Reptiles of South Africa as hosts for tick-borne pathogens and haemoparasites

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"*If the word quit is part of your vocabulary, then the word finish is likely not*"– B.G. Jett.

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2. Peer reviewed publications

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ABSTRACT

Haemoparasites, ticks, and tickborne bacterial microorganisms have been reported in a variety of vertebrate and invertebrate hosts. Reptiles have been documented as hosts for ticks, tick-associated microorganisms, as well as haemoparasites. However, in South Africa, the role of reptiles and their associated invertebrate vectors in the transmission of haemoparasites and tick-borne bacterial microorganisms is lacking, in part, due to limited information on the presence, diversity, and molecular evidence of ticks and their associated pathogens. To increase our knowledge on reptilian haemoparasites, their associated tick species and tick-borne bacterial microorganisms, a combined approach (use of microscopy and polymerase chain reaction) study focusing on the diversity and occurrence of haemoparasites, ticks and tick-borne pathogens was undertaken. Most of the fieldwork took place in Ndumo Game Reserve. However archived samples collected from 2014-2017 in and from the region near the village Paternoster and Bonamanzi Game Reserve were also included.

The study was divided into five main sections for the collection, analysis and reporting of data. The first section was to determine the infestation, morphology and diversity of reptile associated ticks, as well as their potential as vectors of *Hepatozoon*, the second to elucidate the life cycle of *Hepatozoon fitzsimonsi* (Dias 1953) and in conjunction with the former section, third and fourth to determine the morphology, morphometric and phylogenetic analysis of *Hepatozoon* species and haemosporidian species, respectively. Lastly the occurrence and diversity of tick-borne bacterial microorganisms. Ticks present on the reptiles at the time of blood collection were carefully removed using forceps as to ensure that the hypostome remained intact with minimal damage to the tick. Blood was drawn from the subcarapacial sinuses of tortoises, as well as from the caudal vein of squamate reptiles and thin blood smears prepared for screening and morphometrics; the remaining blood fixed in 70% molecular grade ethanol for later molecular analysis. Giemsa-stained blood smears were screened microscopically for the presence of any haemoparsasites. All samples were then further analyzed according to the objectives of the respective chapters. Analyses included both morphological and molecular aspects. Morphology was used for the description and identification of species, and molecular analyses were used to assist with the morphology-based descriptions, as well as to allow for phylogenetic relationship comparisons of the ticks, tick-borne pathogens and haemoparasites.

For chapter 3 of the thesis, a total of 243 collected ticks from these reptiles were used and morphologically identified as *Amblyomma latum* (Koch 1844) (n=43), *Amblyomma exornatum* (Koch 1844) (n=37), *Amblyomma marmoreum* (Koch 1844) (n=113) and *Amblyomma sylvaticum* (de Geer 1778) (n=60), morphological identification was then confirmed molecularly using 12S

rRNA, 16S rRNA, 18S rRNA, ITS2, and CO1 markers. Upon morphological identification, a total number of seven ticks displayed at least one abnormality, such as ectromely and body deformities. These were observed from one of the four species of *Amblyomma* ticks. Phylogenetic relationships of the *Amblyomma* ticks from the current study show that the *Amblyomma* ticks formed a monophyletic clade with closely related *Amblyomma* species, even though amplification of DNA for some of the genes were unsuccessful. A species of *Hepatozoon*, *H. fitzsimonsi* was identified from *A. marmoreum* and *A. sylvaticum* ticks, with an overall prevalence of 4.7%. This study provides the first report of anomalous ticks associated with reptiles in South Africa and represents the second study to describe morphological abnormalities in hard ticks in South Africa. Furthermore, the taxonomic position of *A. sylvaticum* within Amblyomminae have been evaluated for the first time with molecular-phylogenetic methods. Even though ticks have been suggested to be the likely vector of *H. fitzsimonsi*, with this supported by the findings of *Hepatozoon*-like developmental stages in ticks collected off infected tortoises, the present study provides further support for ticks acting as the potential vectors of *H. fitzsimonsi*.

A total of 14 tortoises were collected from Bonamanzi Game Reserve for the purpose of the developmental stages of *H. fitzsimonsi*, one *Kinixys natalensis* and *Kinixys spekii*, seven *Kinixys zombensis* and five *Stigmochelys pardalis.* Three species of *Amblyomma* ticks (Order Acari; Family Ixodidae) were collected from the three *S. pardalis*; *Amblyomma herbraeum, A. marmoreum* and *Amblyomma nuttalli.* The sporogonic stages of *H. fitzsimonsi* were identified in the hemocoel of these *Amblyomma* ticks, whilst merogonic stages were observed in the liver of one *S. pardalis* with no observable stages identified in other organs. The phylogenetic analysis show that the species of *Hepatozoon* from the present study clustered primarily with 18S rDNA sequences of known *H. fitzsimonsi* and within a larger clade containing sequences of *Hepatozoon* of other chelonians.

Two species of varanid lizards and one species of elapid snake were parasitized with *Hepatozoon* species. Two species of *Hepatozoon*, *Hepatozoon* sp. A and *Hepatozoon* sp. B that were morphologically, morphometrically, and molecularly distinct were observed from *Naja mossambica*. The mature gamonts of *Hepatozoon* sp. A are smaller and wider (13.75 × 4.61) than that of *Hepatozoon* sp. B (16.52 × 3.36). According to the phylogenetic analysis, 18S rDNA sequences of *Hepatozoon* sp. A was most closely related to *H. ayorgbor* Sloboda et al. (2007) described from *Python regius* in Ghana, while the sequence of *Hepatozoon* sp. B was most closely related to *Hepatozoon colubri* Börner (1901) that Zechmeisterová et al. (2021) recovered from *Zamenis langissumus* in Iran. Comparison of *Hepatozoon* from this study's monitor lizards with other described species of *Hepatozoon* infecting African varanids show that the *Hepatozoon* described in the present study from South African *Varanus niloticus* and *Varanus albigularis* was

found to be morphologically similar to *Hepatozoon camarai* described by Dias (1954) from a specimen of *V. a. albigularis* in neighbouring Mozambique and *Hepatozoon varani* originally described from *V. niloticus* in South Africa by Laveran in 1905, and later in other up African countries. The sequence of the *Hepatozoon* sp. of monitor lizards from the present study formed a monophyletic clade and clustered with sequences of *Hepatozoon* species of other *Varanus* species and the snake *Liasis fuscus.* This study presents the first formal description of *Hepatozoon* species parasitizing *N. mossambica,* and one more *Hepatozoon* species from *V. albigularis* and *V. niloticus* in South Africa based on morphological description and molecular characterization. Furthermore, the present study represents the first morphological and molecular report of *Hepatozoon* within South African varanids and elapid snakes.

Two tortoises (both *K. natalensis*) were parasitized by a species of *Haemoproteus* (*Haemocystidiumi*). This species did not compare morphologically to the previously described species of *Haemoproteus* (*Haemocystidium*) from South African nor other African tortoises. Four snakes (*N. mossambica* and *Dendroaspis* spp.) were found infected with a species morphologically comparable to *Haemoproteus mesnili* (Bouet 1909) Wenyon 1926. One tick (*A. latum*) was found positive with DNA comparable to the latter when molecularly screened targeting a fragment of the *cyt-b* gene; this most likely due to an incompletely digested bloodmeal. In the phylogenetic analysis, the two sequences of *Haemoproteus* (*Haemocystidium*) from two individuals of *K. natalensis* formed a monophyletic clade with 100% bootstrap support. Furthermore, these sequences clustered in a larger clade with *Haemocystidium* of both terrestrial and aquatic chelonians. Regarding the sequences of *Haemoproteus* (*Haemocystidium*) from snakes and a single tick, these clustered together in a monophyletic clade. With high bootstrap support (99%), this clade formed a sister clade to *Haemoproteus mesnili*, which in turn clustered within a larger monophyletic clade consisting of *Haemocystidium* of snakes and lizards. As such this study provides the first available molecular data for a species of *Haemocystidium* of tortoises in South Africa, and the first combined descriptive approach of *Haemocystidium* species for chelonians.

These *Amblyomma* ticks (n = 253), as well as their reptiles (n=71) were also screened for the presence of various bacterial microorganisms, including *Coxiella, Ehrlichia, Anaplasma, Rickettsia, Borrelia*, as well as haemoparasites such as haemogregarines and haemosporidians by amplification, sequencing and phylogenetic analysis of the 16S rRNA, 23S rRNA, *gltA*, *OmpA,* Flagellin, and 18S rRNA genes, respectively. This study recorded the presence of reptile associated *Borrelia* species and *Coxiella*-like endosymbiont in South Africa for the first time. Furthermore, a spotted fever group *Rickettsia* species was molecularly detected in seven *A. marmoreum* and 14 *A. sylvaticum* from tortoises of genera *Kinixys* and *Chersina*. *Francisella*-like

endosymbiont was molecularly detected in two *A. latum* collected from *N. mossambica*. Moreover, an environmental bacterium genus (*Brevibacterium*) was observed from *A. sylvaticum. Coxiella burnetii, Ehrlichia* spp. and *Anaplasma* spp., were not detected from these ticks. None of the reptilian blood was positive for any of the tick-borne microorganisms. Observations from this study provide indications that reptilian ticks may play a role in the transmission of pathogenic bacteria to homothermic animals. Furthermore, the absence of *Ehrlichia*, *Anaplasma* spp., and *C. burnetii* does not mean that these pathogens should be completely neglected and not screened for in future studies.

In summary, this study explored the occurrence and diversity of reptilian haemoparasites, ticks and tick-borne pathogens infecting reptiles from two provinces (KwaZulu-Natal and Western Cape) of South Africa. Based on the results of the study, four species of ticks were recorded infesting chelonians and squamate reptiles, and the phylogenetic position of *A. sylvaticum* with Amblyomminae was determined for the first time, contributing to studies focusing on the phylogenetic analysis of genus *Amblyomma*, as well as extending the scope of previous studies on ticks infesting squamates in South Africa. Furthermore, in the present study, sporogonic stages in three species of *Amblyomma* ticks were molecularly confirmed to be that of *H. fitzsimonsi*, substantiating that these parasites do act as definitive hosts and strongly suggesting that they are a vector. Moreover, the current study presents the first combined morphological and molecular description of *Hepatozoon* species parasitizing *N. mossambica,* and one more *Hepatozoon* species from *V. albigularis* and both *V. niloticus* in South Africa, respectively. The phylogenetic relationships of species of *Hepatozoon*, *Haemoproteus* (*Haemocystidium*) were also characterized based on comparisons to other available molecular data. The presence of different bacterial microorganisms from the study provides indications that reptilian ticks may play a role in the transmission of pathogenic bacteria to homothermic animals. Furthermore, results of the current study provide a baseline for future studies on taxonomy, diversity and phylogenetic relationships of reptilian haemoparasites, ticks, as well as tick-borne bacterial microorganisms in South Africa and worldwide.

Keywords: Haemoparasites; Molecular characterization; Morphological description; Reptiles; Taxonomy; Tick-borne pathogens.

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PREFACE TO THESIS AND STUDY MOTIVATION

Introduction to Reptilia

Reptiles and their diapsid ancestors are an extremely ancient group of ectothermic vertebrates that evolved approximately 250 million years ago. This evolutionary history is based on early fossil records and molecular phylogeny estimates (Reizs et al. 2011; Böhm et al. 2013). Extant reptiles have been grouped into two subclasses (Archosaura and Lepidosaura) (Zurg et al. 2001). Subclass Lepidosaura is known to contain two orders, Rhynhocephalia and Squamata. The second order includes Sauria, Ophidia, and Amphisbaenia. According to Vidal and Hedges (2009), this subclass appeared over 200 million years ago and diversified into several families over 145 million years ago. Archosaura includes the order Crocodylia that contains crocodylians, gharials and alligators. This order also includes the closest living relatives of birds and it emerged in the Upper Cretaceous (Brochu 2003). Historically, living reptiles have been divided into two phylogenetic branches based on their morphology and fossil records. These branches include Testudines and diapsid (i.e. Lepidosaura and Archosaura) (Hedges and Poling 1999). Throughout the Triassic and Jurassic era, high cladogenesis rates produced a variety of species adapted to terrestrial, freshwater and marine habitats, as well as to almost every temperate, tropical and desert environment (Vidal and Hedges 2009; Böhm et al. 2012).

Even though there is a series of taxonomic revisions on reptiles, the phylogenetic relationships between the primary lineages of Reptilia still remains unclear (Vidal and Hedges 2009; Pyron et al. 2013). Morphological and molecular studies have produced several hypotheses for Reptilia's phylogenetic relationships. It has been confirmed by most studies that Testudines are sisters of the Archosaura, nonetheless, turtles were placed as the sister group to lepidosaurs instead of archosaurs by Rieppel and Reisz (1999). Similarly, between other Lepidosaura, the phylogenetic links of snakes (Ophidia) are uncertain. Some studies place them within Anguimorpha, while others place the snakes as sisters of a clade including all the lizards (Underwood 1970; Lee 2001). In addition, Rhynhocephalia is usually placed as a morphology-based sister group of the Squamata, although analyses of limited molecular data suggest that tuataras are closer to archosaurs and turtles (Rest 2003).

Global diversity of reptiles

Reptiles are one of the most remarkable components of global diversity. There are currently 9546 accepted reptilian species, with approximately 2800 subspecies (Pincheira-Donoso et al. 2013). They are the second richest tetrapods following the approximately 10600 known avian species, and significantly more diverse than the currently known 8648 amphibian species (see Frost 2023) and the approximately 5400 mammalian species (Wilson and Reeder 2005; Reeder et al. 2007). More species and higher numbers of reptiles occur mostly in degraded sites than in sites where

soil and vegetation are well preserved. For example, deserts and semi-deserts harbour a higher diversity of reptiles than rainfall areas (Pianka 1989; Fabricius et al. 2003). The ecological and evolutionary history of these organisms has played a major role in amniote vertebrate's origin and subsequent radiation, and in the function of the modern ecosystem. It has been documented that the evolutionary significant events that occurred in the reptilian past, including the acquisition of water-independent reproduction that resulted in their establishment as the first fully-terrestrial vertebrates, and their universally known Mesozoic proliferation followed by mass extinctions, are among the most important events in vertebrate evolutionary history (Rasmussen et al. 2011; Pincheira-Donoso et al. 2013). Furthermore, reptiles have successfully occupied most areas of the planet including the oceans. In order to cope with the selective demands encountered over hundreds of millions of years ago, they have also accumulated tremendous diversity of defensive, ecological, life history, behavioral and morphological strategies (Pianka and Vitt 2003; Pough et al. 2004; Fry et al. 2006). These and other characteristics have gained reptiles a significant role as model systems for ecological and evolutionary studies (Losos 2009; Vitt and Caldwell 2013).

Diversity of reptiles in South Africa

South Africa is the world's third most biologically diverse country for terrestrial species. It consists of three of the world's 35 terrestrial biodiversity hotspots: the Succulent Karoo, Cape Floristic Region and the Maputaland–Pondoland–Albany region (Biggs et al. 2006). To date, there are 384 documented reptile species (422 species and subspecies, i.e. taxa) in South Africa. This includes 23 chelonian species, 244 lizards, one crocodile and 116 snakes (Bates et al. 2014). In Africa, the richest diversity of geckos and amphisbaenians have been documented from South Africa. The country is also known to have the highest biodiversity of tortoises in the world (Branch 2008). The diversity of snakes in South Africa is lower (116 species) than that of countries from tropical Africa such as DRC (168 species), Cameroon (156 species), and Tanzania (167 species) (Uetz 2011). However, it is higher than that of many other African countries of comparable size, including Ethiopia (107 species) and Nigeria (105 species) (Uetz et al. 2022). It is surprising that the snake diversity of South Africa is much greater than of many tropical African countries and of the 33 species recorded in western Europe (Speybroek et al. 2010), because this country has two temperate-subtropical transitional areas. Additionally, South Africa demonstrates a high level of endemism, with 54.2% (128 of 421) of reptilian species being either endemic (190 taxa) or near endemic (38) (Bates et al. 2014). Despite the high diversity of reptiles in South Africa, only a small number of studies reported the presence of different haemoparasites infecting reptiles (McAllister et al. 2010; Cook et al. 2010, 2014, 2015, 2016,2018; van As et al. 2013, 2016; Harris et al. 2018). Furthermore, a diverse species assemblage of tick-borne pathogens has been identified in lizard species in Portugal, Australia, and the Netherlands, as well as in a variety of reptile species imported into Japan (De Sousa et al. 2012, Whiley et al. 2016; Omondi et al. 2017). These studies

suggest a possible role of reptiles in the epidemiology of diverse tick-borne diseases, including heartwater, which has not been investigated in African endemic settings (Omondi et al. 2017). However, despite the increase of studies on reptilian parasites, the systematics and diversity of these parasites are still poorly understood.

Parasitism

Parasitism in many animals is known to strongly influence individual fitness (Natusch et al. 2018). As highlighted by Arneberg et al. (1996) and Krams et al. (2013), parasites can influence body condition, lower productive output, diminish competitive ability and transmit disease. They can also alter host behavior and spatial ecology (Brown et al. 2016). According to Finnerty et al. (2018), it is sometimes difficult to differentiate whether correlation between parasite loads and host fitness and/or behaviour are a direct impact of parasitism or reflect the fact that a host's ecological or behavioral traits affect its susceptibility to parasites. However, Maia et al. (2016a) indicated that the first step towards differentiating the relative contribution of these factors on parasite diversity and biogeography is by studying parasite infection parameters such as prevalence and parasitaemia. Parasite infection depends on the ability of the parasite to establish successful infection and the host to either tolerate or resist infection, as well as the frequency of encounters among two interacting partners (Poulin 2006). Parasitaemia and prevalence are seen as indicators of parasite-host specificity for parasites with complex lifecycles. According to Poulin (2006), differences in these parameters can assist in differentiating between definite and auxiliary hosts, with the definite host exhibiting higher levels of these parameters. However, when comparing patterns of infection between host species and parasite lineage, one should consider several parasite and host-related factors (Morand and Poulin 2003; Nieberding et al. 2008). Host relatedness is the first factor since distinct host species require a higher adaptive effort for successful parasite establishment, and they are also more likely to differ in their immune defence mechanisms (Poulin and Mouillot 2005). Therefore, parasite species that exhibit complex life cycles, which involve a number of host species, must adapt to different host factors throughout their lifecycle. Ecology is another factor to take into consideration, because as indicated by Wolinska and King (2009), host-parasite interactions may differ when exposed to different habitat and environmental conditions. Actually, recent research has demonstrated that host ecology may be a significant factor driving parasite differentiation as compared to host phylogeny (Pérez-Ponce de Leon and Choudhury 2005; Poulin 2006).

Justification of the study

Reptiles have always been important in human food systems, as research models, for healthy ecosystems, in conservation activities, and as pets, as well as in people's cultures (Huey 1982, Böhm et al. 2013). Additionally, they are ecologically important as predators in food webs, being carnivorous or insectivorous, and as food sources for other animals. Furthermore, some reptiles can act as useful biological indicators for environmental health, making them critical components in the ecosystem; also they provide models that are useful for research concerned with biological and evolutionary processes underlying speciation (Greenberg et al. 1989, Raxworthy et al. 2008). One of the main threats to reptiles is the global trade of these animals, particularly that of the illegal trade (Auliya et al. 2016).

Reptiles are infected with a wide range of parasites, which have been identified as the causative agents of diseases responsible for the decline of reptile populations (Schumacher 2006). Parasites include both blood parasites and tick-borne pathogens (TBPs). Whilst TBPs are purportedly solely transmitted by ticks, blood parasites are transmitted by various haematophagous vectors such as ticks, insects or leeches (O'Donoghue, 2017). Many of these vectors are essential for the development and transmission of these parasites and pathogens, and as such play an important role in these organisms' persistance in the environment and for the potential introduction of these into new and naïve environments (Burridge et al 2001; Burridge and Simmons 2003). Furthermore, some of these vector-borne pathogens such as *Borrelia, Ehrlichia,* and *Babesia*, some zoonotic (*Borrelia*), have been recognized to have a large global impact on animal and human health (Karesh et al. 2005).

South Africa has a rich biodiversity of reptiles that, as previously mentioned, includes 384 species (Bates et al. 2014). However, knowledge on the biodiversity, prevalence and intensity of parasitic and pathogenic infections in these reptiles remains lacking. This knowledge is important because parasitism causes deleterious effects on several aspects of the ecology and evolution of parasite hosts (Smallridge and Bull 2000), and with the legal, but often unregulated trade, as well as the illegal trade in reptiles, the potential transmission and implications for this in naïve hosts, animal and human, are unknown.

As a matter of fact, it is acknowledged that reptiles are hosts for several *Amblyomma* spp. in South Africa (Horak et al. 2006) but a number of poorly studied tick species associated with reptiles still bear the name of this genus, although they may not belong to it. Nonetheless, despite this knowledge, the importance of molecular tools in tick identification and importance of resolving the phylogenetic position of *Amblyomma* ticks, information on tick infestation and molecular data of *Amblyomma* ticks infesting these ectotherms in South Africa appear to be in part lacking. Furthermore, the phylogenetic relationships of *Amblyomma* ticks are not yet resolved, and require special attention, because several poorly studied tick species associated with reptiles still bear

the name of this genus, although they may not belong to it. This study will focus on the molecular evidence and phylogeny of four tick species; *Amblyomma latum* (Koch 1844), *Amblyomma exornatum* (Koch 1844), *Amblyomma marmoreum* (Koch 1844) and *Amblyomma sylvaticum* (de Geer 1778) parasitising South African reptiles. Information on molecular evidence of these tick species as well as their taxonomic positions appear to be lacking, although recently some of these tick species have been included in phylogenetic analyses of both mitochondrial and nuclear genetic markers. At the same time, the taxonomic position of *A. sylvaticum* within Amblyomminae has never been evaluated with molecular-phylogenetic methods, despite the high quality images of their morphological features being published. Therefore, contributing to studies focusing on the phylogenetic analysis of genus *Amblyomma* (Burger et al. 2012; Hornok et al. 2020; Kelava et al. 2021), will be of benefit to the scientific community as new knowledge will be brought to the front burner for future discussions.

During the present study, what appeared to be morphological abnormalities were observed in several ticks. Even though documenting and reporting on these was not in the overall aim or an objective of the study, it was considered important that this study describe these abnormalities. Morphological abnormalities in ticks rarely occurs in nature and as such the existing knowledge on anomalous ticks collected in Africa is extremely sparse and lacking, particularly for those ticks found infesting reptiles. To date, there are only three reports of anomalous ticks in Sub-Saharan Africa, these from hard ticks collected from domestic animals in South Africa, Uganda and Sudan, respectively (Gothe 1967; Balinandi et al. 2019; Shualb et al. 2020). These studies highlight the lack of research in this field and as such emphasizes the need to investigate the occurrence of anomalous ticks in Africa, as a lack thereof may lead to identification mistakes and taxonomic errors (Buczek 2000).. Although it is not clear how morphological anomalies may alter vector competence in ticks, anomalous ticks may be more efficient vectors of pathogens, hence this situation warrants further investigation (Alekseev et al. 2007; Nowak-chmura 2012).

An increasing number of studies have implicated reptiles and their associated ticks as potential reservoirs involved in the transmission cycles of tick-borne pathogens of veterinary importance (Peter et al. 2000; Omondi et al. 2017). This fact is of great concern in tick-borne disease control strategies, as these ectotherms are usually asymptomatic carriers that may serve as reservoirs of the infection. In Africa, *Borrelia*, *Rickettsia* spp*.,* and *Coxiella*-like endosymbionts associated with reptiles have been reported in seven tick species belonging to two genera (*Amblyomma* and *Hyalomma*) (Table S1). *Amblyomma* ticks were recorded to be infected with the highest number of tick-borne bacteria, most of which are zoonotic. Even though reptiles are infested with Argasid ticks, there are no documented tick-borne bacteria infecting these ticks in reptiles from Africa. The aforementioned seven tick species were associated with six host taxa of reptiles (one species of lizard, four species of tortoise and one species of snake), most of these reptiles along with their

ticks exported to exotic countries such as Japan. Although diverse tick-borne pathogens are emerging across the world, with documented effects on the health of humans and livestock, studies into their diversity and specific interactions with ticks and their reptilian host species in South Africa are lacking. In particular, the role of these ectotherms in the epidemiology of TBPs remains unknown, even though exported ticks and reptiles exported from Africa, America, Middle east, and Asia into Japan have been implicated with TBPs of livestock. This leads, therefore, to a lack of knowledge on the diversity of these pathogens in reptiles and whether reptiles can act as reservoir hosts for TBPs infecting domestic animals and humans. This lack of information on tick-borne pathogens of reptilian ticks in South Africa warrants novel research in this area. Prior to this study, only one reptile associated tick; *A. sylvaticum* was documented to harbour *Rickettsia massiliae* in South Africa (Halajian et al. 2018) , and there were no records of reptile associated *Borrelia* in the country.

