-Saharan Africa (Walker, 2000). All life stages of this tick species feed on large animals such as horses, cattle, zebras and eland. However, it has been reported that domestic equids and wild zebras appear to be the preferred hosts (Hoogstraal, 1956). This species has also been collected from other animals such as sheep, goats, impala (*Aepyceros melampus*), African buffalo (*Syncerus scaffer*), blue wildebeest (*Connochaetes taurinus*), and greater kudu (*Tragelaphus strepsiceros*) (Spickett *et al.*, 1992). *Rhipicephalus evertsi evertsi* has been implicated in the transmission of protozoan parasites causing equine piroplasmosis (*Babesia caballi* and *Theileria equi*), anaplasmosis, CCHF virus as well as *Ehrlichia ruminantium* (Hoogstraal, 1956). In the current study *R. evertsi evertsi* (TIC 18A) was collected from

a leopard originating from Namibia, its sequence was closed related to a sequence *R. evertsi mimeticus* (99.75%) that originates from cattle in Angola (Palomar *et al.*, 2021).

Rhipicephalus evertsi mimeticus is said to have minimal economic significance (Spickett *et al.*, 2011); however, it has been attributed to the spread of CCHF fever virus but it is less pathogenic compared to *R. evertsi evertsi* which causes significant paralysis to infected host species (Gothe *et al*., 1986). This species is indigenous to Namibia and Angola and was possibly introduced into South Africa through translocations of animals (Nyangiwe *et al.*, 2017). In the current study, this species was collected from a leopard (*Panthera pardus*) that originated from Namibia and its presence is thus not surprising. Sequence data shows that the species is identical to the reference sequence (OK481095) with 100% identity to *R. evertsi* mimeticus from cattle in Angola (Palomar *et al.*, 2021).

Rhipicephalus follis was collected from a feral Tankwa goat (*Capra hircus*) from the Northern Cape Province. This tick species was reported to occur in the mountainous or higher altitude regions mainly in the eastern half of South Africa (Olwoch *et al.*, 2007). No sequence was obtained for this tick species.

Rhipicephalus simus, commonly referred to as 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). The larvae and nymphs however prefer murid rodents as hosts (Walker, 2003). This species has been reported to be a vector of anaplasmosis and bovine babesiosis in cattle (Olwoch *et al.*, 2007; Booysen *et al.*, 2017). In the current study,

R. simus tick samples were collected from cheetah (*Acinonyx jubatus*), feral Tankwa goat (*Capra hircus*) as well as porcupine (*Erethizon dorsatum*).

In terms of host preference, the findings of the current study are in agreement with what has been reported on preferred host species (Walker, 2000). Kelly, (2001) reported that this tick species is a vector of *R. conorii* which causes Mediterranean spotted fever in humans. Although *R. simus* was identified morphologically, sequence analysis showed that it is similar to three *16S* rRNA gene sequences of *R. gertrudae* MW080139; *R. simus* LC634554- 99% and *Rhipicephalus sp*. KT382478 99%); deposited on Genbank, while *COI* did not amplify thus could not obtain any sequence data.

Rhipicephalus spp *cf*. sp *nr pravus* is a three-host tick. In Ethiopia, adult ticks are most abundant during the rainy season. In the current study, only nymphs were collected for this species, from a cheetah originating from Mpumalanga province. Its presence in this province may be because of the climatic conditions that are almost the same, warm, and high rainfall amounts. (Horak, De Vos and Brown, 1983) and (Flamand *et al*., 1995) reported that hares get infested by all stages of this species, whereas adults commonly infest cattle, sheep, and goats as well as wild ruminants. Its immature stages are normally found on elephant shrews. Bad sequences were obtained for this tick species.

CONCLUSION AND RECOMMENDATIONS FOR FURTHER STUDIES

The combination of morphology and DNA barcoding is practical for identification of arthropods. As compared to morphological identification alone, DNA barcoding is a robust technique that is developing increasingly throughout the world. Other than identification, this technique has a great impact on exploring biodiversity.