Even though several blood parasites have been described infecting reptiles from South Africa, these have not been the result of exhaustive survey studies, and as such the biodiversity of these parasites is expected to be much higher. In addition, although diverse tick-borne pathogens are endemic to Africa, with documented impact on the health of human and livestock, studies into their diversity and specific interactions with invertebrate and vertebrate host species in South Africa are lacking. This lead therefore to a lack of knowledge on the biodiversity of these pathogens in reptiles and whether reptiles can act as reservoir hosts for TBPs infecting domestic animals and humans.

Research on haemoparasites of South African reptiles, highly recommends that revision should not only be on identification of new or previously described species, but phylogenetic relationships between the genera of these parasites should also be revised (Cook et al. 2014; Cook et al. 2016; Cook et al. 2018). However, accurate identification of these parasites using only microscopy is often impossible. It also requires molecular techniques such as PCR, which has proven to be effective in the characterization of blood parasites (Masiga et al. 1992). Molecular data on most reptilian haemoparasites and tick-borne pathogens is lacking, with insufficient data available in GenBank from Africa, and the phylogenetic position of these parasites is yet unknown. Therefore it is imperative to obtain molecular data of parasites not only from reptilian hosts but also from the vectors.

To date, more than 30 species of *Hepatozoon* have been documented from reptiles throughout Africa. In South Africa, these include *Hepatozoon langii* Van As et al. 2013, *Hepatozoon vacuolatus* Van As et al. 2013*, Hepatozoon affluomaloti* Van As et al. 2015 *,* and *Hepatozoon varani* Laveran, 1905 described from four species of lizards, *Hepatozoon bitis* Fantham 1925, *Hepatozoon refrigens* Sambon et al. 1907, *Hepatozoon angeladaviesae* Cook et al. 2018,

Hepatozoon cecilhoarei Cook et al. 2018 and *Hepatozoon* spp. from six species of snakes, and *Hepatozoon fitzsimonsi* (Dias 1953) from five species of tortoises.

Hepatozoon fitzsimonsi, has been described parasitizing tortoises from southern Africa, and has been reported from ticks infesting tortoises from Kenya, East Africa. Even though ticks have been suggested to be the likely vector of this *Hepatozoon* species, with this supported by the findings of *Hepatozoon*-like developmental stages in ticks collected off of infected tortoises (see Cook et al. 2014), a systematic revision by Karadjian et al. (2015) placed this species into genus *Bartazoon*, a genus vectorised by biting insects. As molecular work attempting to link the sporogonic stages in ticks to those gamonic stages in tortoises was unsuccessful during the study of Cook et al. (2014), and with the molecular findings of Mofokeng et al. (2021), further study attempting to resolve the link, if any, between the morphological stages in both the tick and tortoise would be required.This would allow for more informed insight regarding the life cycle of *H. fitzsimonsi* and its vectors, and aiding in informing to which genus it belongs. The present study thus provides further support for ticks acting as the potential vectors of *H. fitzsimonsi* and for this parasite remaining within the genus *Hepatozoon.*

Similarly to the adeleorine apicomplexans such as genus *Hepatozoon*, the order Haemosporida includes haemoprotozoan parasites that infect a range of vertebrate hosts. However, despite their relevance for reptile conservation, the diversity, distribution and taxonomy of these haemosporidian parasites remains poorly investigated in reptiles. As such the contribution of further morphologically and molecularly described taxa for use in phylogenetic analyses would be valuable. Only a few species of haemosporidians have been documented from South African reptiles, including four species from ophidian hosts (*Plasmodium bitis* Fantham and Porter 1950 *, Plasmodium pythonis* Fantham and Porter 1950*, Plasmodium causi* Fantham and Porter 1950*, Plasmodium melanoleuca* Fantham and Porter 1950), three from saurians (*Plasmodium zonuriae* Pienaar 1962; *Plasmodium cordyli* Telford 1987; *Plasmodium intabazwe* van As et al. 2016) and two from chelonians (*Haemoproteus testudinalis* (Laveran 1905) Wenyon 1915; *Haemoproteus natalensis* Cook et al. 2010. Prior to this study there were no sequences of tortoise haemosporidians from South Africa in GenBank.

Overall, the phylogenetic analyses of reptile-associated ticks, these ectoparasites' tick-borne pathogens and haemoparasites will possibly provide an insight into their distribution, transmission as well as their evolution. Additionally, it will also provide more accurate placement in their phylogenies. Furthermore, it is important that we document this parasite diversity in reptiles, so that this knowledge can act as a baseline for future studies into these parasites' transmission routes and modes, as well as in turn assessing their zoonotic potential. This hopefully will contribute to more effective management in regulating both the national and international trade of reptiles along with their ectoparasites.

Aim of the study

The main aim of this study was to record the occurrence, taxonomy, and species diversity of reptile-associated ticks, these ectoparasites' tick-borne pathogens (*Ehrlichia, Anaplasma*, *Rickettsia, Coxiella,* and *Borrelia* spp.), determining if reptiles can act as reservoir hosts for these TBPs, and furthermore the presence, taxonomy and diversity of reptile haemoparasites. Additiionally the aim was to determine potential vectors and elucidate life cycles of haemoparasites (haemogregarines or adeleorine haemoparasites and haemosporidians), where possible, in selected reptiles and their ectoparasites (particularly ticks) in South Africa. Each chapter will have its own objective.

To achieve the aim, the following objectives were formulated:

- ⇒ To identify reptilian ticks morphologically by microscopy, report on any morphological abnormalities observed in these tick species, and molecularly characterise tick species by PCR amplification of both nuclear and mitochondrial markers (ITS2, 12S, COX1, 16S and 18S rRNA), as well as determining their phylogenetic positions and potential to act as vectors of *Hepatozoon fitzsimonsi*.
- ⇒ To provide the description of the tortoise blood parasite, *Hepatozoon fitzsimonsi*, based on observation of its life cycle stages in tortoises and in ticks, as well as the phylogenetic analysis of the 18SrRNA sequence fragments.
- ⇒ To describe haemogregarines microscopically from reptiles and their ticks (using standard morphometric characters) using Giemsa-stained blood smears, and molecularly via amplification of fragments of the 18S rRNA gene, comparing these data with that of previously described haemogregarine species of African reptiles.
- ⇒ To describe haemosporidians microscopically from reptiles and ticks (using standard morphometric characters) using Giemsa-stained blood smears, and molecularly via amplification of the *Cyt-b* gene, comparing these data with that of previously described haemosporidian species of African reptiles.
- ⇒ To determine the occurrence and genetic diversity of tick-borne bacterial microorganisms (*Anaplasma, Ehrlichia, Borrelia, Rickettsia, Coxiella* spp) from reptiles and their associated ticks.

Hypotheses of the study

- ⇒ South African reptiles are host to a diversity of blood parasites and tick-borne pathogens, acting as reservoir hosts for the latter infections.

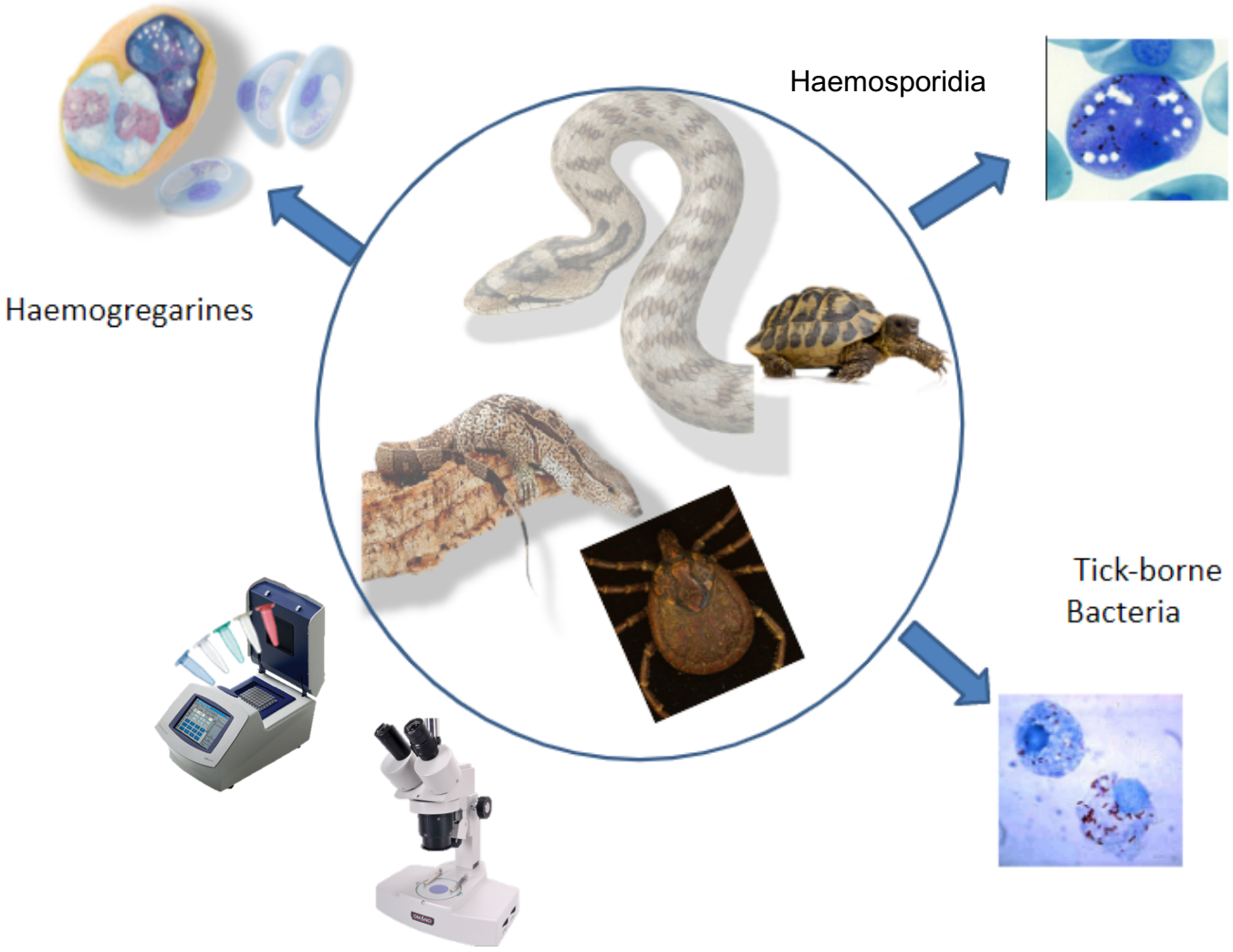
⇒ There is a great genetic diversity among tick-borne pathogens harboured by reptiles in South Africa, and many of these tick-borne pathogens are important agents of zoonotic disease.

Thesis Outline

The above brief introduction is followed by a detailed literature review (Chapter 1) that will include a review on the general information available for ticks, their classification and the role of ticks as vectors/ectoparasites. It will also focus on the importance of tick-borne and haemoparasitic diseases in reptiles. Emphasis will be placed on the diversity of blood parasites and tick-borne pathogens in reptiles, with specific focus on those of South African reptiles. Their epidemiology and pathogenicity are also discussed in this chapter.

Chapter 2 will include a description of the sampling areas, methods of sampling, methods of identifying ticks and the parasites morphologically, methods of detecting the pathogen DNA from the blood as well as other tissue samples and how data was analysed. Chapter 3 will focus on the morphology, morphological abnormalities, molecular and phylogenetic analysis of the *Amblyomma* ticks obtained from the current study, it will further discuss the potential of these ticks to act as vectors of *Hepatozoon fitzsimonsi.* To further support the findings of Chapter 3, the developmental stages and phylogeny of *H. fitzsimonsi* in the blood and liver of tortoises (*Stigmochelys pardalis*) and the haemocoel of ticks of genus *Amblyomma* will be discussed in Chapter 4. Chapter 5 will focus on the taxonomic and molecular characterization of *Hepatozoon* species parasitizing species of *Varanus* and *Naja mossambica*. The diversity of haemosporidian species infecting snakes and tortoises will be discussed in Chapter 6. Chapter 7 will focus on the molecular detection of various tick-borne bacteria from *Amblyomma* ticks collected from reptiles. This chapter will be followed by concluding remarks of the thesis based on the findings of the study. This section will indicate as well whether the results from the research support the hypotheses and whether the aims were achieved accordingly. Lastly, a complete list of references, as well as additional appendices will follow.

CHAPTER 1: LITERATURE REVIEW



1.1. Ticks

Ticks are obligatory blood-feeding ectoparasites infesting a multitude of vertebrates such as amphibians, avians, mammals and reptiles across the world, with more than 900 species being documented (Guglielmone et al. 2014; Sánchez-Montes et al. 2019). This includes 210 soft ticks and 707 hard ticks, with a significant increase in recent years of the species reported from reptiles and amphibians (Barros-Battesti et al. 2015; Sánchez-Montes et al. 2019). These species are divided into three families: the Ixodidae Koch 1844; the Argasidae Koch 1844; and the Nuttalliellidae Schulze 1935, whose evolution and phylogeny remains unsolved and controversial. This is due to the fact that the molecular data for *Nuttalliella namaqua* Bedford 1931 (a single African species, Nutalliellidae) is lacking, and the male as well as the immature stages were somewhat recently discovered and described (Mans et al. 2011; Lattif et al. 2012) Ticks not only cause physical damage to their hosts (sucking blood and skin injury), but most tick species also have the ability to transmit pathogens to their host. They are the most significant vectors of emerging zoonotic pathogens (viruses, bacteria, protozoa and helminths) which can be severe and life-threatening to humans, second to mosquitoes in the number of human diseases they transmit (Kho et al. 2015, Luz et al. 2018). The population of ticks in any region depends upon various factors such as climate, the presence of predators, and competitor species. Some tick species formed a very close relationship with their vertebrate hosts, with a marked preference for reptiles and amphibians. There are other tick species that are regarded parasites of mammals or birds, but they can also parasitize reptiles or amphibians if their natural hosts are not available, such as *Amblyomma mixtum* Koch 1844 and *Amblyomma maculatum* Koch 1844 (Sánchez-Montes et al. 2019). However, in this situation it is not known whether the host shift benefits these species as well.

Ticks usually have several life stages (larvae, nymphs and adults) that are known to remain attached to their reptilian hosts for more than two weeks (Apperson et al. 1993; Padgett and Lane 2001). According to Guglielmone et al. (2010), ticks affect wildlife and domestic animal management across the world, with approximately 896 species of tick in three families being documented. Studies on ticks of South Africa began almost 200 years ago and since then over 80 tick species have been identified and documented (Walker 1991). To date, more than 100 hard and soft tick species belonging to eight genera of the families Ixodidae and Argasidae have been collected from reptiles around the world (Pietzsch et al. 2006). The above tick families have a wide geographical distribution and are described from various vertebrates. However, in many cases reptiles are the definitive hosts of ticks. There are few documented cases when all stages are found exclusively on one reptile species (monoxenous parasitism) (Hoogstraal et al. 1973; Fajfer 2012). In 2001, Parola and Raoult (2001) documented that in the most frequently used classification of ticks, the family ixodidae includes two significant groups, the Prostriata and the

Metastriata, while the Argasidae currently has two subfamilies known as the Argasinae and the Ornithodorinae .

The two groups of Ixodidae differs in their morphology, physiological, phylogenetic, as well as some other characters. According to studies of valid names, A*mblyomma* Koch 1844 was documented as the second largest group of Ixodidae ticks with 130 valid species associated with birds, reptiles, mammals and rarely amphibians as hosts, distributed worldwide, especially in tropical and subtropical areas (Barker and Murell, 2004; Guglielmone et al., 2014). Of the above mentioned number of *Amblyomma* species in the world, nine species, including three species that belonged to the former genus *Aponomma* Neumann 1899 have been reported in South Africa (Walker, 1991; Horak et al., 2006).

The taxonomy of species of the *Amblyomma* genus is currently being revised. It has long been recognized that *Amblyomma* species are closely related to the recently extinct genus "*Aponomma*" Neumann 1899, both belonging to the subfamily Amblyomminae Neumann 1911 (Hoogstraal and Aeschlimann, 1982). Kaufman (1972) proposed that the genus "*Aponomma*" in its traditional concept consists of three well-defined groups: "typical species", "indigenous Australian species" and "primitive species". The author also proposed that the latter two groups might present new genera. This was verified later as "typical *Aponomma*" and have been reassigned to the genus *Amblyomma*; and from the "indigenous Australian species", a new subfamily Bothriocrotoninae was erected (Klompen et al. 2002). In 2020, Hornok et al. (2020) analysed the phylogeny of two reptilian ticks (*Amblyomma transversale* and *Amblyomma exornatum*) based on both mitochondrial and nuclear genes with the reinstatement of genus *Africanella*, and based on the 16S rRNA gene sequences *A. transversale* formed a sister group to Amblyomminae, Haemaphysalinae and Bothriocrotoninae, with poor bootstrap support between the two latter subfamilies while *A. exornatum* formed a sister group to all other members of the subfamily Amblyomminae (Figure 1-1).

Recently, exotic tick species have been introduced into countries where they do not occur naturally through the international trading of reptiles (Burridge 2001; Pietzsch et al. 2006). These exotic ticks have also introduced tick-borne pathogens (TBPs), which have created a public health threat for both humans and domestic animals in these countries. Tick-borne diseases (TBDs) of domestic animals can substantially affect livestock production, food supply, and the economy of many regions worldwide. TBDs cause production losses mainly as a consequence of infertility, abortions, reduced weight gain, decreased quality and production of milk and mortality. Additionally, costs associated with control and preventive measures (dipping, vaccination, chemotherapy, veterinary services, and monitoring) also contribute considerably to economic losses (Brown 1997). In addition, most of these pathogens pose extremely serious zoonotic threats due to the worldwide distribution of ticks and lack of vaccines availability against these

viruses and other pathogens (Clow et al. 2016). Some of these pathogens, such as *Ehrlichia* (*Cowdria*) Moshkovski 1947*, Hepatozoon* Miller 1908 and *Rickettsia* da Rocha-Lima 1916, have been detected in exotic ticks infesting reptiles.

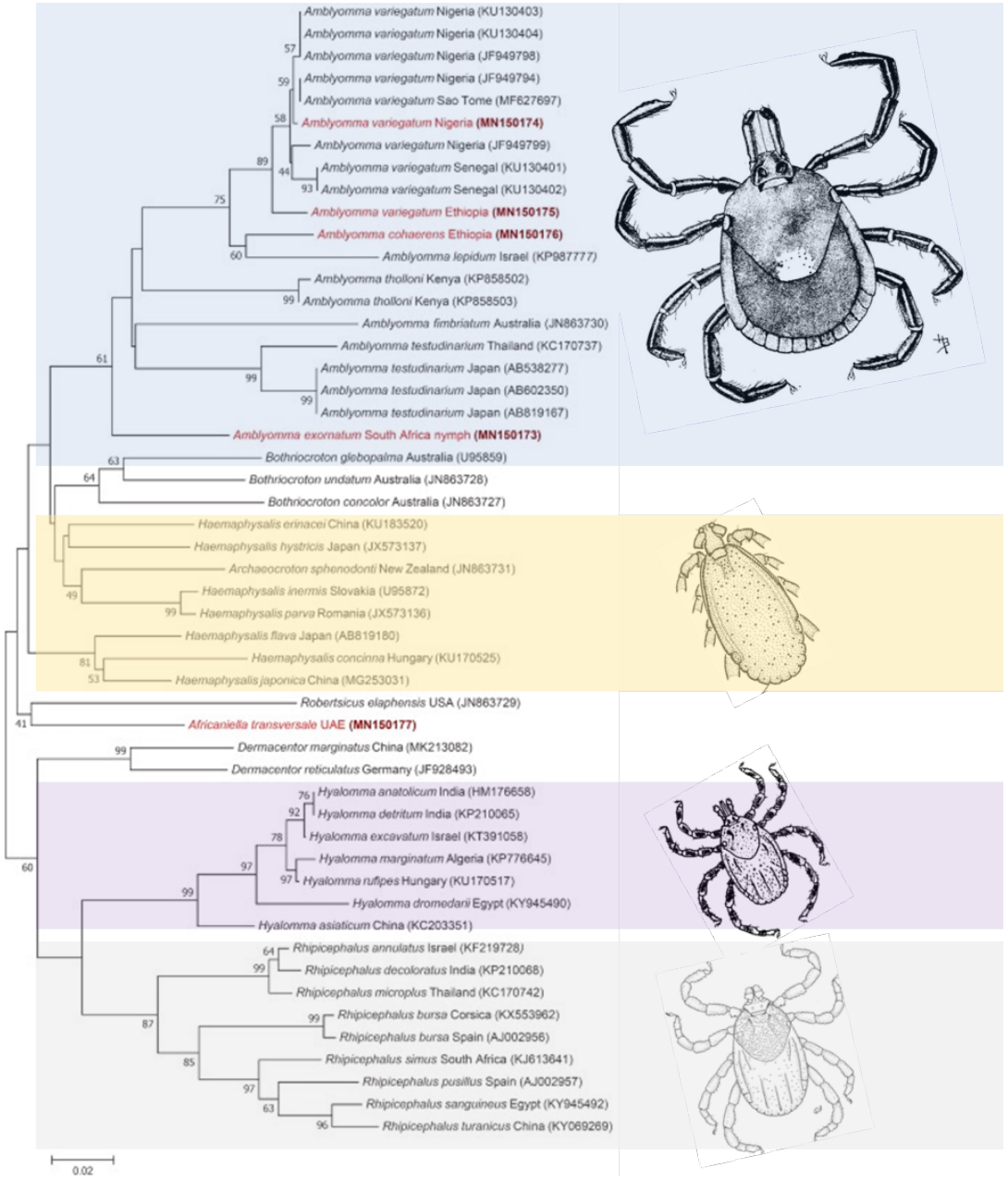


Figure 1-1: The minimum evolution tree of the old word Metastriata based on the 16S rRNA gene sequences (adapted from Hornok et al. 2020).

1.2. Morphological abnormalities in ticks

Morphological abnormalities in ticks are considered peculiar phenomena, and they have been rarely reported in nature (Nowak-Chmura 2012; Keskin et al. 2016). The more frequently observed abnormalities are divided into two groups: General abnormalities (i.e. Dwarfism, gynandromorphism, change in body shape and duplication) and local abnormalities (i.e. asymmetry of spiracle and scutum, malformation of capitulum, and abnormalities of appendages and festoons) (Kar et al. 2015; Keskin et al. 2016). These abnormalities are mainly caused by environmental stress, mutations, host resistance to tick infestation, chemical control of ticks or high relative humidity (Latif et al. 1988; Dergousoff and Chilton 2007; Keskin et al. 2016; Buczek et al. 2019; Shuaib et al. 2020).

Anomalous ticks were first described over 100 years ago with reports of a *Hyalomma aegyptium* Linnaeus 1758 with an extra eye, an unidentified *Amblyomma* sp. Koch 1844 with ectromely, and an *Ixodes hexagonus* Leach 1815 displaying an abnormal tarsus (Neumann 1899). Since then, at low frequencies, different anomalous tick species have been reported from the genus *Amblyomma* Koch 1844*, Dermacentor* Koch 1844*, Hyalomma* Koch 1844*, Ixodes* Latreille1795*,* and *Rhipicephalus* Koch 1844 in Africa, Asia, Europe, and South America (Neumann 1899; Campana-Rouget 1959; Gugliemone et al. 1999; Zharkov et al. 2000; Alekseev et al. 2007; Kar et al. 2015; Keskin et al. 2016; chitimia-Dobler et al. 2017; Balinandi et al. 2019; Shualb et al. 2020; Laatamna et al. 2021; Taank et al. 2021).

1.3. Tick-borne pathogens

Tick-borne pathogens are responsible for some of the most serious emerging infectious diseases facing sub-Saharan Africa (Dunster et al. 2002; Parola et al. 2005; Omondi et al. 2017). The wildlife-domestic-human interaction has the potential to accelerate the spread of zoonotic tick-borne pathogens that cause significant diseases, and eventually death (Uilenberg 1995; Omondi et al. 2017). To date, about 61% of human diseases have been documented to have a zoonotic origin, and wildlife is associated with approximately 75% of emerging zoonotic infections globally (Warwick and Corning 2013). In 2002 and 2003, Perry (2002), and Minjauw and McLeod (2003) have documented that these diseases were ranked high due to their effect on the livelihood of resource-poor farming in developing countries, particularly in parts of Sub-Saharan Africa and other countries where the demand for livestock products is rapidly increasing. Recent studies have implicated reptiles as potential reservoirs involved in the transmission cycles of TBPs (Omondi et al. 2017). *Ehrlichia ruminantium*, considered a pathogen of ruminants responsible for heartwater disease, has been reported in reptile ticks, *Amblyomma sparsum*, *Amblyomma latum* and *Amblyomma exornatum* imported from Africa into Florida, USA (Burridge et al. 2000; Kenny et al. 2004). Reeves et al. (2006) documented a spotted fever group *Rickettsia* in *A. exornatum*

in a reptile breeding facility from the United State, and in 2018, Santodomingo et al. (2018) also found *Rickettsia* in *Amblyomma dissimile* collected from 26 species of reptiles. In addition to these pathogens, *Borrelia* spp. were found in *Hyalomma* ticks and *Ixodes ricinus* associated with a species of *Testudo* tortoise and lizards, respectively (Guner et al. 2003, 2004; Dsouli et al. 2006; Ritcher and Matuschka 2006; Amore et al. 2007). Different *Anaplasma* species were documented in *Amblyomma flavomaculatum,* and *Amblyomma gemma* collected from tortoises and lizards from Poland and Kenya, respectively (Nowak et al. 2010; Omondi et al. 2017). Furthermore, a diverse species assemblage of *Borrelia, Rickettsia, Ehrlichia, Anaplasma,* and *Babesia* have also been identified in lizard species in Portugal, Australia, and the Netherlands, as well as in a variety of reptile species imported into Japan (De Sousa et al. 2012, Whiley et al. 2016; Omondi et al. 2017).