Thus, the current study contributes additional information on the ecology of ticks of wildlife in South Africa. Moreover, data gathered in this study will assist in updating the host and geographic distribution records provided by previous studies since these distributions depend on anthropogenic and environmental conditions and may change as new data becomes available, especially in the context of global climate warming and animal translocations. Further studies should focus on the identification of pathogens, particularly zoonoses, in the ticks identified in this study as continuous monitoring of ticks can help in predicting periods of heavy burden and possible disease outbreaks; thus, providing a chance for early intervention. An effort should also be made in amplifying genes where amplicons could not be obtained due to low DNA concentration or possible mismatches in the *COI* and *16S* primers used in this study. Lastly, this is, to the best of my knowledge, the first study of ticks collected from carcasses of wildlife using data mined from the Wildlife Disease Database conducted in South Africa. It demonstrates that The Wildlife Disease database is a useful resource for research projects on wildlife diseases in southern Africa.

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APPENDIX A: SANBI RESC APPROVAL

SANBI
Biodiversity for Life
South African National Biodiversity Institute

SANBI/RES/P2020/12

01 July 2020

Nozipho Khumalo
Directorate: Foundational Research & Services
SANBI

OUTCOME OF SUBMITTED RESEARCH PROPOSAL

This letter serves to inform you that your submitted research proposal titled "Identification of ticks using morphology and DNA barcoding – a retrospective study" was **approved** by the SANBI NZG Animal Research Ethics and Scientific Committee (RESC).

The following provisos should be taken into consideration:

1. Inform the RESC of completion or termination (with reason) of your research at the SANBI NZG.
2. Submission of an annual progress report in November of each year. Failure to submit a progress report may result in approval to be withdrawn.
3. Submission of a written request for an extension or modification within the research project.
4. The SANBI should be acknowledged in all reports, scientific publications and conference contributions as follows:
   - The SANBI is acknowledged for providing samples/research platform.

The research proposal has been registered on the database as P2020/12. Please use this project number in all future correspondence.

Thank you for making use of the SANBI NZG as a research platform.

Yours sincerely

Prof Antoinette Kotze
Chairperson: SANBI NZG Animal Research Ethics & Scientific Committee

APPENDIX B: UNIVERSITY OF PRETORIA REC APPROVAL

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 4 June 2021

CONDITIONALLY APPROVAL

Ethics Reference No	**REC167-20**
Protocol Title	**Identification of ticks using morphological traits and DNA barcoding**
Principal Investigator	**Miss NL Khumalo**
Supervisors	**Prof MC Oosthuizen**

Dear Miss NL Khumalo,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC167-20) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

NOTE: Conditionally approved (pending obtaining other relevant approvals).

We wish you the best with your research.

Yours sincerely

PROF GT. FOSGATE
Acting Chairperson: Research Ethics Committee

100 YEARS

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Faculty of Veterinary Science
Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

APPENDIX C: DAFF SECTION 20

agriculture, land reform & rural development
Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X250, Pretoria 0001
Enquiries: Mr Herry Gololo · Tel: 012 319 7532 · Fax: +27 12 319 7470 E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/18/1599(HP)

Nozipho Khumalo

National Zoological Gardens

South African National Biodiversity Institute (SANBI)

232 Boom Street

Pretoria

0001

Email: n.khumalo@sanbi.org.za K.Labuschagne@sanbi.org.za

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear researchers

Your fax / memo / letter/ Email dated 31 July 2020, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

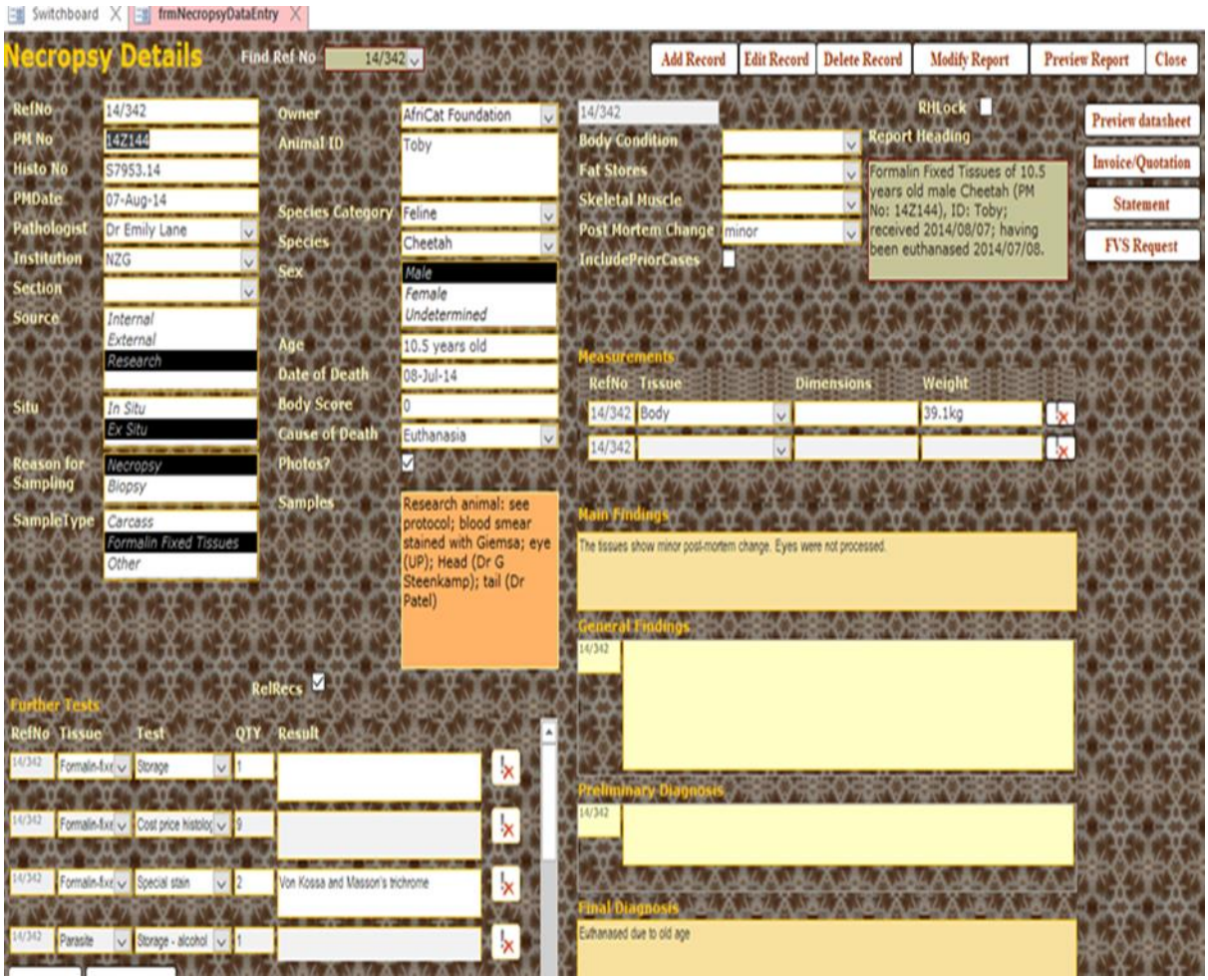
I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All bio-safety and biosecurity protocols as outlined in the application must be followed.
3. Only ticks from the SANBI – NZG, as per the supplied list may be used during the study.
4. Samples must be packaged and transported to the Gertrud Theiler Tick Museum, ARC-OVI and back in accordance with the National Road Traffic Act, 1996 (*Act* No. 93 of 1996);
5. This Section 20 approval is valid until 31 August 2021.