1.3.1. *Borrelia* Swellengrebel 1907

The genus *Borrelia* was originally described in 1907 by Swellengrebel, with *Borrelia anserina* designated as the type species (Skerman et al. 1980; Margos et al. 2018). It comprises three phylogenetic spirochete bacteria groups characterized by reptilian *Borrelia* also known as monotreme associated *Borrelia,* relapsing fever, and the Lyme borreliosis groups (Takano et al. 2010; Mendoza-Roldan et al. 2019). The latter consist of 20 genospecies within the *Borrelia burgdorferi* (*sensu lato*) genogroup worldwide. Nine of these genospecies are recognized to have pathogenic outcomes to humans and/ or animals, and they include *Borrelia afzelii*, *B. bavariensis, B. bissettii, B. burgdorferi (sensu stricto.), B. garinii, B. kurtenbachii, B. lusitaniae, B. spielmanii* and *B. valaisiana.* Lyme borreliosis and relapsing fever genospecies have long been recognized to have different biological, clinical, and epidemiological characteristics, and phylogenetic data is in agreement with this, indicating that these two groups are genetically similar yet distinct, and form independent monophylogenetic sister clades that share a common ancestor (Takano et al. 2011). In 2014, Adeolu and Gupta (2014) proposed the division of *Borrelia* into two genera to reflect the genetic and phenotypic divergence between Lyme *Borrelia* (LB) and Relapsing Fever (RF) species, however, Margos et al. (2017) and Stevenson et al. (2018) indicated that there is inadequate evidence to support this proposal, and it has not been widely utilized in the literature. This proposal was justified according to the identification of conserved signature insertions/deletions and conserved signature proteins that are differentially present in the LB or RF *Borrelia* genogroup, as well as average nucleotide identity values calculated between whole genomes of 18 *Borrelia* species including eight LB species and ten RF species. Although it is uncontested that these differences exist between LB and RF *Borrelia*, Margos et al. (2018) proposed that the methodology used to identify these group-specific differences is subjective and has a highly limited power to delineate these two groups of *Borrelia* into separate genera. The data presented in the methodology employed by Adeolu and Gupta (2014) misrepresents the

extent of genomic divergence between the two groups of *Borrelia* and fails to consider widespread genomic similarities between these two groups. In addition, although Konstantinidis and Tiedje (2005) has previously used average nucleotide identity (ANI) to investigate the taxonomy of prokaryotes, a comprehensive review of this method revealed that although ANI can accurately quantify the genetic relationships between strains belonging to the same species, it was not suitable to differentiate prokaryotic genera. This is due to significant overlapping of intergenera ANI and interspecies ANI values, which leads to unreliability in the method (Qin et al. 2014).

1.3.1.1. Relapsing fever *Borrelia*

In Africa, relapsing fever borreliae are neglected vector-borne pathogens usually transmitted by ticks, except *Borrelia recurrentis*, which is transmitted by louse flies (Cutler et al. 2009). They are responsible for various febrile presentations and are most commonly suspected in malaria-like symptoms. According to Lafri et al. (2017), tick-borne relapsing fever has been recognized as a major cause of disease and mortality in most parts of Africa. To date, in North Africa, *Borrelia crocidurae* and *Borrelia hispanica* are the two documented species of tick-borne relapsing with *Ornithodos erraticus* as a vector of *B. hispanica*, an uncultured bacterium known as *Borrelia merionesi* have also been detected in *Ornithodoros* ticks from Morroco, and Candidatus *Borrelia algerica* from febrile patients in Algeria (Rodhain 1976; Bouattour et al. 2010). In eastern (such as Kenya and Tanzania) and southern Africa (such as Mozambique, Namibia and South Africa), relapsing fever is caused by *B. duttonii* transmitted by *Ornithodoros moubata* (Leeson 1952; Fukunaga et al. 2001; Walker 2003). The two vector species of the *O. moubata* complex (*O. moubata s.s.* and *O. porcinus*) have been documented from various wild animals such as antbears, warthogs and porcupines (Walton 1962). In West Africa, the causative agent of relapsing fever is known as *B. crocidurae* with *O. onsai* as a vector (Schwan et al. 2012).

1.3.1.2. Lyme *Borrelia*

Lyme borreliosis was first described in the 1980s with *Borrelia burgdorferi* as a causative agent (Johnson et al. 1984). The disease is now considered the most important vector-borne disease in most countries, including Europe and USA.

1.3.1.3. Reptile-associated-*Borrelia*

In 2010, Takano et al. (2010) identified reptile-associated-*Borelia* spp. as a novel group of *Borrelia* spp. from imported reptiles and/or their ectoparasites, although this group have been found to be associated with the Monotremata (Panetta et al. 2017; Loh et al. 2017; Gofton et al. 2018). The transmission of these species is thought to be through hard-bodied ticks belonging to genera *Hyalomma* or *Amblyomma,* while they appear to have evolutionarily diverged from an ancestor shared with RF *Borrelia* spp. (Takano et al. 2010). *Borrelia turcica*, an Echidna-Reptile group

(REPG) member was initially discovered in *Hyalomma aegyptum* ticks collected from the order Testudines in Turkey (Güner et al. 2003, 2004) and it was recently detected in blood and multiple organs of reptiles, and in *Amblyomma* and *Bothriocroton* ticks infesting several reptiles (Takano et al. 2010, Takano et al. 2011; Panetta et al. 2017). This group has been demonstrated to form a distinct monophyletic group and showed a relationship with the other two *Borrelia* groups. In recent studies, Güner et al. (2003,2004) and Takano et al. (2010,2011) demonstrated that *Borrelia turcica* ITS7 was distinct but branched off and clustered among the LB and RF *Borrelia* species based on the analysis of 16S rRNA, *flaB* and *gyrB* genes, respectively. Reptile-associated-*Borrelia* isolated from *Amblyomma* ticks clustered with the RF *Borrelia* based on a phylogenetic analysis study by Takano et al. (2011). However, in 2014, based on the analysis of 16S rRNA gene, Adeolu and Gupta (2014) grouped reptilian *Borrelia* species in a third distinct clade (Figure 1-2).

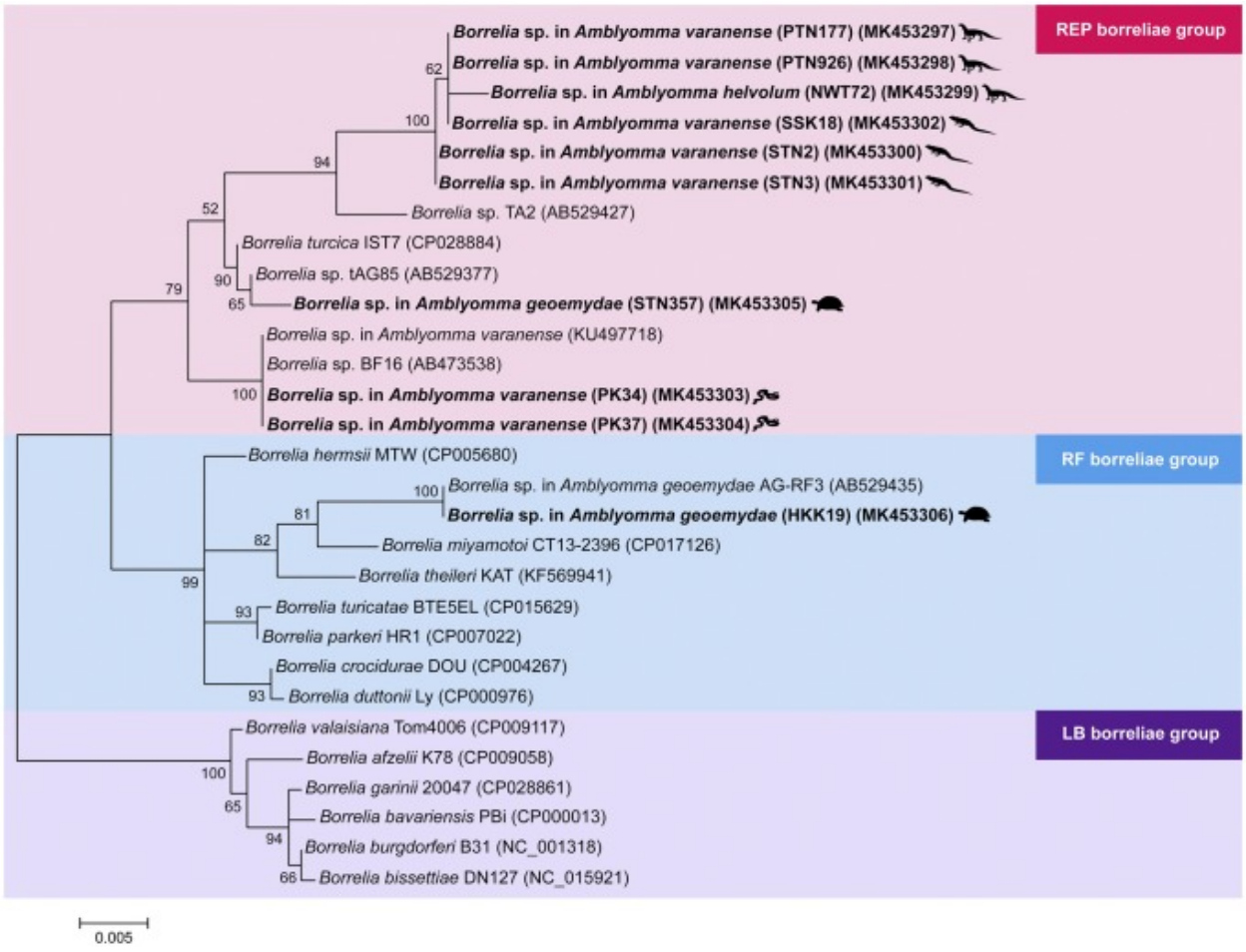


Figure 1-2: A maximum likelihood phylogeny of *Borrelia* species based on 16S rRNA gene sequences. Sourced from Kaenkan et al. (2020), with permission.

1.3.2. *Coxiella* Phillip 1943.

Coxiella burnetii is a causative agent of the zoonotic bacterial pathogen (Q fever) that most commonly infects a variety of mammals throughout the world, except New Zealand. In South Africa, reports on the molecular prevalence of *Coxiella burnetti* are limited, with few studies reporting on the molecular prevalence of this bacteria in various tick species (such as *Amblyomma* spp., *Haemaphysalis* spp., and *Rhipicephalus* spp.) and livestock (Mtshali et al. 2017; Halajian et al. 2018; Mangena et al. 2021; Sadiki et al. 2023). Furthermore, sero-prevalence of *C. burnetti* in South Africa has been reported in domesticated animals (including cats, cattle and sheep), as well as in humans (Gear et al. 1950; Ranking et al. 1950; Summow et al. 1987; Matthewman et al. 1997; Adesiyun et al. 2020; Mangena et al. 2021; De Boni et al. 2022). *Coxiella* is a gram-negative bacterium that replicates as an obligate intracellular pathogen under acidic and microaerophilic conditions in phagolysosome-like compartments of cells, predominantly macrophages (Baca et al. 1994). Transmission routes of *Coxiella* to vertebrate hosts mainly occurs through inhalation of contaminated dust and aerosols (Melenotte et al. 2016), direct contact with milk, urine, faeces, or semen from infected vertebrate hosts, has been reported (Tissot-Dupont and Raoult 2008). During infection, *C. burnetii* affects the mammary gland, and the reproductive system in animals causing stillbirths and miscarriages. Farmers and veterinarians are especially at risk of contracting this disease through contact with infected animals and their products due to the low infective dosage by inhalation (<10 bacterium). (Schimmer et al. 2014; Groten et al. 2020). When exposed to environmental stressors such as heat or desiccation, *C. burnetii* generates spore-like forms that are resistant. As a result, the bacteria can persist in the soil or other dry materials for extended periods of time under unfavorable conditions (Evstigneeva et al. 2007).

Despite the fact that for a long time, *C. burnetii* was the only member of the genus *Coxiella* that was known, recent studies have discovered a group of closely related bacteria known as *Coxiella*-like endosymbionts (CLEs) (Zhong et al. 2012; Seo et al. 2016; Mofokeng et al. 2022). A number of recent studies have indicated that *Coxiella*-like endosymbionts with different genome sizes and gene content shows up to 97% genome identity with the pathogenic *C. burnetii* (Zhong et al. 2012; Seo et al. 2016; Tsementzi et al. 2018). Genetically, CLEs are classified into four clades (A–D), and *C. burnetii* belongs to clade A, which otherwise contains CLE associated with soft ticks (Duron et al. 2015) (Figure1-3).

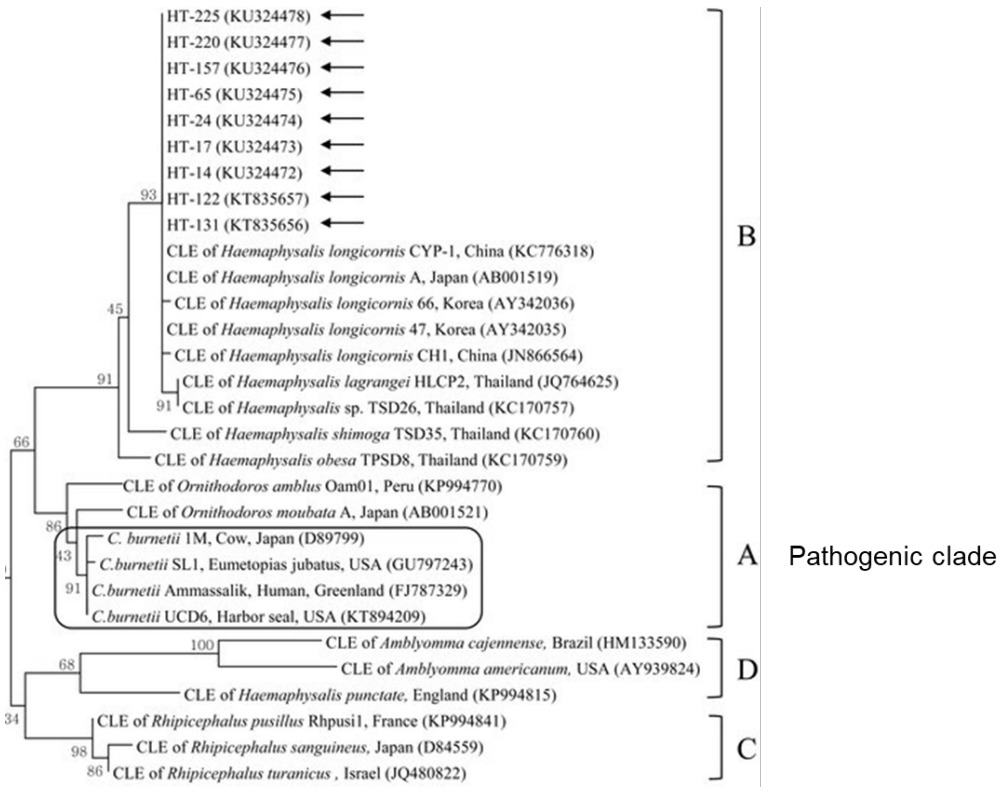


Figure 1-3: A phylogenetic tree construsted using the maximum likelihood method based on the 16S rRNA sequences of the genus *Coxiella.* CLE=*Coxiella*-like endosymbionts (Adapted from Seo et al. 2016).

1.3.3. *Ehrlichia* Moshkoviski 1947

Members of genus *Ehrlichia* (Anaplasmataceae) are obligate intracellular Gram-negative tick-borne bacteria. The bacteria mainly infect granulocytes or monocytes in the peripheral blood of animals and humans, and they are causative agents of veterinary important diseases and emerging human zoonoses on a global scale, collectively known as ehrlichioses (Ndip et al. 2010; Esemu et al. 2011). Over the last few decades, ehrlichial infections have been a subject of increasing interest from both the veterinary and public health pesperctives (Walker and Dumler 1996). According to the latter authors, the identification of *Ehrlichia* began in 1910 when the description of *Anaplasma marginale* was documented by Theiler. Theiler described this species as the etiologic agent of an economically important severe worldwide disease of cattle. To date, the genus comprises of five recognized species namely *Ehrlichia chaffeensis, Ehrlichia ewingii, Ehrlichia canis, Ehrlichia ruminantum,* and *Ehrlichia muris* (Dumler et al. 2001). All these species are atleast 97.7% and 86.3% similar in 16S RNA and *groESL* operon sequences, respectively (Van Vliet et al. 1992; Dumler et al. 2001). When investigated, the organisms have several similar genes encoding surface protein antigens that range in molecular size from about 28–32 kDA. The main characteristics of these species are shown in Table S2.

Ehrlichia species are biologically transmitted by Ixodid ticks. *Ehrlichia canis* is transmitted by *Rhipicephalus sanguineus* ticks, which occurs worldwide (Ndip et al. 2005). However, molecular

evidence of *E. canis* DNA have been recently documented from various other *Rhipicephalus* species ticks (*Rhipicephalus pravus, Rhipicephalus evertsi evertsi* and *Rhipicephalus pulchelus*) sampled from livestock and domestic dogs, and also from an *Amblyomma latum* tick infecting a monitor lizard in Kenya (Omondi et al. 2017). *Ehrlichia chaffeensis* is mainly transmitted by *Amblyomma americanum*. However, the pathogen has also been reported in *H. longocornis*, and *Ixodes persculatus*, *R. sanguineus* from Cameroon, and *Haemaphysalis yeni,* and *Amblyomma testudinarium* ticks from southern China, (Kawahara et al. 1999; Cao et al. 2000; Ndip et al. 2010). *Ehrlichia ewingii* is known to be transmitted by *A. americanum.* Following the detection *E. ewingii* DNA outside its known range, it was postulated that other ticks are also likely to act as vectors of *E. ewingii* (Ndip et al. 2005). *Ehrlichia muris* is transmitted by *H. flava*. Although it is still unknown whether *I. ovatus* is a vector of *E. muris* or not, *E. muris*-like microoganisms have been isolated and/or genetically detected from *I. ovatus* in Japan (Kawahara et al. 1999). Thirteen species of *Amblyomma* ticks have been documented to be the only vectors known to transmit *E. ruminantum* naturally or experimentally. On a global scale, the most important vectors of *E. ruminantum* are the two African ticks (*A. hebareum* and *A. variegatum*) (Bezuidenhout and Jacobsz 1986).

1.3.4. *Anaplasma* Theiler 1910.

Anaplasma species (order Rickettsiales, family Anaplasmataceae) are tick-transmitted intracellular bacteria that infect a multitude of wild and domestic animals, as well as humans (Han et al. 2019). The genus is comprised of various species of veterinary and public health, including *Anaplasma ovis, Anaplsma bovis, Aanaplsma marginale, Anaplasma phagocytophilum, Anaplsma platys,* and *Anaplsma centrale* (Dumler et al. 2001; Li et al. 2015). The main characteristics of these species are listed in Table S3. These pathogens are usually transmitted by Ixodid ticks to mammals; additionally, blood transfusion can be another direct transmission route to both animals and humans (Fine et al. 2016; Marenzoni et al. 2017; Zaid et al. 2019). In terms of phenotypic and genotypical charateristics, *A. marginale* is the most distinct species in the genus, as a matter of fact, some authorities consider it to qualify for a separate genus position (Garrity et al. 2000).

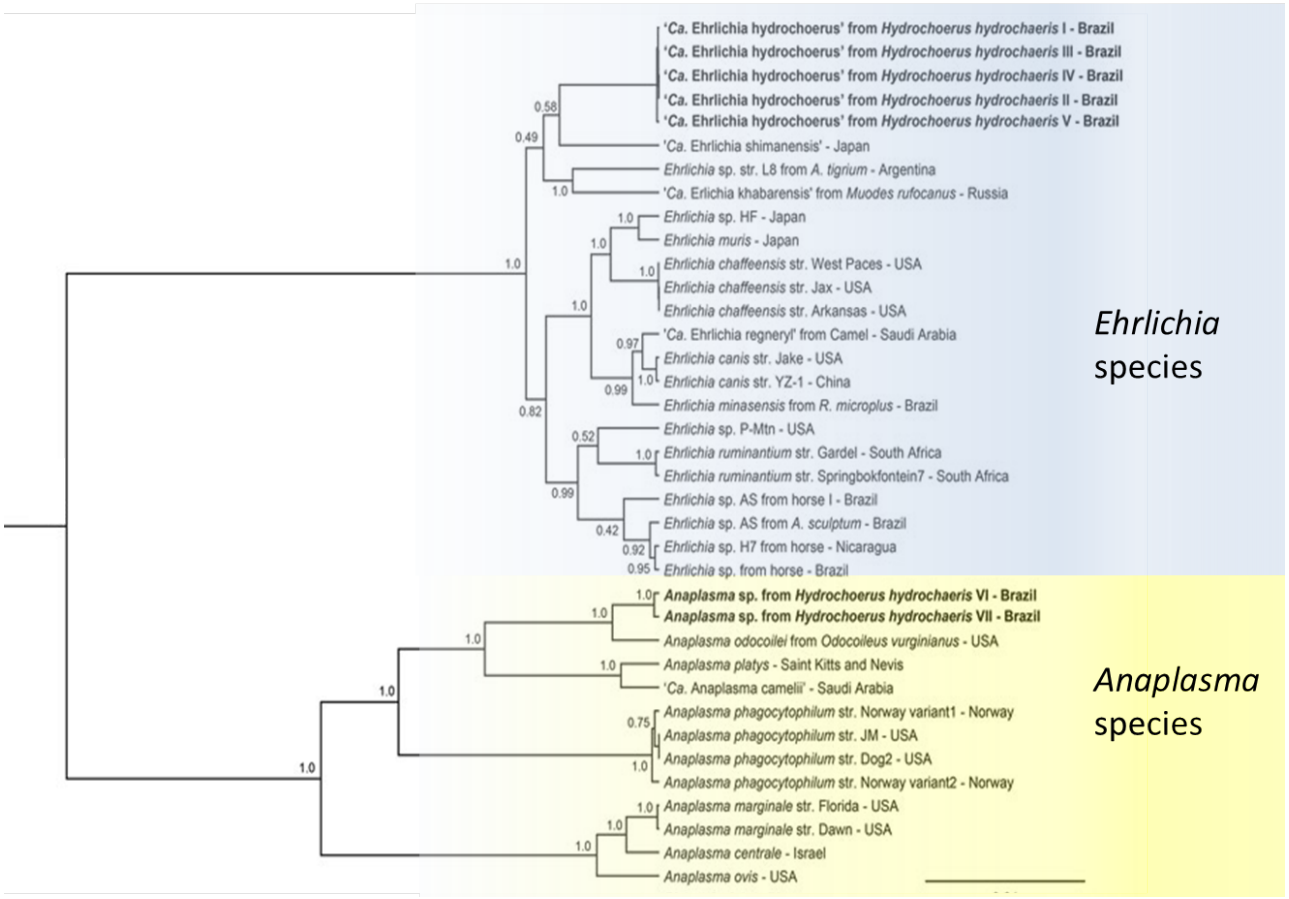


Figure 1-4: A concatenated Bayesian inference phylogenetic analysis of 16S rRNA, *sodB*, and *groEL* partial sequences of *Ehrlichia* and *Anaplasma* species (Adapted from Vieira et al. 2022).