– 1 –

APPENDIX D: WILDLIFE DISEASE DATABASE



APPENDIX E: REFERENCE SEQUENCES USED FOR PHYLOGENETIC ANALYSES

Species	Genbank Accession Number	Host	Country	Reference
***COI* gene**				
Haemaphysalis elliptica	MZ351133	Vegetation	Eswatini	Ledger *et al*. 2021
Hyalomma truncatum	OK481109	Cattle	Angola	Palomar *et al*. 2021
Hyalomma glabrum	KU130597	Livestock and wildlife species	South Africa	Sands *et al*. 2017
Hyalomma glabrum	KU130596	Livestock and wildlife species	South Africa	Sands *et al*. 2017
	KU130598	Livestock and wildlife species	South Africa	
Hyalomma rufipes	JQ737074	*Not indicated	China	Gou *et al*. 2016
Amblyomma (Aponomma) exornatum	MN150166	Rock monitor	South Africa	Hornok *et al*. 2021
Amblyomma (Aponomma) exornatum	MN150167	Rock monitor	South Africa	
Amblyomma marmoreum	MW513958	Reptiles	South Africa	Mofokeng *et al*. 2021
Amblyomma marmoreum	MW513957	Reptiles	South Africa	
Amblyomma marmoreum	KY457515	*Not indicated	South Africa	Mans *et al*. 2019
Otobius megnini	MG582606	Cattle	Iran	Hosseini-Chegeni *et al*. 2019
16S rRNA gene				
Hyalomma truncatum	KY457529	*Not indicated	South Africa	Mans *et al*. 2019
Hyalomma truncatum	KU130478	Livestock and wildlife species	South Africa	Sands *et al*., 2017
Hyalomma marginatum	KT391060	Sheep	*not indicated	Erster *et al*. 2015

Hyalomma glabrum	KU130432	Livestock and wildlife species	South Africa	Sands *et al*. 2017
Hyalomma glabrum	KU130431	Livestock and wildlife species	South Africa	Sands *et al*. 2017
Amblyomma (Aponomma) exornatum	MN150173	Rock monitor	South Africa	Hornok *et al*., 2020
Rhipicephalus sp.	KT382478	Dogs	South Africa	Zemtsova *et al*. 2016
Rhipicephalus evertsi mimeticus	OK481095	Cattle	Angola	Palomar *et al*. 2021
Rhipicephalus sp. GZ-	KT382478	Dogs	South Africa	Zemtsova *et al*., 2016
Rhipicephalus gertrudae	MW080139	Vegetation	South Africa	Bakkes *et al*. 2021
Rhipicephalus simus	LC634554	Vegetation	Zimbabwe	Kobayashi *et al.*, 2021
Rhipicephalus bursa	MT302761	Wild goat	Turkey	Orkun *et al*. 2016
Amblyomma marmoreum	MW290508	Reptiles	South Africa	Mofokeng *et al*. 2021
	MW290507	Reptiles	South Africa	
Amblyomma exornatum	MN150173	Rock monitor	South Africa	Hornok *et al*. 2020
Amblyomma hebraeum	L34316	Not indicated	Not indicated	Black and Piesman., 1994
Otobius Megnini	EF120989	Bovine	Argentina	Nava *et al*., 2009
	L34325	*Not indicated	Not indicated	Black and Piesman., 1994
Haemaphysalis elliptica	HM068961	Cheetah	South Africa	Golezardy *et al*. (unpublished)
	HM068958	Cheetah		
	HM068957	Cheetah		
	HM068956	Cheetah		

APPENDIX F: NUMBER OF BASE PAIR DIFFERENCES FOR THE *COI* GENE

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
TIC19_CO1	1		1	2	4	25	59	59	105	107	108	102	102	102	102	102	104	104	104	138	138
KU130596	2	1		1	5	26	60	60	105	107	108	103	103	103	103	103	104	104	104	137	137
KU130598	3	2	1		6	27	61	61	106	108	109	104	104	104	104	104	105	105	105	137	137
TIC5_CO1	4	4	5	6		27	63	63	105	107	108	100	100	100	101	101	104	104	104	138	138
JQ737074	5	25	26	27	27		59	61	99	100	103	100	100	100	102	102	101	101	103	131	131
TIC11_CO11	6	59	60	61	63	59		2	101	103	103	109	109	109	110	110	102	102	103	145	145
OK481109	7	59	60	61	63	61	2		102	104	104	111	111	111	110	110	104	104	103	145	145
MN150166	8	105	105	106	105	99	101	102		2	27	86	86	86	88	89	107	107	108	114	114
MN150167	9	107	107	108	107	100	103	104	2		29	87	87	87	90	91	108	108	110	116	116
TIC13_CO1	10	108	108	109	108	103	103	104	27	29		93	93	93	89	90	110	110	111	118	118
TIC23A_CO1	11	102	103	104	100	100	109	111	86	87	93		0	0	27	27	98	98	100	116	116
TIC23B_CO1	12	102	103	104	100	100	109	111	86	87	93	0		0	27	27	98	98	100	116	116
KY457515.1	13	102	103	104	100	100	109	111	86	87	93	0	0		27	27	98	98	100	116	116
MW513958	14	102	103	104	101	102	110	110	88	90	89	27	27	27		2	97	97	96	116	116
MW513957	15	102	103	104	101	102	110	110	89	91	90	27	27	27	2		99	99	98	117	117
TIC8_CO1	16	104	104	105	104	101	102	104	107	108	110	98	98	98	97	99		0	13	124	124
MZ351133	17	104	104	105	104	101	102	104	107	108	110	98	98	98	97	99	0		13	124	124
TIC14_CO1	18	104	104	105	104	103	103	103	108	110	111	100	100	100	96	98	13	13		122	122
TIC7_CO1	19	138	137	137	138	131	145	145	114	116	118	116	116	116	116	117	124	124	122		0
MG582606	20	138	137	137	138	131	145	145	114	116	118	116	116	116	116	117	124	124	122	0	

APPENDIX G: NUMBER OF BASE PAIR DIFFERENCES FOR THE *16S* RRNA GENE

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
EF120989	1		1	18	141	141	141	141	140	140	139	139	139	137	137	138	138	130	131	136	133	115	115
L34325	2	1		19	142	142	142	142	141	141	140	140	140	138	138	139	139	131	132	137	134	116	116
TIC_7_16S	3	18	19		141	141	141	141	140	140	140	140	140	134	133	134	134	136	137	142	139	120	120
KU130430	4	141	142	141		0	0	0	1	17	23	22	26	69	71	72	72	64	65	73	62	80	80
KU130431	5	141	142	141	0		0	0	1	17	23	22	26	69	71	72	72	64	65	73	62	80	80
KU130432	6	141	142	141	0	0		0	1	17	23	22	26	69	71	72	72	64	65	73	62	80	80
TIC_5_16S	7	141	142	141	0	0	0		1	17	23	22	26	69	71	72	72	64	65	73	62	80	80
TIC_19_16S	8	140	141	140	1	1	1	1		17	23	22	26	68	70	71	71	63	64	72	61	80	80
KT391060	9	140	141	140	17	17	17	17	17		14	13	12	70	71	72	72	62	63	71	63	78	78
KY457529-RC	10	139	140	140	23	23	23	23	23	14		1	5	68	69	70	70	62	63	71	62	76	76
KU130478_Hyalomma_truncatum_isolate	11	139	140	140	22	22	22	22	22	13	1		4	67	68	69	69	61	62	70	61	76	76
TIC_11_16S	12	139	140	140	26	26	26	26	26	12	5	4		67	68	69	69	61	62	70	63	76	76
MT302761	13	137	138	134	69	69	69	69	68	70	68	67	67		17	18	18	47	48	55	46	80	80
TIC_18A_16S	14	137	138	133	71	71	71	71	70	71	69	68	68	17		1	1	46	47	53	45	80	80
OK481095	15	138	139	134	72	72	72	72	71	72	70	69	69	18	1		0	47	48	54	46	81	81
TIC_18B	16	138	139	134	72	72	72	72	71	72	70	69	69	18	1	0		47	48	54	46	81	81
TIC_21_16S	17	130	131	136	64	64	64	64	63	62	62	61	61	47	46	47	47		1	9	10	78	78
KT382478	18	131	132	137	65	65	65	65	64	63	63	62	62	48	47	48	48	1		10	11	79	79
MW080139	19	136	137	142	73	73	73	73	72	71	71	70	70	55	53	54	54	9	10		19	85	85
LC634554	20	133	134	139	62	62	62	62	61	63	62	61	63	46	45	46	46	10	11	19		80	80
HM068956	21	115	116	120	80	80	80	80	80	78	76	76	76	80	80	81	81	78	79	85	80		0
HM068961	22	115	116	120	80	80	80	80	80	78	76	76	76	80	80	81	81	78	79	85	80	0	
TIC_14_16S	23	115	116	120	80	80	80	80	80	78	76	76	76	80	80	81	81	78	79	85	80	0	0
HM068957	24	115	116	120	80	80	80	80	80	78	76	76	76	80	80	81	81	78	79	85	80	0	0
HM068958	25	115	116	120	80	80	80	80	80	78	76	76	76	80	80	81	81	78	79	85	80	0	0
TIC_8_16S	26	117	118	121	80	80	80	80	80	80	78	78	78	79	79	80	80	81	82	88	81	4	4
TIC_13_16S	27	126	127	130	83	83	83	83	82	80	79	78	78	71	76	77	77	82	83	90	81	67	67
MN150173	28	150	151	152	110	110	110	110	109	104	104	103	101	99	105	106	106	112	113	120	112	91	91
MW290508	29	122	123	110	86	86	86	86	86	80	79	78	78	77	80	81	81	84	85	93	84	69	69
MW290507	30	122	123	110	86	86	86	86	86	80	81	80	80	76	78	79	79	85	86	94	84	65	65
TIC_23A_16S	31	121	122	109	84	84	84	84	84	78	79	78	78	78	78	79	79	83	84	92	83	64	64
TIC_23B_16S	32	121	122	109	84	84	84	84	84	78	79	78	78	78	78	79	79	83	84	92	83	64	64
TIC_23D_16S-RC	33	120	121	108	85	85	85	85	85	79	80	79	79	77	77	78	78	82	83	91	83	63	63
L34316	34	120	121	106	107	107	107	107	107	102	107	106	104	89	94	95	95	104	105	112	106	91	91
TIC_20_16S	35	120	121	106	107	107	107	107	107	102	107	106	104	89	94	95	95	104	105	112	106	91	91
TIC_28_16S	36	120	121	106	107	107	107	107	107	102	107	106	104	89	94	95	95	104	105	112	106	91	91