1.3.5. *Rickettsia* da Rocha-Lima 1916

Rickettsia species (Order Rickettsiales, Family Rickettsiaceae) are gram-negative bacteria that have an obligate association with eukaryotic cells. Phylogenetically, they are a well-defined group of bacteria in the class α-proteobacteria (Roux and Raoult 1995; Kichuki et al. 2002). This bacterial group consist of both animal and human pathogens transmitted mostly through arthropod bites, mainly infect endothelial cells, and can cause a range of mild to fatal diseases such as epidemic typhus and Rocky Mountain spotted fever. Phylogenetically, *Rickettsia* species are divided into two groups: the typhus group (TG), which includes *Rickettsia typhi* and *Rickettsia prowazekii,* and their transmission is associated with fleas, and contact with infected faeces of the human body louse, respectively; The spotted fever group (SFG) consists of *Rickettsia aeschlimannii, R. akari*, *R. africae, R. felis, R. conorri, R. australis, R. helvetica, R. honeii, R. japonica*, *R.massiliae*, *R.montanensis*, *R. parkeri*, *R. peacockii*, *R. rhipicephali*, *R. slovaca* , *R. sibirica* and *R. rickettsii* (Bouyer et al. 2001). In South Africa, four tick-borne spotted fever group (SFG) *Rickettsia* spp. (*Rickettsia africae, R. conorii conorii, R. aeschlimannii,* and *R. sibirica mongolitimonae*) have been associated with human diseases (Pretorius and Birtles 2002, 2004; Parola et al. 2013) (Figure 1-5). Stothard et al. (1994) documented that the last group is a representative of early divergent lineages within the genus, thus referred to as the ancestral group, which consists of the AB male-killing bacterium, *R. bellii* and *R. canadensis*. Reportedly, *R. honei*, the etiological agent of Spotted fever of Flinders Island was the first *Rickettsia* species recorded to be associated with amphibians and reptiles. It was detected in several *Bothriocroton hydrosauri* collected from *Tiliqua nigrolutea*, *Austrelaps superbus*, and *Notechis scutatus* from Australia (Stenos et al. 2003). Following the latter findings, records of *Rickettsia* species documented from ticks feeding on reptiles and amphibians has increased 10 times (Sanchez-Montes et al. 2019). One species from the ancestral group (*R. bellii*), nine from the spotted fever group such as *R. africae* and *R. honei*, and a typhus group member (*R. typhi*) were included from these records.

Notwithstanding the considerable increase in the number of named SFG species, there is no consensus corncerning the criteria used to define *Rickettsia* species. The use of 16S rRNA gene show 97.2–99.9% similarity among the SFG rickettsiae. Thus, according to the standards used to classify other bacteria, many designated species of SFG rickettsiae actually might be genotypes or strains of a single species (Garrity et al. 2000).

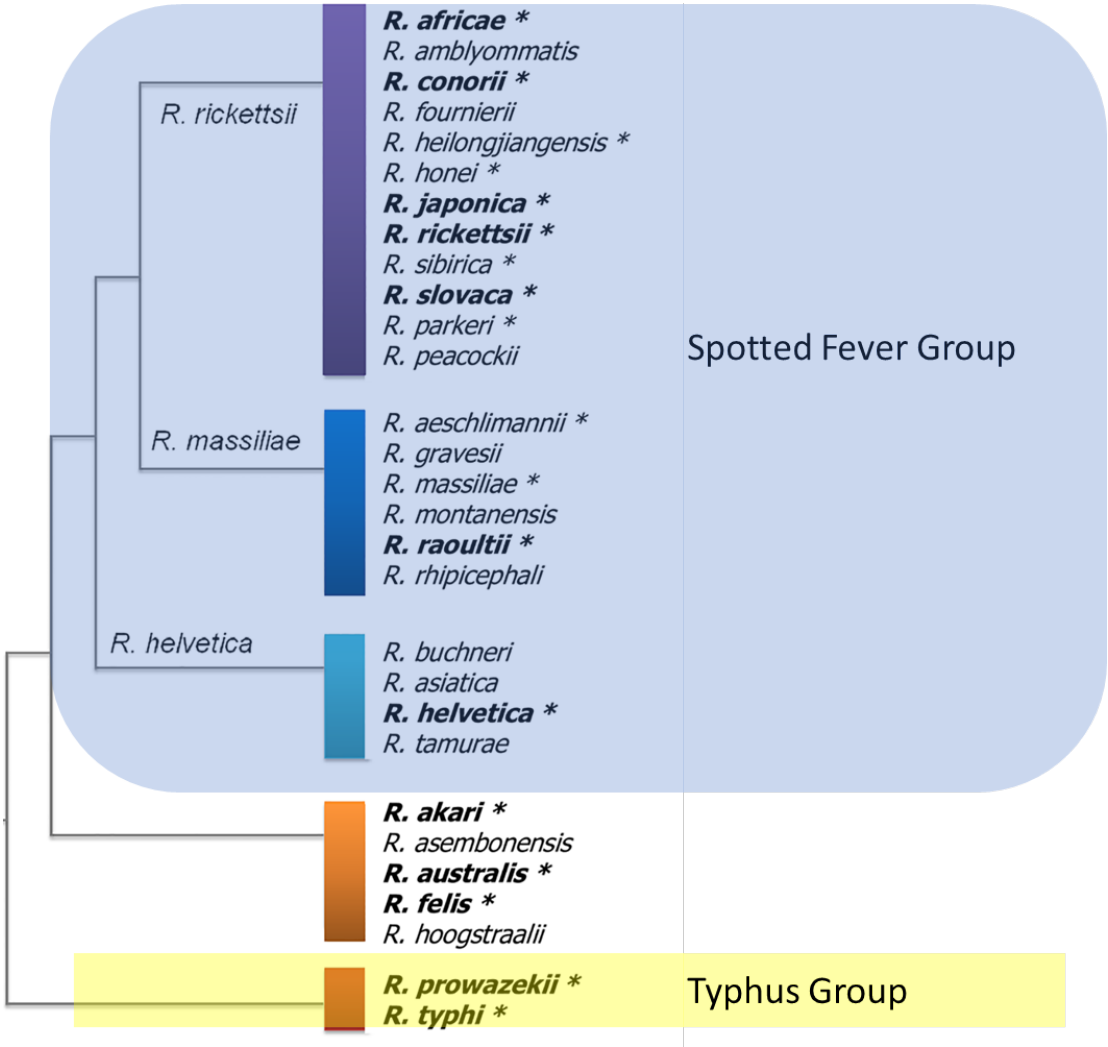


Figure 1-5: A concatenated phylogenetic tree of gltA and sca4 gene sequences of Rickettsia species. Asterisks represent pathogenic *Rickettsia* while bold indicates *Rickettsia* causing central nervous system infections (adapted from Sekeyová et al. 2019).

1.4. Tick-borne endosymbionts

Ticks are extensively studied due to their roles as vectors of zoonotic pathogens (Dantas-Torres et al. 2012). However, in addition to pathogens, most tick species also harbor a diverse microbiome that includes symbiotic and commensal bacteria (Duron et al. 2017; Daveu et al. 2021; Kobayashi et al. 2021; Mofokeng et al. 2022). Tick endosymbionts have gained interest over the last decade and are now being investigated due to their physiological significance in ticks (Kobayashi et al. 2021). Transovarial transmission is the main route of transmission for symbionts, however, horizontal transfer through co-feeding also plays an important role (Nardi et al. 2021). Moreover, closely related species of ticks and even different individuals of the same species, may harbor different bacterial endosymbionts (Duron et al. 2017). These endosymbionts are involved in the reproduction and growth of ticks by providing these arthropods with necessary compounds that are absent in their haematophagous diet (Rio et al. 2016; Nardi et al. 2021). Furthermore, they are also involved in the maintenance of pathogens in arthropods (Moreira et al. 2009). To date, atleast ten genera of symbionts have been documented in ticks, three genera of these symbionts (*Coxiella*-like endosymbiont, *Franciella*-like endosymbionts, and *Midichloria*) have been documented to be exclusive to ticks. During their persistent co-ecolution with their hosts, symbionts have developed strategies to multiply within their hosts, and some of these symbionts are mutualistic, whereas others may be facultative (Diaz-Sanchez et al., 2019; Daveu et al. 2021).

1.5. Haemoparasites

Haemoparasites are a phylogenetically diverse group of organisms parasitizing different vertebrate hosts across the world, and are important human and animal pathogens (Concannon et al. 2005). The reptilian haemoparasites represent a rather unexplored field; however, in 2009, Telford (2009) indicated that the diversity in recorded infections is greater than the haemoparasite infections of other vertebrates such as mammals and birds. Major factors contributing to greater taxonomic diversity often include restricted habitats and lower vagility of terrestrial reptiles, greater phyletic age of reptiles may be another factor influencing this diversity (Telford 2009).

According to Akande et al. (2010), haemoparasites are responsible for the destruction of red blood cells, which results in anorexia, infertility, anaemia, and jaundice. Infections with these blood parasites are very common in reptiles across the world (Smallridge and Bull 2000; Cook et al. 2010). Not only have parasites of the phylum Apicomplexa and class Kinetoplastea been recorded to occur in reptiles, but also filarid nematodes have frequently been reported in lizards, snakes, and chelonians (Tenter and Schnieder 2006; Ursula et al. 2014). In South African reptiles, a number of taxa of Apicomplexa have been described from the genera *Plasmodium*, *Haemoproteus*, *Hepatozoon*, *Hemolivia*, *Karyolysus* and *Haemogregarina* (Pienaar 1962;

Telford 2009; van As et al. 2013, 2016; Cook et al. 2010, 2014, 2015, 2016, 2018). According to Pienaar (1962), *Plasmodium zonuriae* was the first saurian malaria parasite described in South Africa from cordylid lizards collected in North West province. However, by 2009, Telford (2009) described and reported two species of *Plasmodium* known to parasitize cordylid lizards of South Africa, namely *P. zonuriae* from both *Pseudocordylus microlepidotus* and *Cordylus vittifer*, and *Plasmodium cordylii* from *Cordylus vittifer*. Van As et al. (2016) also described and reported a new *Plasmodium* species parasitizing cordylid lizards of South Africa, namely *Plasmodium intabazwe* from *Pseudocordylus melanotus*. Only two species of *Haemoproteus* have been described to date from South African reptiles, *Haemoproteus natalensis* (Cook et al. 2010), and *Haemoproteus testudinalis* (Laveran 1905) from species of *Kinixys* and *Stigmochelys* (Cook et al., 2010). To date, only a few species of *Hepatozoon* have been described and reported from reptiles of South Africa. These include *Hepatozoon langii* and *Hepatozoon vacuolatus* (van As et al. 2013) from *Pseudocodylus langi* (Cordylidae), *Hepatozoon affluomaloti* van As et al. (2013) from *Pseudocodylus melanotus* and *Pseudocodylus subviridis* (Cordylidae), *Hepatozoon varani* (Laveran 1905) from *Varanus niloticus,* and *Hepatozoon fitzsimonsi* (Dias 1953) from five different species of tortoises including *Kinixys lobatsiana, Kinixys zombensis, Stigmochelys pardalis, Chersina angulata* and *Kinixys natalensis.* The only species of *Hepatozoon* described to date from South African snakes are *Hepatozoon refrigens* (Sambon and Seligmann 1907) from *Pseudaspis cana, Hepatozoon bitis* (Fatham 1925) from *Bitis arietans, Hepatozoon angeladaviesae* (Cook et al. 2018) from three species of Colubridae including *Philothamnus semivariegatus, Philothamnus hoplogaster* and *Philothamnus natalensis natalensis,* and *Hepatozoon cecilhoarei* (Cook et al. 2018) from *Philothamnus natalensis natalensis.* To date, only a single species of *Hemolivia* and *Karyolysus* respectively has been described from African reptiles, *Hemolivia parvula* (Dias 1953) from *K. zombensis* (Cook et al. 2015) and *Karyolysus paradoxa* (Dias 1954) from *Varanus niloticus* and *Varanus albigularis* (Cook et al. 2016). *Haemogregarina*, even though at one stage a genus with many members infecting reptiles, many of these species were transferred to the genus *Hepatozoon*, leaving only one species described from South African reptiles, *Haemogregarina pelusiensi* Pienaar 1962 from *Pelusios sinuatus* (Smith 1996; Telford, 2009). Regarding the class Kinetoplastea, only one species has been described infecting reptiles, *Trypanosoma mocambicum* (Pienaar, 1962) from *P. sinuatus* (Dvořáková et al. 2015). With regard to filarial nematodes, McAllister et al. (2010) documented that the first record of reptilian nematode, *Ascaris attenuate* (currently *Polydelphis anoura*) was reported by von Linstow (1899) from *Bitis arientans*. In 2009, La Grange et al. (2009) reported *Trichinella zimbabwensis* from *Codylus niloticus* in South Africa. To date, to our knowledge, there are currently 53 species of nematodes within nine families reported to occur in South Africa (Hering-Hagenbeck and Boomker 2000; Mc Allister et al. 2010).

1.5.1. *Hepatozoon* Miller 1908

Members of *Hepatozoon* (Miller 1908) are very common intracellular protozoans found in squamates and other vertebrates such as mammals and amphibians (Telford 2009; Harris et al. 2011). To date, more than 30 species of *Hepatozoon* have been documented from reptiles throughout Africa (Van As et al. 2015; Cook et al. 2016; Cook et al. 2018). Over the past decade several phylogenetic studies have provided useful insight into the evolutionary relationships of adeleorinid parasites, as well as better capability to distinguish between species, using 18S rRNA gene sequences. Recent estimated relationships using the 18S rRNA gene sequences have indicated that *Hepatozoon* is paraphyletic, with genus *Karyolysus* as well as genus *Hemolivia* forming a lineage within *Hepatozoon* (Barta et al. 2012; Haklová-Kočíková et al. 2014; Kvičerová et al. 2014; Cook et al. 2016; Netherlands et al. 2018) (Figure 1-6). Almost a decade ago, a new genus, *Bartozoon* Karadijian et al. 2015, was proposed as a part of taxonomic revision in an effort to resolve the polytomies between predicted phylogeny and the current taxonomy of these haemogragarines (Karadijian et al. 2015; Borges-Nojosa et al. 2017; Netherlands et al. 2018; Cook et al. 2018). This new erected genus was proposed for *Hepatozoon* species previously regarded to parasitize amphibians, marsupials, avian, rodents and reptiles, and was proposed to be solely transmitted by biting insects. However, it has been documented that the life history of some species such as *Hepatozoon fitzsimonsi* (Dias 1953) within the new genus does not correlate with the defining characteristic of *Bartazoon* (Cook et al. 2014; Karadijian et al. 2015; Netherlands et al. 2018). As Maia et al. (2016b) has also pointed out, it is possible that *Hepatozoon perniciosum* Miller 1908, the type species of *Hepatozoon,* may in fact form part of the newly erected genus *Bartazoon*, as do most other rodent haemogregarine species. Furthermore, several analyses using a single gene continues to identify new genetic lineages, indicating that *Bartazoon* is not a well-supported group (Maia et al. 2016a; Tomé et al. 2016). Thus, according to Leveille et al. (2014) and Netherlands et al. (2018), the deeper taxonomic revision of haemogregarines based on their phylogenetic affinities and life histories requires more studies. These studies should involve the use of fast-evolving markers such as mitochondrial genes encoding cytochrome c oxidase subunit 1 (CO1) and cytochrome B (cyt-b), elucidating life cycles, and building larger datasets. The sequence variability of these fast-evolving markers is adequate enough to efficiently delineate recent speciation events, and may complement nuclear genes such as 18S rDNA, which are known to be more-slowly divergent (Pawlowski et al. 2012; Leveille et al. 2014). However, as suggested and recommended by Maia et al. (2016b) and Borges-Nojosa et al. (2017), we will conservatively continue to refer throughout this thesis to species parasitizing reptilian hosts as species of *Hepatozoon* and not *Bartozoon*.

Within the family Hepatozoidae, the majority of *Hepatozoon* species are characterized by the presence of intraleucocytic and intraerythrocytic gamonts in the vertebrate host, including reptiles.

Merogonic division lacking within the red blood cells, but usually occur in vascular endothelial cells (Telford 2009). Furthermore, another defining characteristic of *Hepatozoon* species is the syzygy of gamonts in the vector following a blood meal. The development of sporogony and formation of large oocysts ultimately occur following the penetration of the paired gamonts through the gut wall to the haemocoel (see van As et al. 2015). These large polysporocytic oocysts contains the infective stages (sporocysts containing four to 16 or more sporozoites), which erect upon ingestion by the vertebrate hosts and produce macro- and micromeronts typically in the liver (Smith 1996; Barta 2000; Van As et al. 2015).

The transmission of *Hepatozoon* sp. to the vertebrate host occurs through either ingestion of infected vector or through predation on another vertebrate host with tissue cysts (Davis and Johnston 2000). Most studies on the life cycle of *Hepatozoon* species parasitizing reptiles have been conducted using cullicidae (*Culex, Aedes*, and rarely *Anopheles* species) as possible definitive hosts. There are apparently eight vector species that have been documented to be naturally infected by either *Hepatozoon* oocysts or sporocysts of reptilian hosts (Telford 2009). These documented invertebrate vectors include siphonapterans, ixodid and argasid ticks, dipterans, hirudineans, anopleurans, mites, and assassin bugs. Prior to studies by Sloboda et al. (2007) and Netherlands et al. (2014), most descriptions of *Hepatozoon* sp., particularly those parasitizing reptiles and amphibians were based on the morphology of peripheral blood gamont stages. However, a number of *Hepatozoon* species are difficult to assess just by the use of morphological identification methods, because many of these species are morphologically identical and are able to infect various hosts (Telford 1984, Harris et al. 2011). Unfortunately, many of these descriptions without an account of a corresponding life cycle are controversial. In particular, little research has been done to identify the final host of haemogregarines from reptiles, for example, haemotophagous arthropods or their route of transmission among hosts (Telford et al. 2012; Van As et al. 2015).

1.5.2. *Hemolivia* Petit et al. (1990)

Members of *Hemolivia* Petit et al. 1990 have only been recently discovered as compared to other haemogregarine genera of different families (Haemogregarinidae, Hepatozoidae and Karyolysidae) (Cook et al. 2015). To date, only four species have been documented from ectothermic vertebrates. These include *Hemolivia mariae* from *Tiliqua rugosa, Hemolivia stella* (the type species) from *Rhinella marina, Hemolivia. mauritanica* from Testudo graeca, and *Hemolivia parvula* from *Kinixys zombesis* (Sergent and Sergent 1904*;* Petit et al. 1990; Smallridge and Paperna 1997; Cook et al. 2015). Even though the genus *Hemolivia* is well-defined both morphologically and biologically, its position within the haemogregarines remains unclear due to the fact that it has been largely neglected in terms of phylogenetic analysis (Kvičerová et al. 2014). To date, no obvious clinical signs of disease caused by genus *Hemolivia* have been observed,

however liver degeneration and nephrotic syndrome may develop in cases of prolonged invasion (Słodki et al. 2011). Barta et al. (2012) presented a phylogenetic tree in which *Hm. marinae* clustered within the genus *Hepatozoon* as a sister group of *Hepatozoon* species (GenBank acc. No. EU430236) from *Liasis fuscus*. This alignment raises some serious questions regarding the monophyly and phylogenetic position of *Hemolivia*. The short distance between *Hemolivia* and closest *Hepatozoon* is an indication that there is a relatively recent split between these two lineages. According to Kvičerová et al. (2014), this is an implication that the genus *Hemolivia* with its current distribution and diversity, is either a new group or a non-monophyletic assemblage of morphologically defined organisms. However, the monophyly of genus *Hemolivia* can certainly not be considered as impossible based on the position of one sample. Furthermore, it was admitted by Barta et al. (2012) that the closest relative to *Hemolivia mariae* in their tree may have been determined incorrectly and could present another *Hemolivia* rather than *Hepatozoon*. So, to date, there are various possible situations regarding the evolution and phylogeny of genus *Hemolivia*. These situations include *Hepatozoon* and *Hemolivia* as two monophyletic sister groups, polyphyly of *Hemolivia,* and the paraphyly of genus *Hepatozoon* with respect to a monophyletic genus *Hemolivia* (Kvičerová et al. 2014).

The genus *Hemolivia* is characterized by occurrence of merogony in the reticulo-endothelial cells and red blood cells while formation of cysts exists in the parenchymatous organs such spleen, liver and kidney of their vertebrate host, with gamonts occurring in the peripheral blood erythrocytes and developing into stain-resistant capsules on maturity (see Široký et al. 2007; Cook et al. 2014). Additional characteristics include the presence of typical star-shaped oocysts within the intestinal cells of the vector during sporogony (Davies and Johnston 2000). However, for the African species, *Hemolivia mauritanica,* this characteristic has not yet been demonstrated (Telford 2009; Cook et al. 2015). *Hm. mauritanica* was first described as *Haemogregarina mauritanica* Sergent and Sergent 1904, from *Testudo graeca.*

According to Petit et al. (1990) and Davies and Johnston (2000), the parasites of genus *Hemolivia* can be transmitted through all the known transmission modes of haemogregarines. These include ingestion of sporocyst-infected invertebrate hosts, predation on vertebrate host with cysts containing cystozoites, or directly from a sporocyst-infected environment. Transmission through the bite of a vector (*Amblyomma limbactum*) has also been observed by Smallridge and Paperna (1997). For many years, ticks have been considered as invertebrate vectors of this parasite, mainly because the type species *Hemolivia stellata* Petit et al. (1990) in the toad *Rhinella marina* (syn. *Bufo marinus*) is transmitted by *Amblyomma rotundatum* Koch 1844 (Telford 2009). Other studies of *Hemolivia* species in reptiles, such as *Hemolivia mariae* in Scinid lizard, transmitted by *Amblyoma limbatum* and *H. mauritanica* in terrestrial tortoise, transmitted by *Hyalomma aegyptium* support this. So far, only a single species of *Hemolivia, Hm. mauritanica* (Sergent and

Sergent 1904) have been described from African terrestrial tortoises (Cook et al. 2015). However, Cook et al. (2015) recommended that *Haemogregarine parvula* should be reassigned to genus *Hemolivia*, *Hm. parvula* (Dias 1953). This was the first species of the genus *Hemolivia* documented from southern African tortoises.

1.5.3. *Karyolysus* Labbé 1894

Members of genus *Karyolysus* Labbé 1894 (Apicomplexa: Adeleida: Karyolysidae) are representatives of protozoan haemogregarines commonly found to parasitize Saurians (Haklová-Kočíková et al. 2014; Cook et al. 2016). Parasites of this genus have been considered to infect Palearctic lizards. These are mainly reported from European countries including Romania, Hungary, Spain and Slovakia (Alvarez-Calvo 1975, Haklová-Kočíková et al. 2014), but also from Scandinavia (Svahn 1974) and Russia (Beyer and Sidorenko 1984). Recently, Tomé et al. (2019) documented seven new species in the Canary Islands. However, India, Kenya, and recently also South Africa are changing this view as there were reports on the presence of *Karyolysus* (de Mello and de Meyrelles 1973; Mutinga and Dipeolu 1989; Cook et al. 2016). Lacertid lizards of the genera *Lacerta, Zootoca, Gallotia* and *Podarcis* are the prevailing vertebrate host of species of *Karyolysus*. However, the known host range of this genus has broadened, as there are documented reports of it from Scincid, geckonid and varanid lizards (Mutinga and Dipeolu; Cook et al. 2016; Tomé et al. 2019). *Karyolysus* sp. have been mainly reported to occur in Asia and Europe (Haklová-Kočíková et al. 2014; Cook et al. 2016). Currently only 18 species of genus *Karyolysus* are recognized (Svahn 1974; Smith 1996; Van As et al. 2013; Haklová-Kočíková et al. 2014; Tomé et al. 2019),. This includes *Karyolysus lacertae* Danilewsky 1887, *K. lacazei* Labbé 1894, *K. zuluentai* Reichenow 1920, *K. biretortus* Nicolle 1904, *K. subtilis* Ricci 1954, *K. octocromosomi* Alvarez-Calvo 1975, *K. minor* Svahn 1975, *K. latus* Svahn 1975, *K. poleensis* Mutinga and Dipeolu 1989, *K. bicapsulatus* Franca 1910,*K. paradoxa* (Dias 1954) (Cook et al. 2016), *K. galloti* Tomé et al. 2019, *K. canariensis* Tomé et al. 2019, *K. stehlini* Tomé et al. 2019, *K. gomerensis* Tomé et al. 2019, *K. atlanticus* Tomé et al. 2019, *K. tinerfensis* Tomé et al. 2019 and *K. geckonis* Tomé et al. 2019.

Parasites of genus *Karyolysus* have a complex life cycle in which merogony takes place in a vertebrate host, while sporogony and gametogony takes place in the gut of an invertebrate host (Telford 2009). Mites have been documented to be the main intermediate host involved in the transmission of the parasite. These parasites are strictly obligate and can utilize taxonomically related vertebrate hosts such as lizards and snakes (Haklová-Kočíková et al. 2014). The transmission of *Karyolysus* species to their vertebrate hosts (lizards) is considered to involve ingestion of mites that contains infectious sporozoites, without the typical arrangement of sporocysts. Meronts can be seen in capillary endothelium of the liver, lungs, heart, and spleen, while gamonts parasitize red blood cells in peripheral blood of lizards (Telford 2009; Haklová-

Kočíková et al. 2014). Transovarian transmission occurs through the eggs of an infected female mite to the larvae and represents the next generation infection. Gamonts leave their host cells quickly after being ingested by the mites thus forming a pair a day post feeding (Svahn 1974). Micro and macrogametes pair and divide to produce two biflagellate microgametes, with one fertilizing the microgamete. In 1921, Reichenow (1921) described that the resulting zygote forms oocysts in the epithelial cells of the mite stomach (*Karyolysus lacertae*) or in the podosomal region of the haemocoel (*Karyolysus lacazei*), and this was later confirmed by Svahn 1974. The latter author also found that the *Karyolysus* oocysts developed in the stomach epithilium but could also be found in the anterior part of the haemocoel (Svahn 1974; Telford 2009). While the transmission of *Hepatozoon* involves a wide range of vectors, including ticks, in an experimental study by Svahn (1974), the presence of *Karyolysus* from infected *Lacerta agilis* to larvae and nymphs *of Ixodes ricinus* ticks was not demonstrated, despite the fact that these ticks are more abundant than mites on these lizards (Majláthová et al. 2010).