	3	24	25	26	27	28	29	30	31	32	33	34	35	36
1	115	115	115	117	126	150	122	122	121	121	120	120	120	120
2	116	116	116	118	127	151	123	123	122	122	121	121	121	121
3	120	120	120	121	130	152	110	110	109	109	108	106	106	106
4	80	80	80	80			86	86	84	84	85	107	107	107
5	80	80	80	80			86	86	84	84	85	107	107	107
6	80	80	80	80			86	86	84	84	85	107	107	107
7	80	80	80	80			86	86	84	84	85	107	107	107
8	80	80	80	80			86	86	84	84	85	107	107	107
9	78	78	78	80			80	80	78	78	79	102	102	102
10	76	76	76	78			79	81	79	79	80	107	107	107
11	76	76	76	78			78	80	78	78	79	106	106	106
12	76	76	76	78			78	80	78	78	79	104	104	104
13	80	80	80	79			77	76	78	78	77	89	89	89
14	80	80	80	79			80	78	78	78	77	94	94	94
15	81	81	81	80	77	106	81	79	79	79	78	95	95	95
16	81	81	81	80	77	106	81	79	79	79	78	95	95	95
17	78	78	78	81	82	112	84	85	83	83	82	104	104	104
18	79	79	79	82	83	113	85	86	84	84	83	105	105	105
19	85	85	85	88	90	120	93	94	92	92	91	112	112	112
20	80	80	80	81	81	112	84	84	83	83	83	106	106	106
21	0	0	0	4	67	91	69	65	64	64	63	91	91	91
22	0	0	0	4	67	91	69	65	64	64	63	91	91	91
23		0	0	4	67	91	69	65	64	64	63	91	91	91
24	0		0	4	67	91	69	65	64	64	63	91	91	91
25	0	0		4	67	91	69	65	64	64	63	91	91	91
26	4	4	4		67	91	70	66	65	65	64	91	91	91
27	67	67	67	67		60	67	66	66	66	65	82	82	82
28	91	91	91	91	60		93	92	91	91	90	106	106	106
29	69	69	69	70	67	93		10	10	10	11	61	61	61
30	65	65	65	66	66	92	10		8	8	9	59	59	59
31	64	64	64	65	66	91	10	8		0	1	59	59	59
32	64	64	64	65	66	91	10	8	0		1	59	59	59
33	63	63	63	64	65	90	11	9	1	1		58	58	58
34	91	91	91	91	82	106	61	59	59	59	58		0	0
35	91	91	91	91	82	106	61	59	59	59	58	0		0
36	91	91	91	91	82	106	61	59	59	59	58	0	0	