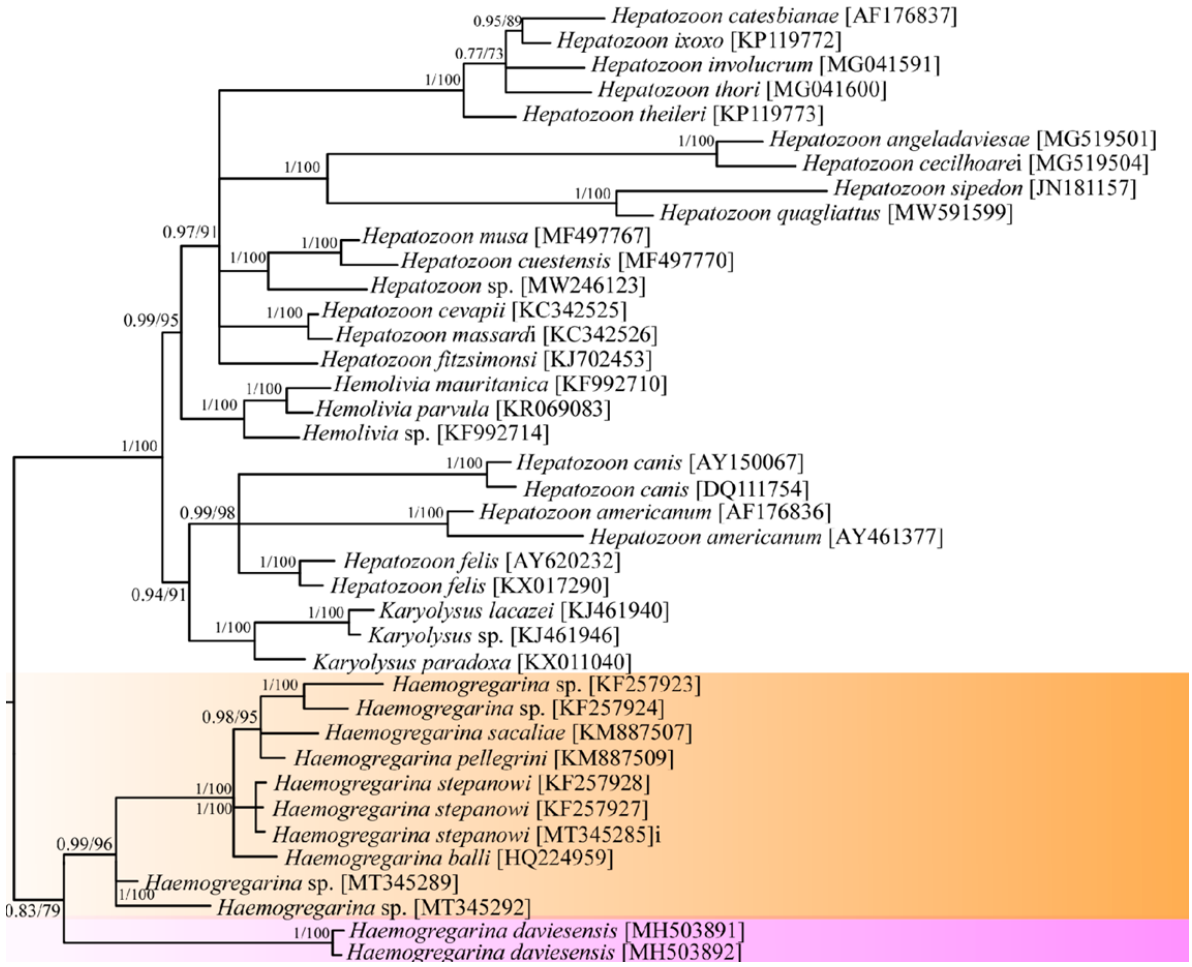


Figure 1-6: Bayesian (BI) and Maximum likelihood (ML) phylogenetic tree of haemogregarines based on the 18S rRNA gene sequences (adapted from Úngari et al. 2023).

1.6. Haemosporidians

Haemosporidians (Phylum Apicomplexa) are vector-borne parasites parasitizing a vast range of vertebrate hosts including reptiles, avians, amphibians, fish and mammals across the world (Valkiūnas 2004; Nourani et al. 2018; Van Hemert et al. 2019). This group includes the causal agent of malaria, *Plasmodium* parasites, the genera *Fallisia* and *Leucocytozoon*, as well as the species rich genus *Haemoproteus* with its two subgenera *Haemoproteus* and *Parahaemoproteus*. They are notable for their diversity and cosmopolitan distribution (Valkiūnas 2005; Toscani Field et al. 2018). Most available information about haemosporidians is with regard to the genera with most anthropogenic significance, such as genus *Plasmodium*. However, over the last decade, research investigating this and the other common genera (*Haemoproteus* and *Leucocytozoon*) has increased in wild avian hosts (Hellgren et al. 2004; Valkiūnas 2005; Bensch et al. 2009; Valkiūnas et al. 2010). There are currently four recognised families in the order Haemosporida, and this include Haemoprotidae characterized by the presence of haemozoin pigment with no erythrocytic merogony; Plasmodiidae characterized by the presence of both haemozoin pigment and erythrocytic merogony; Garniidae characterized by the presence of erythrocytic merogony but the haemozoin pigment is absent; and *Leucocytoidae* characterized by the absence of these two characteristics (Perkins 2014; Maia 2015). As documented by Valkiūnas (2005) and Perkins (2014), haemosporidians were traditionally classified according to their morphological characters, life history traits, geographical distribution and host taxa. There have been many redecriptions with regard to the haemosporidian's taxonomy, especially with the use of molecular tools that allowed to greatly increase the number of identified species compared to the original microscopically identified species (Bensch et al. 2004; Hellgren et al. 2004). Several subgenera have been formulated to distinguish these parasites according to their morphological similarities and genetic distinctiveness of some of the clades using molecular tools (Table S4).

Until 2015, only three hypotheses that aimed to explain the evolutionary history of Haemosporidia existed. According to Perkins (2014), the main difference between these hypotheses is the number of parasite taxa included in each analysis and the outgroup used to root the phylogenies. Hypothesis one was proposed in 2002, and it was generated based on 21 mammals, 11 lizards and 18 avian species, rooted with both *Lecucytozoon* and *Theileria* (Perkin and Schall 2002). The main discovery of this study was the identification of two clades (mammalian parasite clade and reptile/bird clade). The generation of hypothesis 2 was based on 11 mammalian parasite species, 7 lizards and 39 lineages from avian hosts. The phylogeny was rooted with *Leucocytozoon*. This study found a similar overall topology, but the main discovery was that the major cladogenic

events of Haemosporida seemed to be related to host switches between vectors. This indicates that the parasite might occasionally have been ingested by an unusual vector, which was suitable for parasite development that resulted in continued transmission and subsequent establishment, radiation, differentiation and speciation (Perkins 2014). The last hypothesis was generated based on both previous datasets plus additional sequences available on public databases (Outlaw and Ricklefs 2011). The uniqueness of this study was that their approach did not use an outgroup and this provided striking findings, such as the fact that *Leucocytozoon* was a derived and not an ancestral lineage. Therefore, these findings suggests that this genus is not a suitable outgroup for rooting the Haemosporida tree for the cyt-b gene, and that mammalian Polychromophilus was more closely related with bird and reptile haemosporidians than with other mammalian haemosporidians. However, in 2016, a study by Borner et al. (2016) using a multi-gene approach found an alternative hypothesis. This hypothesis included *Leucocytozoon* as basal to *Haemoproteus* and *Plasmodium*, therefore making this a suitable outgroup, and that mammalian *Plasmodium* was a sister taxa to bird and reptile *Plasmodium*. Furthermore, in 2013, Pineda-Catalan et al. (2013) have genetically characterized chelonian parasites and the phylogenetic results of the study showed that reptilian hemoproteids are distinct from Avian hemoproteids (Figure 1-7). This emphasized the crucial need to take reptilian parasites into consideration when estimating the phylogenetic position and taxonomy of parasites.

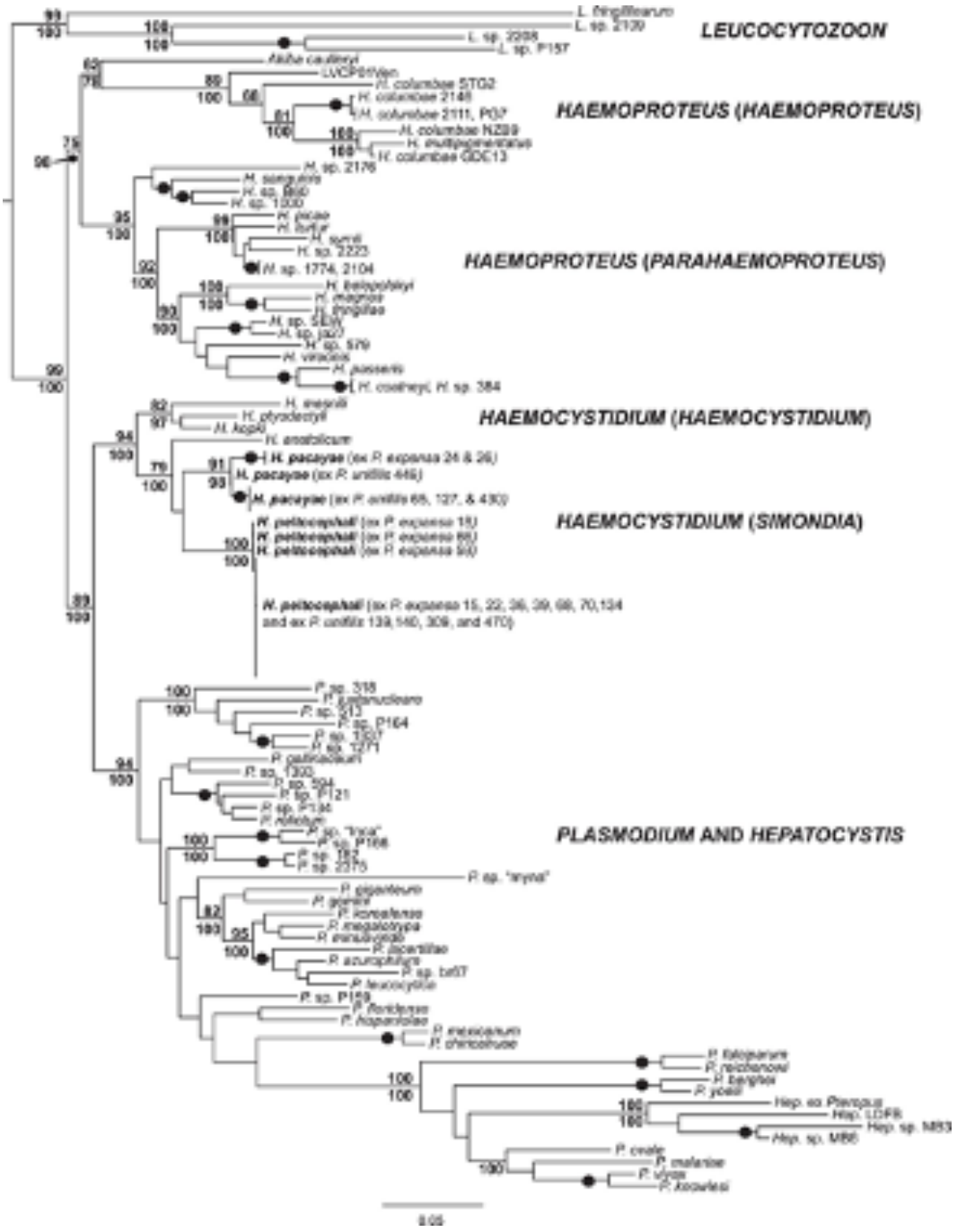


Figure 1-7. A concatenated maximum likehood phylogeny (Cytb [989 bp], COX1 [836 bp], ClpC [373 bp]) of haemosporidians. *Leucocytozoon* species were used as an outgroup. Sourced from Pineda-Catalan et al. (2013), with permission.

1.6.1. Family Haemoproteidae Doflein 1916

Members of family Haemoproteidae infect leucocytes and erythrocytes, and they are characterized by the appearance of a “halter shape” that stretches lengthways or curves around the nucleus of a host cell and that produce haemozoin pigment by digestion of haemoglobin (Lainson and Naiff 1998, Cook et al. 2010, Orkun and Güven 2013). The family comprises species of *Haemoproteus* Kruse 1890, with gametocytes inside the red blood cells but without a division stage in the peripheral blood cells and appear to have an affinity for the pulmonary tissue, especially endothelial cells of the pulmonary cells of the pulmonary vessels. *Haemproteus metchnikovi*’ schizonts are, however, primarily found in the infected turtle’s spleen. Reptilian *Haemoproteus* represent a rather neglected group as compared to avian *Haemoproteus* (Javanbakht et al. 2015). Most studies done so far on Haemoproteus of Testudines has been taxonomic and descriptive, based solely on morphology of their blood stages (Laison and Naiff 1998; Telford 2009; Cook et al. 2010). In avian hosts, these parasites have been identified as *Haemoproteus*, while in reptilian hosts they may be reffered to as *Haemocystidium* (Maia 2015). Several subgenera were proposed for each of these genera, and they were proposed according to their intermediate and vector hosts, and their diversity. Genus *Haemoproteus* is divided into two subgenera (*Haemoproteus* and *Parahaemoproteus*) based on their vector differences. Subgenus *Haemoproteus* is transmitted by louse flies (Hippoboscidae) while *Parahaemoproteus* is known to be transmitted by biting midges (Culicoides) (Orkun and Güven 2013). However, in 1973, DeGiusti et al. (1973) found sporozoites in the salivary glands of a tabanid fly (*Chryops callidus*). When these sporozoites are inoculated in a turtle under laboratory conditions, gametocytes typical of *Hp. metchnikovi* appeared inside the red blood cells. Tabanid flies can thus be introduced as a vector group, which further complicates the systematics and biology of haemoproteids (DeGiusti et al. 1973; Fallis and Desser 1977). Recently, in addition to the vector differences in avian haemoproteids, the difference in sporogony stage, the production of oocysts in the invertebrate host and, in particular, the knowledge provided by molecular phylogeny has added distinct lineages (Valkiünas 2005; Martisen et al. 2008; Valkiünas et al. 2008; Santiago-Alarcon et al. 2010; Valkiünas et al. 2010; Levi et al. 2011).

In 1901, Simond (1901) described the first chelonian haemoproteid from the blood of *Chitra indica* (Trionychidae) as *Haemamoeba metchnikovi*. Since its description, identifications using numerous genera such as *Haemocystidium, Simondia, Haemamoeba* and *Haemoproteus* were employed for chelonian haemoproteids and several taxonomic revions have been made (Mühlens 1931; Wenyon 1965; Lainson and Naiff 1998). The position of these parasites among *Haemoproteus* was eventually accepted (Levine and Campbell 1971; Levine 1985; Lainson and Naiff 1998; Telford 2009). A decade ago, a taxonomic review by Pineda-Catalan et al. (2013)

using molecular-genetic markers suggested that *Haemoproteus* should be divided into four genera. This division placed reptilian haemoproteids in the genus *Haemocystidium*.

Four of the 12 described chelonian hemoproteids have been reported from African terrestrial tortoises: *Haemoproteus testudinalis* (Laveran 1905), Wenyon 1915, in *Stigmochelys pardalis* and *Kinixys lobatsiana*, respectively from South Africa (Cook et al. 2010); *H. roumei* (Bouet 1909), Wenyon 1915, in *Kinixys belliana belliana* from West Africa (Bouet 1909; França 1911; Joyeux 1913); a *Haemoproteus* sp. in captive West African *K. b. belliana*, *Kinixys erosa* and *K. homeana* (Plimmer 1912); and *Haemoproteus balazuci* Dias 1953 in *K. b .belliana* from Mozambique (Dias 1953). Cook et al. (2010) recommended that *Hp. balacuzi* should be used as a junior synonym of *Hp. testudinalis*. The authors also described a new haemoproteid (*Haemoproteus natelensis*) in captive *Kinixys natalensis* from KwaZulu-Natal, South Africa.

1.6.2. Family Plasmodiidae Mesnil 1903

Family Plasmodiidae is the most studied family within the Haemosporida, since it includes the etiological agents of human malaria and inhabit all major groups of terrestrial vertebrates (Garnham 1966, Valkiünas 2005). There are atleast 100 recognized species parasitizing reptiles, and they have been described based on morphological characters (Garnham 1966; Telford 1984; Matta et al. 2018). In 1966, Garnham (1966) proposed 3 subgenera within this family to describe the 23 known species parasitizing lizards up to that moment: *Carinamoeba* for parasites with very small erythrocytic meronts, *Sauromoeba* for those with large erythrocytic meronts, and *Ophidiella* for *Plasmodium* species parasitizing snakes. A decade later, seven subgenera were proposed for *Plasmodium* species infecting reptiles (*Ophidiella, Larcetamoeba, Paraplasmodium, Garnia, Asiamoeba, Sauramoeba* and *Carinamoeba*) (Telford 1988). However, this proposal is controversial. Subgenus *Garnia* is sometimes regarded as a distinct family, Garniidae with its own three genera (*Fallisia, Garnia*, and *Progarnia*) (Table 2). This is because these parasites do not produce the haemozoin pigment, and *Fallisia* parasites are only found in the white blood cells and platelets (Lainson et al.1971,1974; Valkiūnas 2005).

In case of *Plasmodium* in African reptiles, the first saurian *Plasmodium* species, *Plasmodium agamae* and *Plasmodum mabuiae*, were described in 1909 by Wenyon (1909) from an agamid lizard and from a scincid lizard in North Africa, respectively. Globally, the discovery and description of *Plasmodium* species increased slowly until the 1960s when research on these species greatly increased (Garnham 1966; Telford 1994). To date, there are atleast 27 *Plasmodium* species described from African lizards, three of these species, *Plasmodium zonuriae* (Pienaar 1962), *Plasmodium cordyli* (Telford 1987), and *Plasmodium intabazwe* (Van As et al. 2016), have been described from African cordylid lizards. In 2019, Omonona et al. (2019) observed *Plasmodium* sp. in *Varanus niloticus* from Nigeria. Three species of *Plasmodium* have

also been described from African snakes, and two of these species, *Plamodium bitis* Fantham and Porter 1950 and *Plasmodium pythonis* Fantham and Porter 1950, have been documented in South Africa from *Bitis arietans* and *Python sebae*, respectively (Fantham and Porter 1950).

1.7. Viruses

Many different species of reptiles have been reported to harbour a variety of viruses, including arboviruses that typically infect avian and mammalian hosts, as well as reptile-specific viruses (Marschang 2011). A number of studies have demonstrated that various arboviruses can infect a variety of different reptile species, and the development of viremia in these ectotherms is mainly influenced by temperature. It has also been further demonstrated that these viruses may survive in reptiles over winter, which may impact the epidemiology of these viruses (Marschang 2011). The viruses that have been commonly detected in reptiles include herpesviruses, reoviruses, paramyxoviruses, picornaviruses, iridoviruses and ranaviruses. Most species of reptiles (Testudines, Squamata, Crocodylia) have been found molecularly and serologically positive for a number of arboviruses (Steinman et al. 2003). It has also been documented that tortoises and their associated *Hyalomma aegyptium* play a role in the transmission cycle of Bunyavirales (Crimean-Congo hemorrhagic fever) (Kar et al. 2020). Even though a number of viruses have been documented in reptiles and their associated vectors, they are not a focus in the current study.

1.8. Methodologies for parasite detection and identification

According to Poulin and Mouillot (2005), the first step towards understanding the ecological interactions in the relationships between parasites and hosts is to assess the prevalence and intensity of infection of parasites in vertebrates. The use of light microscopy and the indirect immuno-fluorescent antibody tests (IFATs) have been the traditional and “gold standard” methods for detection and identification of parasites (Valkiūnas, 2005; Telford, 2009), however, this improved to different kinds of molecular based tests that involves the amplification of genes using sets of primers and a slot blotting hybridization technique to real time (qPCR) for both their sensitivity and accuracy for detecting parasites (Collins et al. 2002).

Observing the morphological characters and developmental stages of a parasite through microscopy allows an understanding of the course of infection itself, as well as to increase the knowledge about a parasite’s life cycle. Infections with most apicomplexans such as haemosporidians and hemogregarines are detected by identifying gamonts inside the blood cells of their vertebrate hosts (see Cook et al. 2014). However, correct identification and characterization of new parasite species often depend on the characterization of the developmental stages of the parasite in the definitive host (Cook et al. 2014; Maia 2015), where sexual reproduction occurs. Hence, microscopy is a valuable tool as it allows to observe

morphological characters of taxonomic and evolutionary importance, to assess prevalence and intensity of infection, to identify mixed infections and in which cells or tissue the parasites occur, to perform inexpensive routine screenings, and to identify double gametocyte infections in single host cells (Moody 2002; Jovani et al. 2004; Valkiūnas et al. 2008; Cook et al. 2014).

More than a decade ago, in some studies microscopy has shown to be less accurate and less sensitive than molecular techniques in the determination of parasite prevalence and intensity, especially where the parasitemia level is very low (Richard et al. 2002; Perandin et al. 2004; Mangold et al. 2005; Valkiūnas et al. 2008). Nonetheless, there are reports comparing different methodologies that show different levels of sensitivity. This may be an indication that the examiner/technician experience and quality of the samples and materials or protocols used are key issues. Hence, a priori assessment on the performance of different methodologies is often necessary (Maia 2015).

1.8.1. Molecular techniques

Molecular techniques are used in parasite species identification for various reasons such as assessing the diversity and search for cryptic species, termed “cryptic species prospecting, and linking morphologically indistinguishable early lifestages with later stages of known parasite species (Jousson et al. 1999; Bartoli et al. 2000; Criscione et al. 2005). According to Nadler and De León (2011), these techniques provide an opportunity to uncover parasite biodiversity by characterizing their genetic diversity, differentiation and population structure.

Quantitative and conventional PCR are the two main molecular techniques used in parasitological studies. Kaltenboeck and Wang (2005) documented that the sensitivity and specificity of the techniques makes them easy for scientists to detect microorganisms that are difficult to culture or to confirm their presence by analysing their products. Conventional PCR techniques basically detect the presence or absence of parasite DNA when compared to the quantitative PCR methods which are able to provide an indication of the level of parasite load (Desquesnes and Dávila 2002). The primary advantage of conventional PCR is that it offers genetic information with considerably greater length and simplicity as compared to qPCR methods, which can be used to determine the phylogenetic relationships between parasites. In a situation where a specific genetic sequence is known, PCR can also be used to genotype by designing primers that can only amplify the targeted region of an organism. Even though this has been the preferred method of diagnosis to date, drawbacks such as the risk of post-amplification contamination are highly possible. However, PCR is highly recommended in various research centres for diagnosis of infectious diseases (Harmon et al. 2007).

The use of quantitative PCR techniques has exhibited to be of potential value for a number of parasitic infections and due to the fact that there is no need for gel electrophoresis to visualize

the PCR products, this makes qPCR convenient, more rapid and advantageous than convectional PCR (Salih et al. 2015). This technique is simple, fast and automatized amplification system responsible for reducing the risk of cross contamination; it has been used to detect various parasites including trypanosomes in many countries across the world (Gasser 2006; Sharma et al. 2012).

However, due to its high sensitivity, any form of sample contamination can produce false positive or false negative results (Smith and Osborn 2009). Additionally prior sequence data is needed when designing primers for performing PCR analyses, making this technique useful when only detecting the presence and absence of an already known pathogen or gene. Therefore, PCR can only be used to identify the presence or absence of a known pathogen or gene. Furthermore, the PCR primers can anneal non-specifically to target DNA sequences that are similar, but not identical. Moreover, while at a very low rate, the DNA polymerase can include incorrect nucleotides into the PCR sequence (Hajia 2018).

1.8.2. Phylogenetic analysis

Different evolutionary tree inference methods including Maximum Parsimony, Bayesian Inference and Maximum Likelihood can be used to test hypothesis for molecular sequence data (Maia 2015). These methods analyze character-state data, and may employ complex and realistic substitution models (Nadler and De León 2011). These models can be tested to select the best-fit substitution model and parameters for each data set (Posada and Crandall 1998; Posada 2008; Darriba et al. 2012). The large number of potential characters available to infer relationships is one of the advantages of sequence data, though its resolution is limited by several factors including the choice of an outgroup, taxon sampling, alignment methodology, selecting genes and the size of the sequence (Smith 1994; Nadler 1995; Sanderson and Shaffer 2002; Rich and Xu 2011). Recently, there's a development of models that are able to estimate independent rates of molecular evolution for each lineage and outgroup-free approaches to estimate phylogenetic relationships, and they may open new perspectives for studying evolutionary history of some parasite groups (Outlaw and Ricklefs 2011; Drummond et al. 2012). Additionally, the increase in taxon sampling may uncover unexpected relationships between parasite taxa (e.g. polyphyly and paraphyly) that challenge traditional taxonomy, especially when including previously unavailable parasite species or genera (Putaporntip et al. 2010; Pineda-Catalan et al. 2013; Haklová-Kocíková et al. 2014).

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1. Study area

The following sites were chosen so that a diverse population of reptiles and their ticks could be sampled, and so that the natural occurence and intensity of parasites can be assessed. The samples were collected from the sites below:

Ndumo Game Reserve

Ndumo Game Reserve, KwaZulu-Natal (KZN), South Africa (26°54′18.5″S, 32° 19′24.7″E) is situated on the South African border with Mozambique and according to Grant and Thomas (1998) (Fig. 2-1), it covers an area of 10117ha. The reserve is characterized by a rich diversity of microhabitats such as subtropical vegetation and sand forest (De moor et al. 1977).

Bonamanzi Game Reserve

Bonamanzi Game Reserve, KZN (28°03′25″S 32°17′42″E) (Fig 2-1) has warm summer. The vegetaion type in the reserve consists of wooded grassland and mainly compound-leaved, short woodland. It also has a mean rainfall range of 444–1200mm.

Paternoster, Western Cape

The region near the village of Paternoster, Western Cape (WC) (17◦51′56″ E; 32◦49′2″ S) (Fig. 2-1) is surrounded by farmlands and bordering the reserve area of cape Columbine and West Coast national park. It is characterized by limestone and sedimentary geology, as well as strandveld and coastal fynbos.

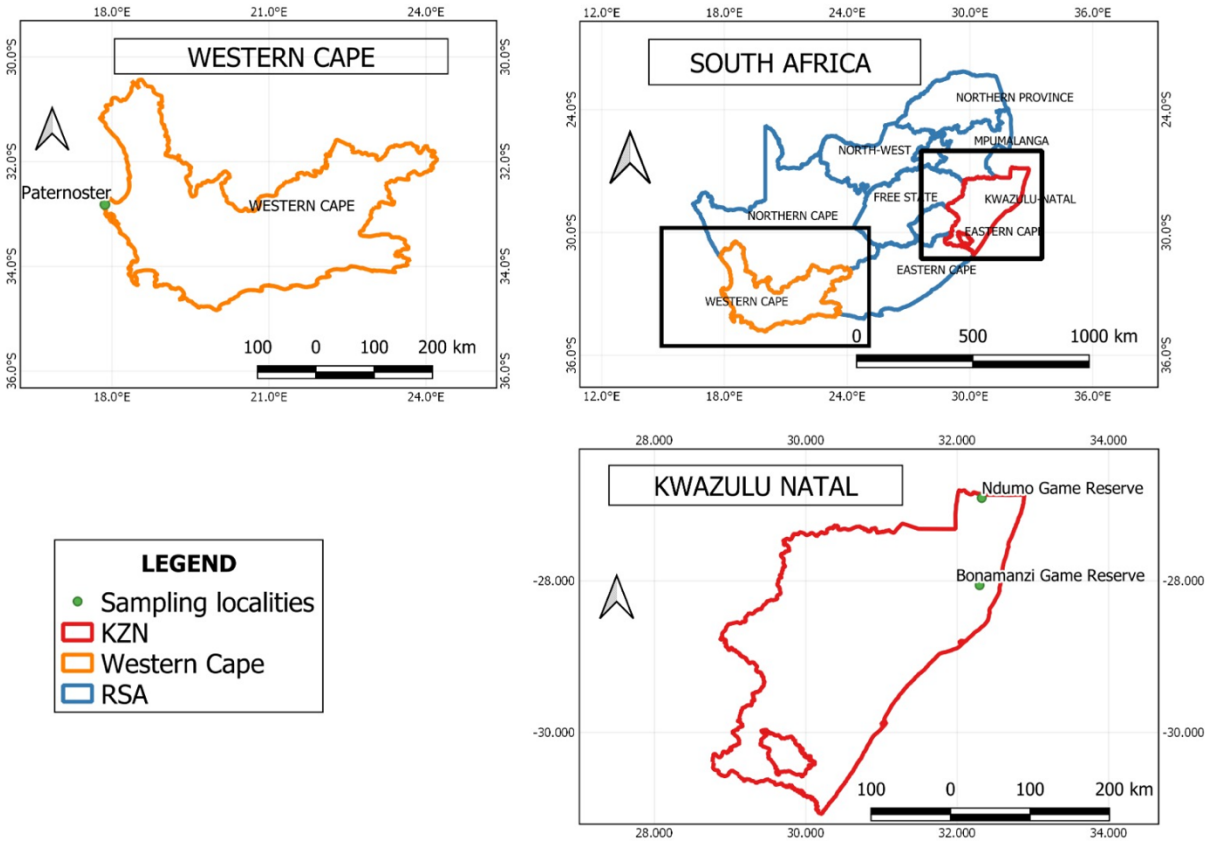


Figure 2-1: Map of the sampling localities in KwaZulu-Natal and Western Cape, South Africa.

2.2. General sample collection

A total of 71 reptilian blood samples, including 30 from snakes, 25 from land tortoises and 16 from monitor lizards (table 2-1), as well as 253 ticks were collected in and around the Ndumo Game Reserve (NGR), KwaZulu-Natal (32°18’49” E;26°54’33” S) (2014–2017), Bonamanzi Game Reserve (BGR), KwaZulu-Natal (32°17'8.772"E, 28°4'19.092"S) (2017), and the region near the village of Paternoster, Western Cape (WC) (17◦51′56″ E; 32◦49′2″ S) (2012) using active sampling techniques dependent on species. Reptiles were identified to species level using the field guides by Branch (1998) and Marais (2004). Active sampling included hand capture in the case of monitor lizards and tortoises, along with the use of a grab- and hook-stick for snakes. In the case of venomous snake handling, the use of clear Perspex tubes were used to restrain the head, whilst blood was drawn and the body checked for ticks. Blood was collected from the caudal vein of snakes and monitor lizards, and from the subcarapacial sinuses of tortoises using sterile 1ml syringes fitted with the appropriate sized gauge sterile needle dependent on reptile size. Depending on the species and health status of the animal, blood (0.5–1% of the body weight) and ticks were collected from reptiles. Blood collected was used to prepare thin blood smears and a

small volume of blood was preserved in 70% molecular grade ethanol (Sigma-Aldrich, St. Louis, MI, USA) and stored in a -80°C freezer for long-term storage and a -20°C freezer when the samples were to be processed. Ticks were collected in situ whilst the reptiles were rstrained by hand. Upon collection, the ticks were placed in falcon tubes with a damp piece of cottonwool, kept in a dark, cool environment, and allowed to digest their blood meals for approximately 10–20 days (Desser et al 1995; Mofokeng et al. 2021). Thereafter, they were fixed in molecular grade 70% ethanol. Sampling was carried out under the relevant permits (KZN: OP 839/2014, OP 4374/2015, OP 4092/2016, OP 4264/2017; WC:0035-AAA004-00383) and was approved by the North-West University AnimCare Research Ethics Committee, South Africa (NWU-00571-19-A5).

2.3. Methods specific to *Hepatozoon fitzsimonsi* in tortoises and ticks.

For developmental stages of Hepatozoon fitzsimonsi *in tortoises and ticks:* (Chapter 4 of the thesis)

Tortoises were collected from 2016–2017 from Bonamanzi and Ndumo Game Reserve, KZN, and identified to species level using field guides (Branch 1998, 2008; Boycott and Bourquin 2000). Collection permits did not allow for euthanasia, however, in one case a tortoise was found recently dead, the result of a roadkill incident.

Blood was collected from the subcarapacial sinuses (McArthur et al. 2004) and thin blood smears were prepared, and allowed to air dry before being fixed in absolute methanol and stained in a solution of Giemsa's stain (FLUKA, Sigma-Aldrich, Steinheim, Germany) for 20 minutes (see Cook et al. 2014). Once stained and dried, these were stored in a dustproof container for later screening. A small volume of blood was preserved in 70% ethanol and kept in the freezer (-20°C) until used for molecular work. Samples from the roadkill individual were taken from the kidney, heart, liver, lung, and spleen during dissection. Impression and squash smears were prepared on clean microscope slides, and these fixed and stained following the same method as described for the blood smears. A small section of each organ was preserved in 70% ethanol.

Tick collection, with preparation of tick impression slides

Ticks present on tortoises at the time of blood collection were carefully removed using forceps so as to ensure that the hypostome remained intact with minimal damage to the tick. Ticks were placed in plastic tubes and these tubes labelled according to the individual tortoise from which they were collected. Ticks were allowed to digest their blood meals for approximately 10–20 days (Desser et al. 1995; Smith 1996). Thereafter, adult ticks were dissected according to Edwards et al. (2009) and visceral impression slides were prepared on clean microscope slides. The remaining tick and its viscera were immediately fixed in 70% ethanol and stored in a -20°C freezer until further molecular analysis. Slides were allowed to dry and followed the same fixing and

staining protocol as that of the blood and organ smears. Morphological identification to species level was done with the aid of a Nikon AZ100M microscope and guidelines for tick identification as provided by Theiler (1945) and Theiler and Salisbury (1959).

Microscopical screening of tortoise blood and tissue smears and tick impression slides

Stained blood smears were screened at 100× oil immersion objective, and micrographs of parasites were taken on a calibrated Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, Netherlands) using the imaging software NIS Elements. All measurements were in micrometres (µm) unless otherwise indicated. Measurements comprised the parasite's length (including recurved tail when present) and width within its parasitophorous vacuole (PV), and the parasite's nucleus length and width (see Cook et al. 2014). Subsequently, the morphometric data of parasites was used in comparison to previous descriptions of *H. fitzsimonsi*.

Impression slides were screened using the 20×, 60× and 100× oil immersion objective on a calibrated Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, Netherlands) using the imaging software NIS Elements. Micrographs of sporogonic stages were taken using both the 20× and 100× objectives, depending on the size of the parasite stage. Sporogonic stages were identified and measured according to Cook et al. (2014), with the aid of the ImageJ version 1.47 software program (Wayne Rasband National Industries of Health, USA) (Rasband 2014) (http://imagej.nih.gov/ij) and subsequently compared to those observed in ticks from Cook et al. (2014). The number of sporozoites within each sporocyst were estimated by counting their nuclei (Cook et al. 2014).

Table 2-1. List of specimens collected for the current study.

Animal species	Common name	Number	site	Age	Tissue
Atractaspis bibronii	Bibron's Stiletto Snake	2	Ndumo Game Reserve	Adult	Blood
Bitis arietans	Puff adder	3	Ndumo Game Reserve	Adult	Blood
Dendroaspis angusticeps	Green mamba	2	Region surrounding Durban	Adult	Blood
Dendroaspis polylepis	Black mamba	4	Region surrounding Durban	Adult	Blood
Dipsadoboa aulica	Marbled tree snake	1	Ndumo Game Reserve	Adult	Blood
Dispholydus typus	Boomslang	1	Ndumo Game Reserve	Adult	Blood
Gonionotophis capensis capensis	Cape file snake	1	Ndumo Game Reserve	Adult	Blood
Haemachatus haemachatus	Rinkhals	2	Region surrounding Durban	Adult	Blood

K. spekii	Speke's hinge-back tortoise	1	Ndumo Game Reserve	Adult	Blood
K. zombensis	Bell's Hingeback Tortoise	12	Ndumo Game Reserve	Adult	Blood
Kinixys natalensis	Natal hinge-back tortoise	1	Ndumo Game Reserve	Adult	Blood
Naja mossambica	Mozambique spitting cobra	3	Ndumo Game Reserve & region surrounding Durban	Adult	Blood
P. natalensis	Natal green snake	2	Ndumo Game Reserve	Adult	Blood
P. semivariegatus	Spotted bush snake	2	Ndumo Game Reserve	Adult	Blood
Philothamnus hoplogaster	Green water snake	2	Ndumo Game Reserve	Adult	Blood
Psammophis brevirostris	Short-snouted grass snake	1	Ndumo Game Reserve	Adult	Blood
Python natalensis	Southern African rock python	3	Ndumo Game Reserve	Adult	Blood
Stigmochelys pardalis	Leopard tortoise	11	Ndumo Game Reserve	Adult	Blood
Thelotornis capensis	Savanna vine snake	1	Ndumo Game Reserve	Adult	Blood
V. niloticus	Nile monitor	3	Ndumo Game Reserve	Adult	Blood
Varanus albigularis	Rock monitor	13	Ndumo Game Reserve	Adult	Blood
Total		**71**			

2.4. Processing of samples and microscopy screening

2.4.1. Blood and other tissue samples

Thin blood smears were prepared on clean glass slides, air-dried, fixed for 10 minutes in absolute methanol and stained using Giemsa-stain (FLUKA, Sigma-Aldrich, Steinheim, Germany) for 20 minutes as indicated by Cook et al (2014, 2016). Stained blood smears were screened with the 100× oil immersion objective, and micrographs of parasite stages were taken on a calibrated Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, Netherlands) using the imaging software NIS Elements. All measurements were in micrometres (µm) unless otherwise indicated. Measurements comprised the parasite's length (including recurved tail when present) and width within its parasitophorous vacuole (PV), and the parasite's nucleus length and width. Measurements of the PV length and width, and the length from mid-nucleus to both anterior and posterior end of the parasite were also taken. Parasitaemia was calculated per 100 erythrocytes, with ~10^4 erythrocytes examined per blood smear, following previous methods (see Cook et al. 2014, 2016). Subsequently, the morphometric data of parasites was used in comparison to previously described blood parasites of reptiles from Africa.

2.4.2. Tick identification

Morphological identification of ticks to species level was done with the aid of a Nikon stereo-microscope. Briefly, each tick specimen was placed on a glass slide and images were taken on a calibrated Nikon SSZ1500 stereo microscope (Nikon, Amsterdam, Netherlands), captured with an attached Nikon digital camera and accompanying software. The external features of the ticks (legs and spurs, positions and shapes of the anal groove, festoons, hypostome, genital aperture and spiracles) were recorded for species identification, and used in drawing conclusions according to the guidelines for tick identification as provided by Theiler (1945), Theiler and Salisbury (1959), and Horak et al. (2018). In addition to tick species, the sex, life stage, host, and location of sample collection were also noted. In case of tick morphological abnormalities, the identification of the anomalous tick specimen was based on the morphology and structure of different body parts, such as basis capituli, scutum/conscutum, spiracles, legs, festoons, etc. All microscopically identified tick samples from the study areas were also used for molecular studies.

2.5. DNA extraction, PCR amplification, and phylogenetic analyses

2.5.1. DNA extraction

Blood and tick samples from parasitised reptile specimens used for molecular work.

Genomic DNA of blood parasites and tick-borne pathogens was extracted from the blood samples using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa). Total genomic DNA was also extracted from whole individual tick specimens used in this study. Briefly, tick specimens were sterilized in ethanol solution and then washed twice in sterile distilled water. Afterwards, the individual tick specimen was dissected into pieces and placed in a micro-centrifuge tube filled with 180 µl lysing buffer solution supplied by a DNeasy Tissue Kit (Qiagen, Germany) and then homogenized for 10 minutes with a Tissue Lyser. The homogenate was centrifuged at room temperature and the supernatant fluid was further processed by a DNeasy Tissue Kit, as instructed by the manufacturer. After filtration, the filtrate was collected and stored at -20ºC. Once extracted, DNA was used for polymerase chain reaction (PCR) amplification.

2.5.2. PCR amplification of ticks, tick-borne bacterial micro-organisms and haemoparasites

2.5.2.1. Amplification of ticks, tick bacterial microorganisms, and haemoparasites

PCR and nPCR was conducted for molecular identification of ticks, detection of tick-borne pathogens, as well as haemoparasites, targeting different genes by using the primers and PCR conditions shown in table 6.

Conditions for PCR were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles, entailing a 95°C denaturation for 30 s, annealing as indicated (Table 6) for 30 s with an end extension at 72°C for 2 minutes, and following the cycles a final extension of 72°C for 10 min. PCR reactions were performed with volumes of 25 µl, using 12.5 µl Thermo Scientific DreamTaq PCR master mix (2×) (final concentration: 2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM $MgCl_2$), 1.25 µl (10 µM) of each of the primer sets mentioned above, and at least 2 µl DNA. The final reaction volume was made up with PCR-grade nuclease free water (Thermo Scientific). Reactions were undertaken in a Bio-Rad C1000 Touch™ Thermal Cycler PCR machine (Bio-Rad, Hemel Hempstead, UK). Resulting amplicons were visualized under ultraviolet light on a 1% agarose gel stained with gel red using a Bio-Rad GelDoc™ XR+ imaging system (Bio-Rad, Hemel Hempstead, UK).

Positive PCR products from each sample were sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) for purification and sequencing in both directions. Resultant sequences were assembled, and chromatogram-based contigs were generated and trimmed using Geneious Ver 7.1 (http://www.geneious.com, Kearse et al., 2012). Sequence and species identity were identified using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/). Some sequences obtained in the current study were deposited in the NCBI GenBank database, while others will be deposited once the manuscripts are submitted for publication. For phylogenetic analysis, comparative sequences of ticks, tick-borne pathogens and haemoparasites were downloaded from GenBank and aligned to the sequences generated within this study. Sequences were aligned using the Clustal W alignment tool under the default settings and implemented in MEGA Ver 11.1 (Tamura et al. 2021). To infer phylogenetic relationships, Maximum likelihood (ML) and Bayesian inference (BI) methods were used. Prior to the analyses, a model test was performed to determine the most suitable nucleotide substitution model, according to the Akaike information criterion (AIC) using jModelTest 2.1.7 (Guindon and Gascuel, 2003; Darriba et al., 2012). For the ML analysis nodal support was assessed using 1000 rapid bootstrap inferences. The BI analysis was implemented from within Geneious Prime® 2022.2.2 using MrBayes 3.2.2 (Huelsenbeck and Ronquist 2001). The analysis was run twice over 10 million generations for the Markov Chains Monte Carlo (MCMC) algorithm, with a subsampling frequency of 200 generations and a ‘burn-in’ of 25%. Pairwise distances were generated by Mega-X 10.2.4 with a Kimura 2-parameter substitution model with transitions and transversions included (Kimura 1980; Kumar et al. 2018) as in Zechmeisterová et al. (2022).

Table 2-2. List of primer sequences used for detection and identification of bacteria, ticks and haemoparasites

Bacterial primers						
Species	Assay	Primer sequence	Annealing (ºC)	Product size	Target gene	Reference
Universal bacterial primers	PCR	TCAAAKGAATTGACGGGGGC GCCCGGGAACGTATTCAC	50	~478 bp	16S rRNA	Relman et al., 1992
Anaplasma spp.	PCR	CAGAGTTTGATCCTGGCTCAGAACG GAGTTTGCCGGGACTTCTTCTGAA	50	467 bp	16S rRNA	Stuen et al., 2003.
Borrelia spp.	PCR	AGAGCAACTTACAGACGAAATTAAT CAAGTCTATTTTGGAAAGCACCTAA	54	482 bp	*Flagellin* gene	Skotarczak et al., 2002.
Borrelia spp	PCR	TGCGTCTTAAGCATGCAAGT GTACAAAGGCCCGAGAACGTA	56	1344 bp	16S rRNA	Loh et al., 2017
	nPCR	CATGCAAGTCAAACGGAATG ACTGTTTCGCTTCGCTTTGT	56	1205 bp	16S rRNA	Loh et al., 2017
Rickettsia spp.	PCR	GGGGGCCTGCTCACGGCGG ATTGCAAAAAGTACAGTGAAC	48	382 bp	*gltA* gene	Dupont et al., 1994.
Rickettsia spp.	PCR	TGGCGAATATT TCTCCAAAAA GTTCCGTTA ATGGCAGCATCT	46	631 bp	*ompA* gene	Roux et al., 1996

Table 2-2 continues

Species	Assay	Primer sequence	Annealing (ºC)	Product size	Target gene	Reference
Coxiella spp	PCR	CGTAGGAATCTACCTTRTAGWGG GCCTACCCGCTTCTGGTACAATT	52	832-939 bp	16S rRNA	Duron et al. 2015
	nPCR	TGAGAACTAGCTGTTGGTTAGT	52	624-627 bp	16S rRNA	Duron et al. 2015
Coxiella spp	PCR	GCCTGCGAWAAGCTTCGGGGAG CTCCTAKCCACASCTCATCCCC	56	694-1188 bp	23S rRNA	Duron et al. 2015
	nPCR	GATCCGGAGATWTCYGAATGGGG TCGYTCGGTTTCGGGTCKACTC	56	583-867 bp	23S rRNA	Duron et al. 2015
Coxiella burnetii	PCR	ACT CAA CGC ACT GGA ACC GC TAG CTG AAG CCA ATT CGC C	50	257 bp	IS1111	Stein and Raoult, 1992

Table 2-2 continues

Tick identification						
Species	Assay	Primer sequence	Annealing (ºC)	Product size	Target gene	Reference
Tick identification	PCR	CATTAAATCAGTTATGGTTCC CGCCGCAATACGAATGC	52	~780	18S rRNA	LV et al. 2014
	PCR	CTGCTCAATGATTTTTTAAATTGCTGT GG CCGGTCTGAACTCAGATCAAG T	50	~460	16S rRNA	Black and Piesman 1994
	PCR	AAACTAGGATTAGATACCCT AATGAGAGCGACGGGCGATGT	52	337-355	12S rRNA	Beati and Keirans 2001
	PCR	YTG CGA RAC TTG GTG TGA AT TAT GCT TAA RTT YAG SGG GT	50	900-1200	ITS2	Abdigoudarzi et al. 2011
	PCR	GGTCAA-CAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	47	~710	COX1	Folmer et al. 1994
Haemoparasites						
Species	Assay	Primer sequence	Annealing (ºC)	Product size	Target gene	Reference
Haemosporidians	PCR	CAT ATA TTA AGA GAA TTA TGG AG AGA GGT GTA GCA TAT CTA TCT AC	50		*Cytochrome-b (cyt-b)*	Waldenstöm et al. 2004.
Haemosporidians	nPCR	ATG GTG CTT TCG ATA TAT GCA TG GCA TTA TCT GGA TGT GAT AAT GGT	50		*Cytochrome-b (cyt-b)*	Waldenstöm et al. 2004.

Table 2-2 continues

Species	Assay	Primer sequence	Annealing (ºC)	Product size	Target gene	Reference
Haemogregarines	PCR	GCCAGTAGTCATATGCTTGTC CAAATCTAAGAATTTCACCTCTGAC	60		18S rRNA	Criado-Fornelio et al. 2006; Ujvari et al. 2004.
Haemogregarines	PCR	GTTTCTGACCTATCAGCTTTCGACG TGATCCTTCTGCAGGTTCAC	60		18S rRNA	Ujvari et al. 2004; Mathew et al. 2000.

2.6. Statistical analysis

Parasite prevalence was calculated according to the equation [(no. of parasitised hosts)/ total no. of examined hosts)] × 100, and the exact 95% confidence interval (95% CI) was also computed.

CHAPTER 3: MORPHOLOGICAL AND MOLECULAR SCREENING OF *AMBLYOMMA* TICKS AND THEIR POTENTIAL TO ACT AS VECTORS OF *HEPATOZOON FITZSIMONSI*

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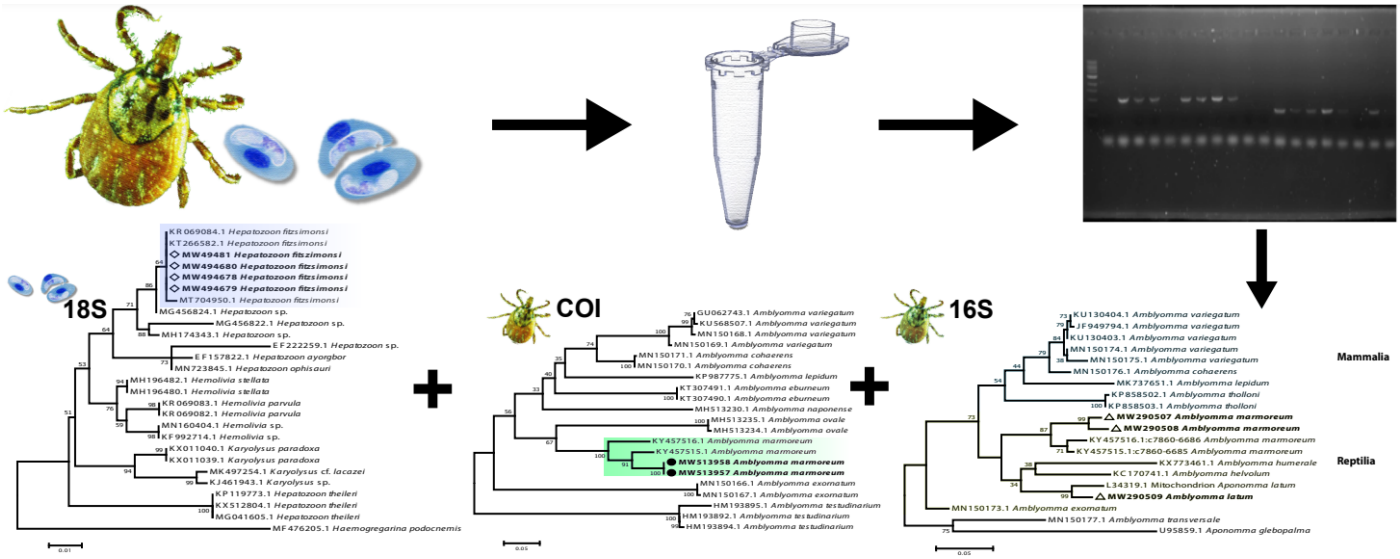


3.1. Introduction

Ticks are obligate blood-feeding ectoparasites infesting a multitude of vertebrates across the world, with more than 900 species being documented (Vredevoe 2006; Sanchez-Montes et al. 2019). They are the most significant vectors of emerging zoonotic pathogens that can be severe and life-threatening to humans, livestock, and other vertebrate hosts (Kho et al. 2015; Luz et al. 2018; Onyiche et al. 2022). The genus *Amblyomma* is the third largest genus in the family Ixodidae, with 130 described species to date (Guglielmone et al. 2010; Kelava et al. 2021), however, only nine of these, including three species that belonged to the former genus *Aponomma* Neumann 1899 have been reported in South Africa (Walker 1991; Horak et al. 2006).

The taxonomy of species of the *Amblyomma* genus has been uncertain and has been continuously revised since 1972 when Kaufmann (1972) first proposed the three main groups of the subgenus *Aponomma*. This author proposed that "*Aponomma*" in its traditional concept is polyphyletic, and consists of three well-defined groups: "typical species", "indigenous Australian species" and "primitive species". The author also proposed that the latter two groups might present new genera. This was confirmed later as "typical *Aponomma*" have been reassigned to the genus *Amblyomma*; and from the "indigenous Australian species", a new subfamily Bothriocrotoninae was erected (Klompen et al. 2002).

Conventionally, tick systematics rely mostly on morphological descriptions, and molecular characterization and phylogenetic analyses have been proposed and used widely in tick systematics to try and resolve confusion between closely related taxa (Mans et al. 2011; Hornok et al. 2020; Kleva et al. 2021; Senbill et al. 2022). The use of different genetic markers including the complete genome, nuclear and mitochondrial genes are currently considered as well-defined DNA sequences to give accurate tick identification (Hornok et al. 2020; Kleva et al. 2021).

Morphological abnormalities in ticks are considered peculiar phenomena, and they have been rarely reported in nature, (Norwak-Chmura 2012; Keskin et al. 2016). The more frequently observed abnormalities are divided into two groups: general abnormalities (i.e. dwarfism, gynandromorphism, change in body shape and duplication) and local abnormalities (i.e. asymmetry of spiracle and scutum, malformation of capitulum, and abnormalities of appendages and festoons) (Kar et al. 2015; Keskin et al. 2016). These abnormalities are mainly caused by environmental stress, mutations, host resistance to tick infestation, chemical control of ticks or high relative humidity (Latif et al. 1988; Dergousoff and Chilton 2007; Keskin et al. 2016; Buczek et al. 2019; Shuaib et al. 2020).

To date, there are only three reports of anomalous ticks in sub-Saharan Africa, with all of these originating from hard ticks collected from domestic animals in South Africa (Gothe 1967), Uganda (Balinandi et al. 2019) and Sudan (Shualb et al. 2020). These studies highlight the lack of research or paucity of reports in this field and as such emphasize the need to investigate the occurrence of anomalous ticks in Africa for the benefits of the scientific community involved in acarology research, as a lack thereof may lead to identification mistakes and taxonomic errors (Buczek 2000). On the other hand, it may not necessarily be a lack of research on the occurrence of these abnormalities but may be attributed to the paucity of reports and thus highlights the need to share this rare information for the benefits of the scientific community involved in acarology research. Although it is not clear how morphological anomalies may alter vector competence in ticks, anomalous ticks may potentially be more efficient vectors of pathogens, hence this warrants further investigation (Alekseev et al. 2007; Nowak-chmura 2012).

Species of *Hepatozoon* Miller 1908 are frequently observed as intraerythrocytic or intraleucocytic haemoparasites of reptiles (Smith 1996; Telford 2009). These adeleid haemogregarines display a heteroxenous life cycle requiring a vertebrate intermediate host and an invertebrate vector or definitive host (Smith 1996; Telford, 2009; Karadjian et al. 2015). The systematics and diversity of species of apicomplexans such as *Hepatozoon* are still poorly understood, the genus *Hepatozoon* itself being paraphyletic based on estimated relationships using sequences of the 18S rRNA gene (see Barta et al. 2012, Haklová-Kočíková et al. 2014, Kvičerová et al. 2014, Cook et al. 2016). In an attempt to aid in the resolution of this, a new genus *Bartazoon* Karadjian et al. 2015 was erected, this genus included all species of reptiles, previously assigned to *Hepatozoon*. One of the characteristics of the genus *Bartazoon* is that the vectors of all members are biting insects, whilst species of *Hepatozoon* have ticks and mites as vectors (Karadjian et al. 2015). To date, the genus *Bartazoon* has not been widely accepted as the monophyly of this genus is not well supported (Maia et al. 2016; Borges-Nojosa et al. 2017). Furthermore, the taxonomic revision which established *Bartazoon* did so based on the use of a single gene – the 18S rRNA gene. As such, the taxonomic changes associated with this new genus was considered premature (Maia et al., 2016; Borges-Nojosa et al. 2017; Cook et al. 2018). Therefore, as suggested and recommended by Maia et al. (2016) and Borges-Nojosa et al. (2017), we will conservatively continue to refer to species parasitizing reptilian hosts as species of *Hepatozoon* Miller 1908 and not *Bartozoon*. In South Africa, species of *Hepatozoon* infecting reptiles have been described from four species of lizards, two species of snake and five species of tortoise (Cook et al. 2018). That in the tortoises includes only a single species *Hepatozoon fitzsimonsi* Dias 1953 (Cook et al. 2014; Cook et al. 2018). This species of haemogregarine was originally described as belonging to *Haemogregarina* (*sensu stricto*) but subsequently reassigned to *Hepatozoon* based on a study into the morphology of its peripheral blood stages, findings of potential life cycle stages in

Amblyomma ticks found feeding on tortoises, as well as phylogenetic analysis of the 18S rDNA sequence fragments from microscopically positive tortoise blood (Cook et al. 2014).

Although it is acknowledged that reptiles are hosts for several *Amblyomma* spp. in South Africa (Horak et al. 2006), the importance of molecular tools in tick identification and importance of resolving the phylogenetic position of *Amblyomma* ticks, information on tick infestation and molecular data of *Amblyomma* ticks infesting these ectotherms in South Africa appear to be in part lacking. Therefore, contributing to studies focusing on the phylogenetic analysis of genus *Amblyomma* (Burger et al. 2012; Hornok et al. 2020; Kelava et al. 2021), as well as extending the scope of previous studies on ticks infesting squamates in South Africa (Hornok et al. 2020), will provide valuable information for and stimulate future discussions. In this sense, the current study focused on the use of morphological and molecular tools to identify and characterize species of ticks collected infesting reptiles in South Africa, as well as to screen these arthropods for *Hepatozoon* spp. Additionally, the phylogenetic position of these sequences of *Amblyomma* species was inferred during this study with respect to other *Amblyomma* ticks from other parts of the world based on the analysis of both nuclear and mitochondrial genes.This study provides molecular findings of *H. fitzsimonsi* in species of *Amblyomma*, with some comments on the potential of these acarine parasites as potential hosts for this haemogregarine. Furthermore, we also provide the first report on morphological abnormalities associated with these tick species from in South Africa.

3.2. Materials and methods

A total of 253 *Amblyomma* ticks from reptiles were morphologically identified using microscopy, and molecularly confirmed by PCR and sequencing. The ticks were further screened for the presence of *H. fitzsimonsi*. Tick identification and characterization, as well as screening for *H. fitzsimonsi* was performed as indicated in chapter 2. Sequences obtained in the current study were deposited in GenBank under the following accession numbers: [(OP650054–OP650061; MW290507–MW290509)16S rRNA; (OP650048–OP650053) 18S rRNA; (OP677562-OP677565) ITS2; (OP677566-OP677568) 12S rRNA; and (MW513957–MW5139580) CO1] for tick identification, and [(MW494678–MW494681) 18S rRNA] for *Hepatozoon* species.

3.3. Results

3.3.1. Tick prevalence, morphology and phylogeny

Tick prevalence

In total, 71 reptiles, including 16 monitor lizards, 30 snakes, and 25 tortoises were captured in this study. Overall, we recorded 43 *Amblyomma latum* (10 females; 33 males) from 30 snakes representing four of 10 different species, 37 *A. exornatum* (5 females; 2 larva; 30 males) from 16

lizards representing one of two different species, 113 *A. marmoreum* (31 female; 13 larva; 69 males) from tortoises and one lizard, and 60 *A. sylvaticum* (56 males; 4 females) from tortoises (Table 3-1). The overall prevalence of tick parasitism on reptiles was 34/71 47.89%. Considering the reptile orders, the prevalence was 37.5% in *Sauria,* 40% in *Ophidia,* and 64% in testudines. It is observed that ticks found on reptiles were predominantly at the adult stage, both males and females, with a few larval stages. Ticks were never found on some species (Table 3-1). The number of ticks found on each animal ranged from one to a maximum of 23 individuals.

Table 3-1. Number of reptiles parasitized by different stages of *Amblyomma* ticks

Animal species	Parasitized hosts	Prevalence	Number of parasitized animals[b] by each tick species and stage									
			A. exornatum			*A. latum*		*A. marmoreum*			*A. sylvaticum*	
	No. parasitized[a]/ No. examined	(%)	M	F	L	M	F	M	F	L	M	F
Bitis arientans	1/3	33.3	0	0	0	1	0	0	0	0	0	0
Chersina angulata	9/9	100	0	0	0	0	0	5	5	0	9	0
Dendroaspis angusticeps	1/2	50	0	0	0	0	1	0	0	0	0	0
Dendroaspis polylepis	3/4	75	0	0	0	1	2	0	0	0	0	0
Haemachatus haemachatus	0/2	0	0	0	0	0	0	0	0	0	0	0
K. spekii	1/1	100	0	0	0	0	0	0	0	0	0	1
Kinixys natalensis	1/1	100	0	0	0	0	0	0	1	0	0	0
Kinixys zombesis	8/12	66.7	3	3	3	3	3	8	8	0	3	3

Table 3-1 continues

Animal species	**Parasitized hosts**	**Prevalence**	**M**	**F**	**L**	**M**	F	**M**	**F**	**L**	**M**	**F**
Mehelya capensis	0/1	0	0	0	0	0	0	0	0	0	0	0
Naja mossambica	7/10	70.0	0	0	0	7	2	0	0	0	0	0
Philothamnus hoplogaster	0/3	0	0	0	0	0	0	0	0	0	0	0
Philothamnus semivariegatus	0/2	0	0	0	0	0	0	0	0	0	0	0
Psammophis brevirostris	0/1	0	0	0	0	0	0	0	0	0	0	0
Python sebae	0/2	0	0	0	0	0	0	0	0	0	0	0
Stimochelys pardalis	2/2	100	0	0	0	0	0	2	2	2	0	0
Varanus albigularis albigularis	6/13	46.2	6	1	1	0	0	0	1	0	0	0
Varanus niloticus	0/3	0	0	0	0	0	0	0	0	0	0	0

[a]Number of reptiles with at least one *Amblyomma* tick

[b]Number of reptiles infested with at least one life stage of *Amblyomma* tick

Morphological analysis of Amblyomma *ticks from reptiles*

One hundred and thirteen (113) ticks were identified as *Amblyomma marmoreum*, forty-three (43) as *Amblyomma latum,* thirty-seven (37) as *Amblyomma exornatum*, sixty (60) as *Amblyomma sylvaticum* based on their morphology (Fig. 3-1). The characteristics of the adult *Amblyomma* ticks are shown in Table 3-2 and described as indicated by Theiler (1945), Theiler and Salisbury (1959), and Horak et al. 2018, respectively.

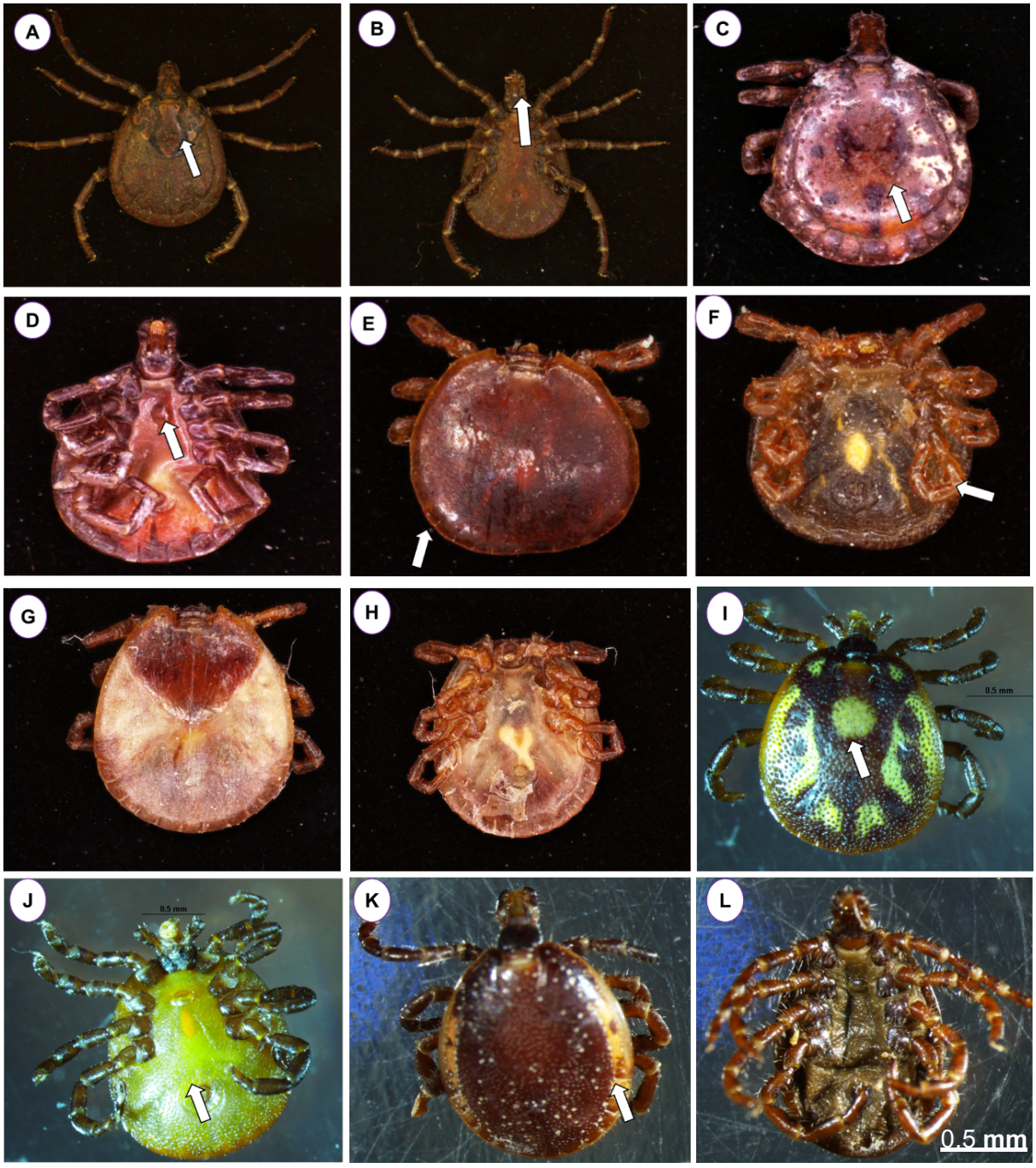


Figure 3-1: Morphology of *Amblyomma* ticks: (A-D) dorsal and ventral view of both male and female *A. marmoreum*, A- arrow showing yellow scutum with brown stripes, B- arrow showing fairly stout palps. C- Arrow showing brown ovoid and convex body, D- Arrow showing geninal pore opposite coxa II; (E-F) dorsal and ventral view of male *A. latum,* E- Festoons picked out with brown lines, F- Robust legs; G-H Dorsal and ventral view of female *A. latum,* I-J dorsal and ventral view of male *A. exornatum,* I & J- yellow-Copper coloured patches and yellow ventral side K-L dorsal and ventral view of *A. sylvaticum,* K- Ivory-coloured ornamentation. Scale bar: 0.5 mm.

Table 3-2. Morphological chacteristics of *Amblyomma* ticks collected from reptiles in the current study

Anatomical feature	*Amblyomma marmoreum*	*Amblyomma latum*	*Amblyomma exornatum*	*Amblyomma sylvaticum*
Capitulum	longer than wide	longer than wide	longer than wide	longer than wide
Hypostome	stout, mildly club-shaped	long, narrow, mildly club-shaped	long midly club-shaped	long, mildly club-shaped
Palps	long, robust	long relative to the size of the tick	long relative to the size of the tick	long
Scutum (Female)	wide, cordiform, widest slightly posterior to eye level,	wide, broadly cordiform; yellowish-brown to light brown, inornate	-	-
Conscutum	wide, broadly ovate, widest in posterior third, MG present	yellowish-brown, inornate, sub-circular to broadly ovate, MG present	broadly ovate to sub-circular, MG absent	wide, broadly ovate, widest at posterior third of its length, MG present
Cervical groove	crescentic, deep, short	absent/ small shallow depressions	deep, crescentic	

Table 3-2 continues

Anatomical feature	*Amblyomma marmoreum*	*Amblyomma latum*	*Amblyomma exornatum*	*Amblyomma sylvaticum*
Eyes	present- oval, flat, and inconspicuous	absent	Absent	present- small, dark, hemispherical, orbited
Spriracular plates	sub-triangular with rounded angles, with long and narrow DP (Male) sub-triangular with rounded angles, with initially broad DP (Female)	elongate, sub-oval with long and moderately thin DP (Male) sub-triangular with short DP (Female)	sub-triangular with rounded angles, with long and narrow DP (Male) sub-oval with long and narrow DP (Female)	broadly sub-oval with long, curved, initially broad DP (Male) sub-triangular with rounded corners, with initially broad DP (Female)
Shape of basis capitula	sub-pentagonal	broadly quadrangular (Male) broadly sub-pentagonal (female)	sub-quadrangular	sub-pentagonal
Cornua (male)	very short with rounded tips	absent	very short	absent
Festoons	large, prominent, considerably longer than wide	picked out by short light brown lines, not delineated anteriorly from the conscutal surface	rectangular, their anterior margins not clearly delineated from conscutal surface.	
Legs	long and robust	robust relative to the size of the tick	robust, uniformly brown in colour.	

Morphological abnormalities of Amblyomma *ticks*

Among the 253 ticks collected from the reptile hosts, five (1.97%) tick specimens were found with anomalies in morphological features. These morphological abnormalities were observed from *Amblyomma latum* infesting snakes, and all anomalies recognized and described are summarized in Table 3-3. The anomalous female *Amblyomma latum'* scutum was ±1.48 mm long by ±2.01 mm wide while male *Amblyomma latum*' conscutum was ±2.47 mm long by ±2.40 mm. Morphological abnormalities were not observed from *Amblyomma marmoreum, Amblyomma sylvaticum and Amblyomma exornatum.* Atrophy of the third left leg and mouthparts turned ventrally from the basis capitula (Fig 3-2A) was observed from one female *A. latum*. In addition, body deformation and asymmetry as well as deformation of the anal groove and mouthparts turned ventrally from the basis capitula (Fig 3-2B). Furthermore, ectromely was recorded in two female *Amblyomma latum* ticks occurring at the 4th left leg (Fig. 3-2C), and 4th right leg (Fig. 3-2E) associated with body asymmetry. Finally, a deformed festoon as well as slight bifurcation was also observed (Fig. 3-2D).

Table 3-3. Number of anomalies in Ixodid ticks collected from reptiles

Host	Tick species	No and gender	Morphological abnormalities
Dendroaspis polylepis	*Amblyomma latum*	1F	Atrophy of the third left leg, mouthparts turned ventrally from the basis capitula (Fig. 3.2A)
Dendroaspis polylepis	*Amblyomma latum*.	1F	Body deformation and asymmetry, deformation of the course of anal groove, mouthparts turned ventrally from the basis capitula(Fig. 3.2B)
Dendroaspis angusticeps	*Amblyomma latum*	1F	Ectromely of the fourth left leg and body asymmetry (Fig. 3.2C)
Naja mossambica	*Amblyomma latum*	1M	Deformed festoon and partial bifurcation (Fig. 3.2D)
Dendroaspis polylepis	*Amblyomma latum*	1F	Ectromely of the fourth right leg, Body deformation and asymmetry, mouthparts turned ventrally from the basis capitula (Fig. 3.2E)

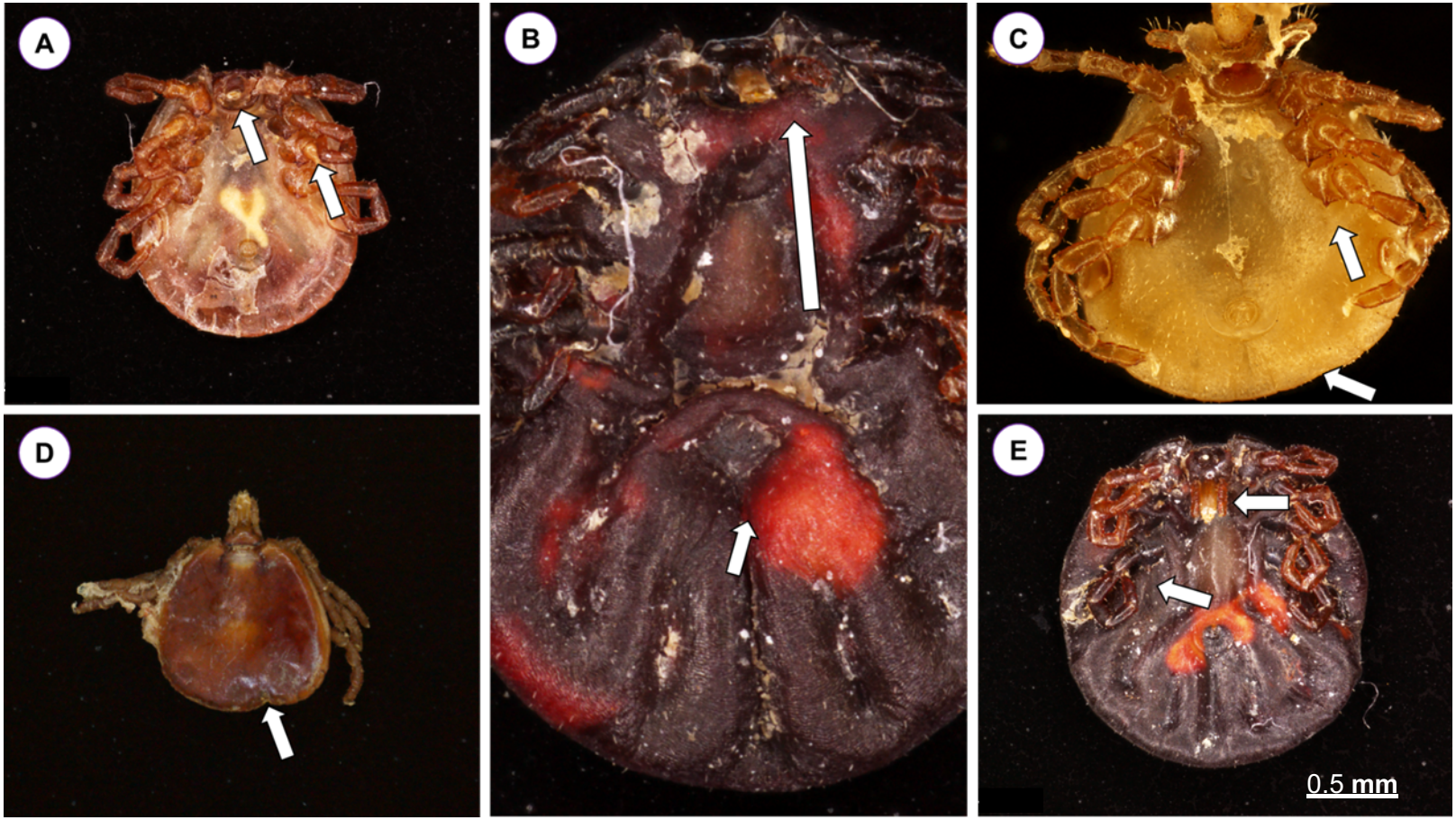


Figure 3-2: Morphological abnormalities of *Amblyomma latum* ticks. Arrows showing: (A) atrophy and ventrally turned mouthparts; (B) body and anal groove deformation, ventrally turned mouthparts; (C) Body asymmetry and ectromely of the 4th left leg; (D) deformed festoon and partial bifurcation; (E) body asymmetry, ectromely of the 4th right leg, and ventrally turned mouthparts. Scale bar: 0.5 mm.

Molecular and phylogenetic analysis of Amblyomma *ticks*

Sequence data between 411–431 bp of the 16S rRNA gene were obtained for *A. marmoreum*, *A. exornatum* and *A. latum*. No good quality sequences were obtained for *A. sylvaticum* with the 16S rRNA gene. According to the BLASTn analysis of the 460 bp of the 16S rRNA gene, the *A exornatum* from the current study showed an identity similarity of 91.73–-97.63% with A*. exornatum* (Accession number: MN032120.1-MN032122.1 & MN150173.1) from DR Congo and South Africa, respectively. However, the *A. latum* from the current study showed an identity percentage of 94.51–99.28% with another published *A. latum* sequences (Accession number: LC34319.1 & MW290509.1). Blast results for sample sequences show that all *A. marmoreum* had 92–95% similarity to a complete genome of *A. marmoreum*, GenBank accession numbers KY457515.1 and KY457516.1, and 99.05% similarity with *A. marmoreum* (Accession number: OM462845.1-OM462862.1) from Italy.

In the 16S rRNA phylogenetic analysis, *A. exornatum* belonged to the sister group of all other members of Amblyomminae, and with high bootstrap value (93%, p= 0.06-0.07), with the South African *A. exornatum* and the DRC *A. exornatum* forming two separate clades. With high support (99%, p=0.04), the *A. latum* from this study clustered in a larger clade consisting of *A. latum* ticks. The *A. marmoreum* from the current study constitute a monophyletic clade (93% bootstrap value) closely affiliated to the same lineage group of *A. marmoreum* (Fig. 3.3). The overall number of variable sites among the sequences of *A. latum* and *A. exornatum* was 75. The highest number of variable sites among the *A. latum* sequences was 18, while for *A. exornatum* sequences was 27. The variable sites of *A. latum* from the current study ranged from 0–1 and it was 2 for *A. exornatum*.

On the other hand, according to the BLASTn analysis of the ~780 bp of the 18S rRNA gene, the *A. exornatum* from the current study showed an identity score of 99.61% with *Amblyomma exornatum* (MT299737.1) from South Africa, while *A. latum* had an identity score of 99.22% with *Aponomma latum* (L76347.1). the *A. marmoreum* had an identity score of 98.65% with *Amblyomma sparsum* (MF102090.1 & OM2810150.1) and *A. marmoreum* (KY457491.1 & KY457492.1). Moreover, the *A. sylvaticum* had an identity similarity of 95.94% with *Amblyomma neumanni* (FJ464424.1) from Argentina. The overall number of variable sites among the sequences of *A. latum* and *A. exornatum* was 26. The highest number of variable sites among the *A. latum* sequences was 5, while for *A. exornatum* sequences was 3. The highest number of variable sites among sequences was 21. In the 18S rRNA phylogenetic analysis, *A. exornatum* from the current study constitutes a monophyletic clade (99% boostrap value; p=0.00) closely affiliated to the same lineage group of varanid ticks. Furthermore, with a weak support (47%; p=0.03), both *A. latum* and *A. exornatum* clustered in a larger clade

consisting of ticks infesting squamate reptiles (Fig. 3-4). The *A. sylvaticum* belonged to a sister group of all other Amblyomminae, while *A. marmoreum* clustered within Amblyomminae with all other *A. marmoreum* sequences.

Sequence identity for *A. exornatum* following BLASTn query of the 900–1200 bp sequences obtained from the ITS2 region had 85.11% with the GenBank entry for *Amblyomma paulopanctatum* (MT000649.1), while *A. latum* showed a similarity score of 86.59% with *Amblyomma tholloni* (MT000684.1). The *A. marmoreum* sequences had a similarity score of 98.94–99.05% with *A. marmoreum* (OM320578.1 & KY457491.1-KY457492.1) sequences published in GenBank. The ITS2 region was not able to amplify the *A. sylvaticum* tick species. Based on the phylogenetic analysis of the ITS2 region of nuclear ribosomal DNA, both *A. latum* and *A. exornatum* belonged to the sister group of all other Amblyomminae, while *A. marmoreum* clustered in one large clade with the same lineage group of *A. marmoreum* (Fig. 3-5).

The 337–355 bp of the 12S rRNA genes and 92.26% homology respectively and *Amblyomma exornatum* (MT586108.1 & MT586109.1), and 86.43% and *Haemaphysalis tibetensis* (OM368296.1) respectively. The 12S rRNA could not amplify both *A. marmoreum* and *A. sylvaticum.* According to the BLASTn analysis of the ~710bp of the CO1 gene, the *A. sylavticum* had an identity score of 86.91% with *A. marmoreum* (KY457516.1) and 86.61% *A. testudinarium* (LC554763.1), while *A. marmoreum* had an identity score of 94.16%–99.85% with *A. marmoreum* (KY457515.1, OL339476.1). The CO1 gene could not amplify the DNA of both *A. latum* and *A. exornatum.* Based on the phylogenetic analysis of the CO1 gene, with 100% boostrap value, the *A. marmoreum* from the current study formed a monophyletic clade affiliated with closely related *A. marmoreum* group, while *A sylvaticum* belonged to a sister group of *A. marmoreum* clade, forming its own monophyletic clade within the Amblyomminae (Fig. 3-6).

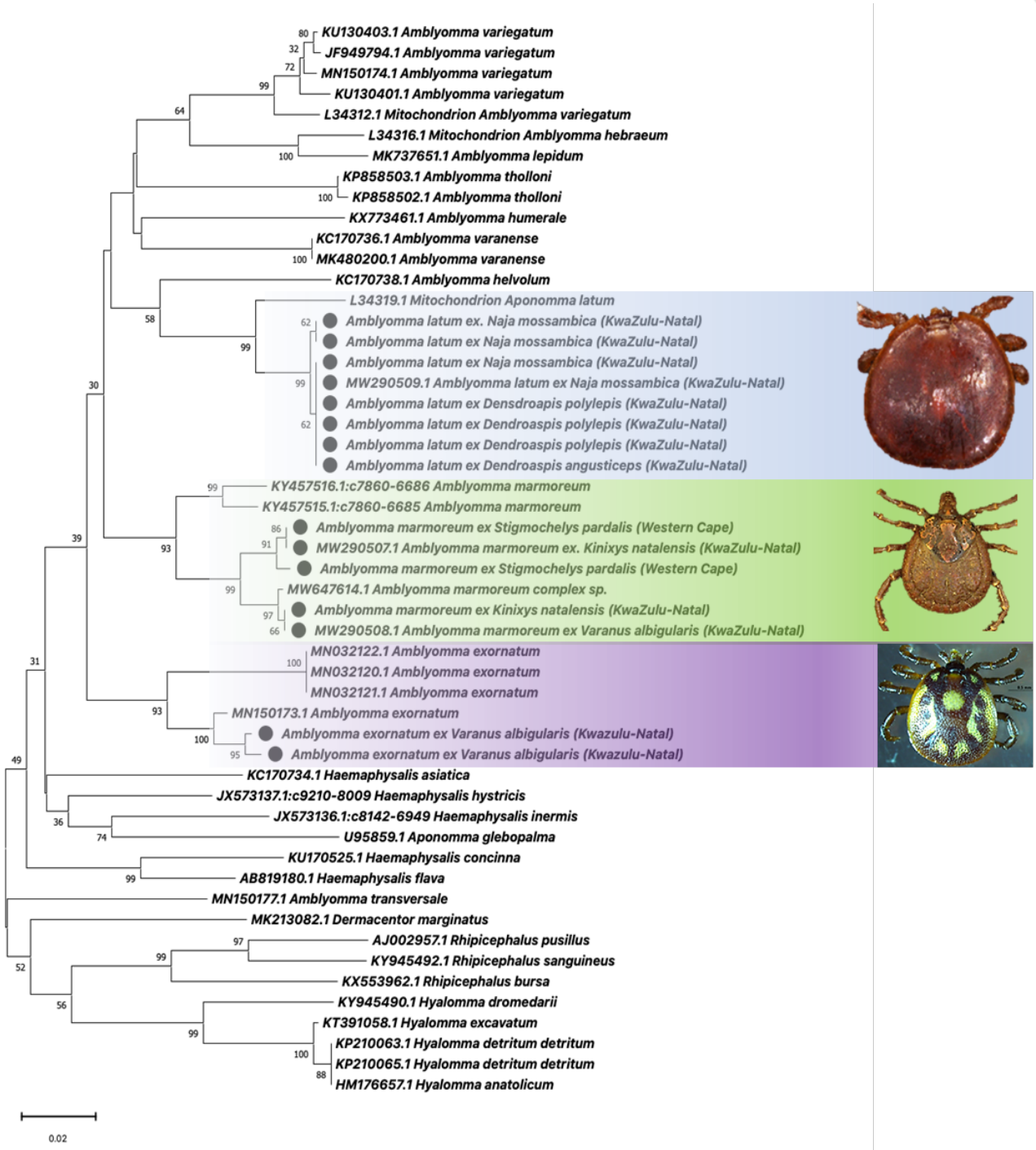


Figure 3-3: Minimum evolution analysis of *Amblyomma* tick species based on the 392 bp gene16S rRNA sequences of 16S rRNA usinn Jukes Cantor + Gamma distribution model. Bootstrap values at the major nodes are of percentage agreement among 1000 replicates. The branch scale represents substitutions per site. Sequence data for *A. sylvatiicum* could not be obtained with the 16S rRNA gene.

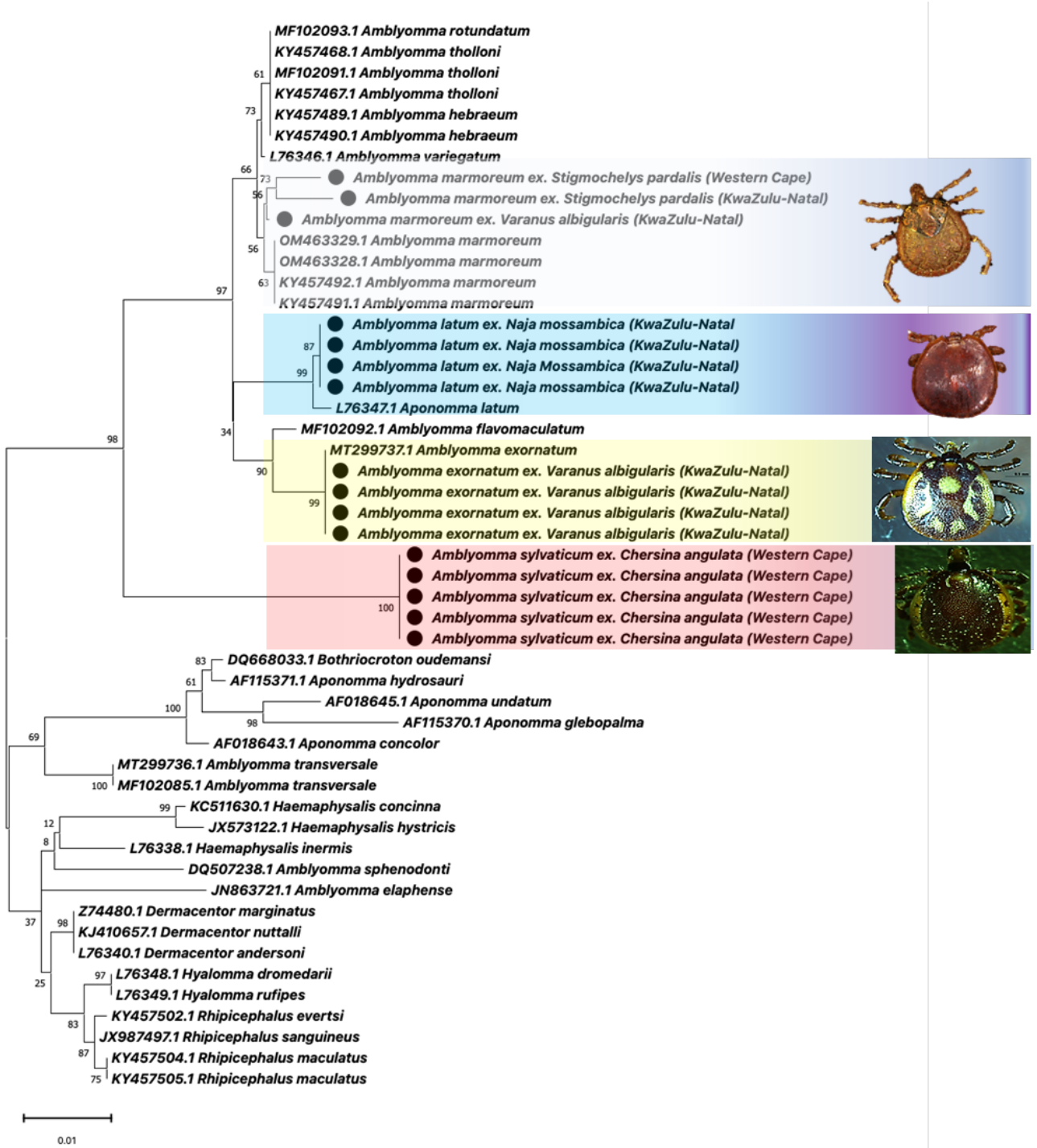


Figure 3-4: Minimum evolution analysis of *Amblyomma* tick species based on the 712 bp gene sequences of 18S rRNA using K2 +G model. Bootstrap values at the major nodes are of percentage agreement among 1000 replicates. Sequences from the study are indicated with black dots. The branch scale represents substitutions per site.

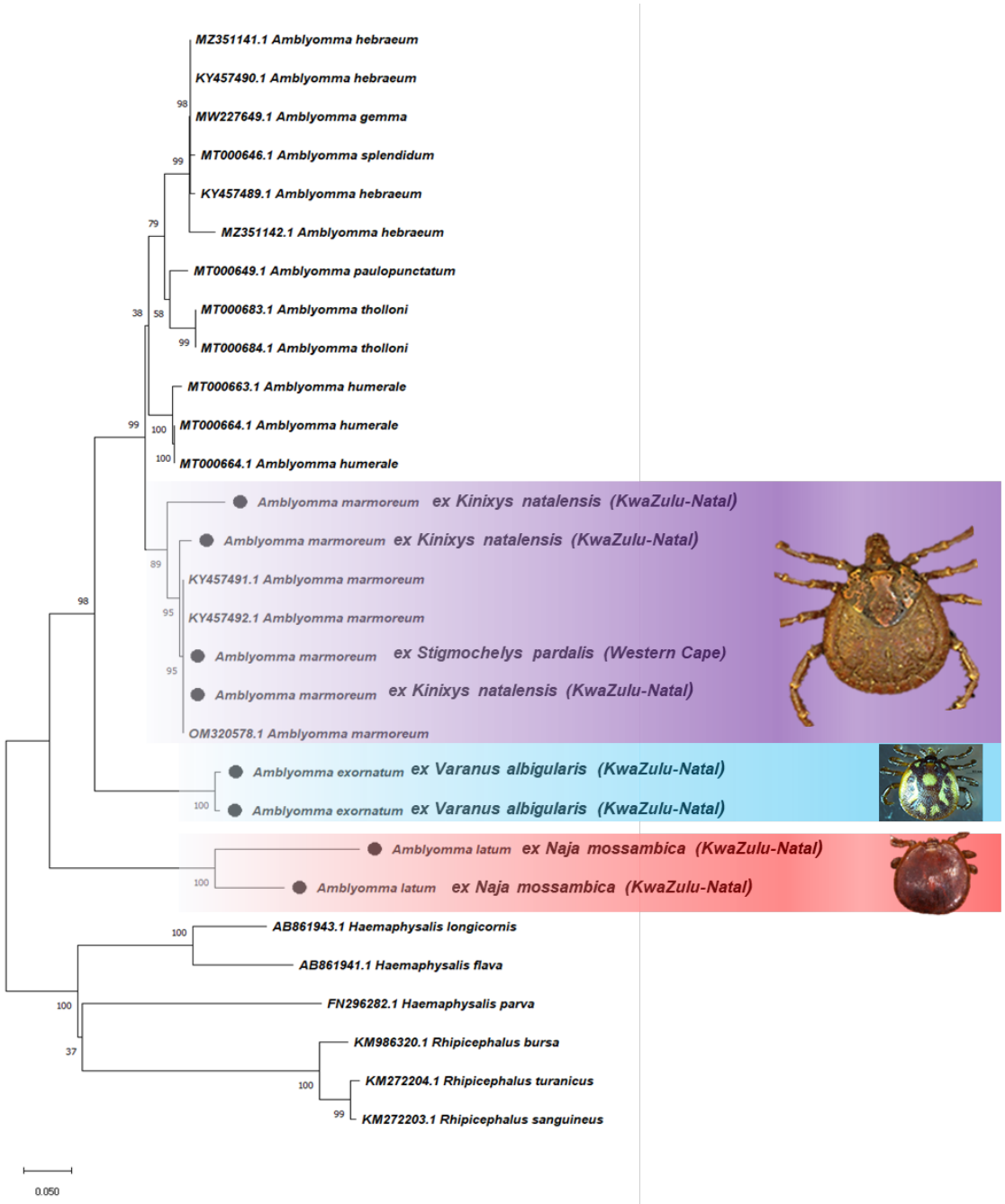


Figure 3-5: Maximum likelihood analysis of *Amblyomma* tick species based on the 612 bp of the ITS2 spacer sequences using TN93+G model. Bootstrap values at the major nodes are of percentage agreement among 1000 replicates. The branch scale represents substitutions per site. Sequence data for *A. slyvaticum* could not be obtained with ITS2 spacer.

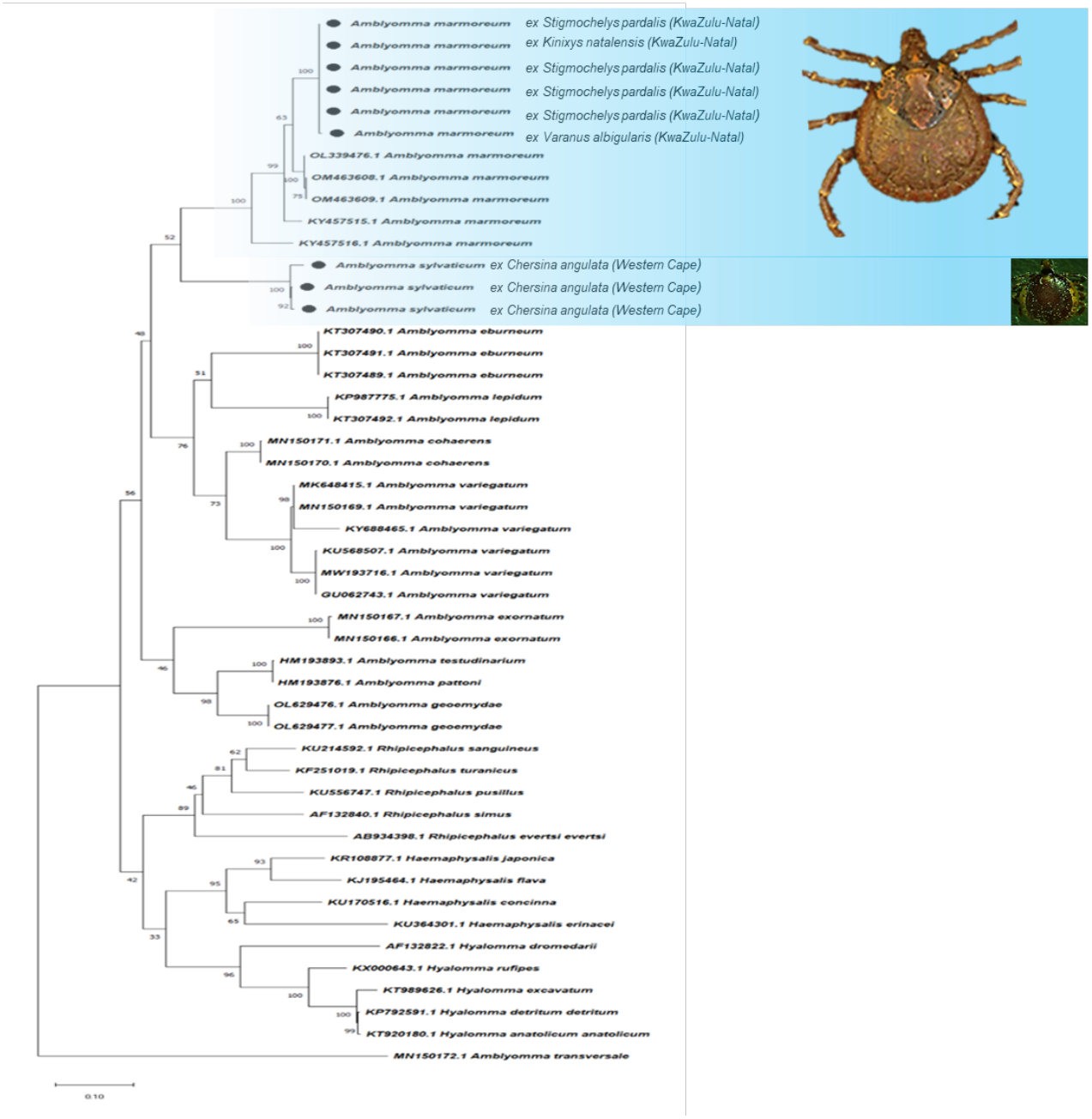


Figure 3-6: Maximum likelihood analysis of *Amblyomma* tick species based on the CO1 sequences. Bootstrap values at the major nodes are of percentage agreement among 1000 replicates. The branch scale represents substitutions per site. Sequence data for *A. latum* and *A. exornatum* could not be obtained with the CO1 gene.

Molecular and phylogenetic analysis of Hepatozoon *from* Amblyomma *ticks*

The overall prevalence of *Hepatozoon* was 14/253 (5.53%). It was detected from 1/4 (25%) *A. marmoreum* parasitizing *Varanus albigularis* and 13/173 (7.51%) *A. marmoreum* and *A. sylvaticum* parasitizing tortoises. No *Hepatozoon* was detected from *A. latum*. The BLASTn analysis for the 430 bp 18S rRNA of the *Hepatozoon* sequences had a 99% similarity with *H. fitzsimonsi* accession number KR069084.1 from South African tortoises. According to the evolutionary divergence estimates these *Hepatozoon* gene sequences were most closely related to the *H. fitzsimonsi* gene sequence (GenBank: KT266582.1) from *Amblyomma sparsum* collected off *Stigmochelys pardalis* from Kenya (P=0.01), than to gene sequences of *H. fitzsimonsi* from *Kinixys* spp. from South Africa (GenBank: KR069084.1) (P=0.02–0.03) and Nigeria (GenBank: MT704950.1) (P=0.02–0.04). All the 18S rRNA *Hepatozoon* sequences from the study clustered in a single clade with *H. fitzsimonsi* from Africa, this clade clustered within a larger monophyletic clade, sister to a clade containing *Hepatozoon* spp. from *Ctenosaura pectinata* (Mexico) and *Ornithoros actamensis* (Chile), respectively (Figure 3-7).

Table 3-4. Evolutionary differences of some species of *Hepatozoon* Miller 1908 from reptiles and their associated ticks. Expressed as standard error (bottom left) and pair-wise distance (p distance) (top right).

	Accession number	***Hepatozoon* sp**	**Host spp & Country**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1.	**MW494678**	***Hepatozoon fitzsimonsi***	***Amblyomma marmoreum***		0.02	0.03	0.02	0.01	0.03	0.03	0.04	0.06	0.03
2.	**MW494679**	***Hepatozoon fitzsimonsi***	***Amblyomma marmoreum***	0.01		0.03	0.01	0.01	0.03	0.03	0.05	0.06	0.03
3.	**MW494680**	***Hepatozoon fitzsimonsi***	***Amblyomma marmoreum***	0.01	0.01		0.03	0.01	0.02	0.02	0.04	0.05	0.03
4.	**MW494681**	***Hepatozoon fitzsimonsi***	***Amblyomma marmoreum***	0.01	0.01	0.01		0.01	0.03	0.04	0.05	0.06	0.04
5.	KT266582.1	*Hepatozoon fitzsimonsi*	*Amblyomma sparsum*- Kenya	0.00	0.00	0.00	0.00		0.01	0.01	0.01	0.03	0.00
6.	KR069084.1	*Hepatozoon fitzsimonsi*	*Kinixys zombesis*- South Africa	0.01	0.01	0.01	0.01	0.00		0.00	0.02	0.03	0.01
7.	MT704950.1	*Hepatozoon fitzsimonsi*	*Kinixys* spp.-Nigeria	0.01	0.01	0.01	0.01	0.01	0.00		0.02	0.04	0.01
8.	MH174343.1	*Hepatozoon* sp.	*Ornithodoros atacamensis*-Chile	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.02	0.01
9.	MG456822.1	*Hepatozoon* sp.	*Sauromalus* spp-Mexico	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.03
10.	MG456824.1	*Hepatozoon* sp.	*Ctenosaura pectinata*-Mexico	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	

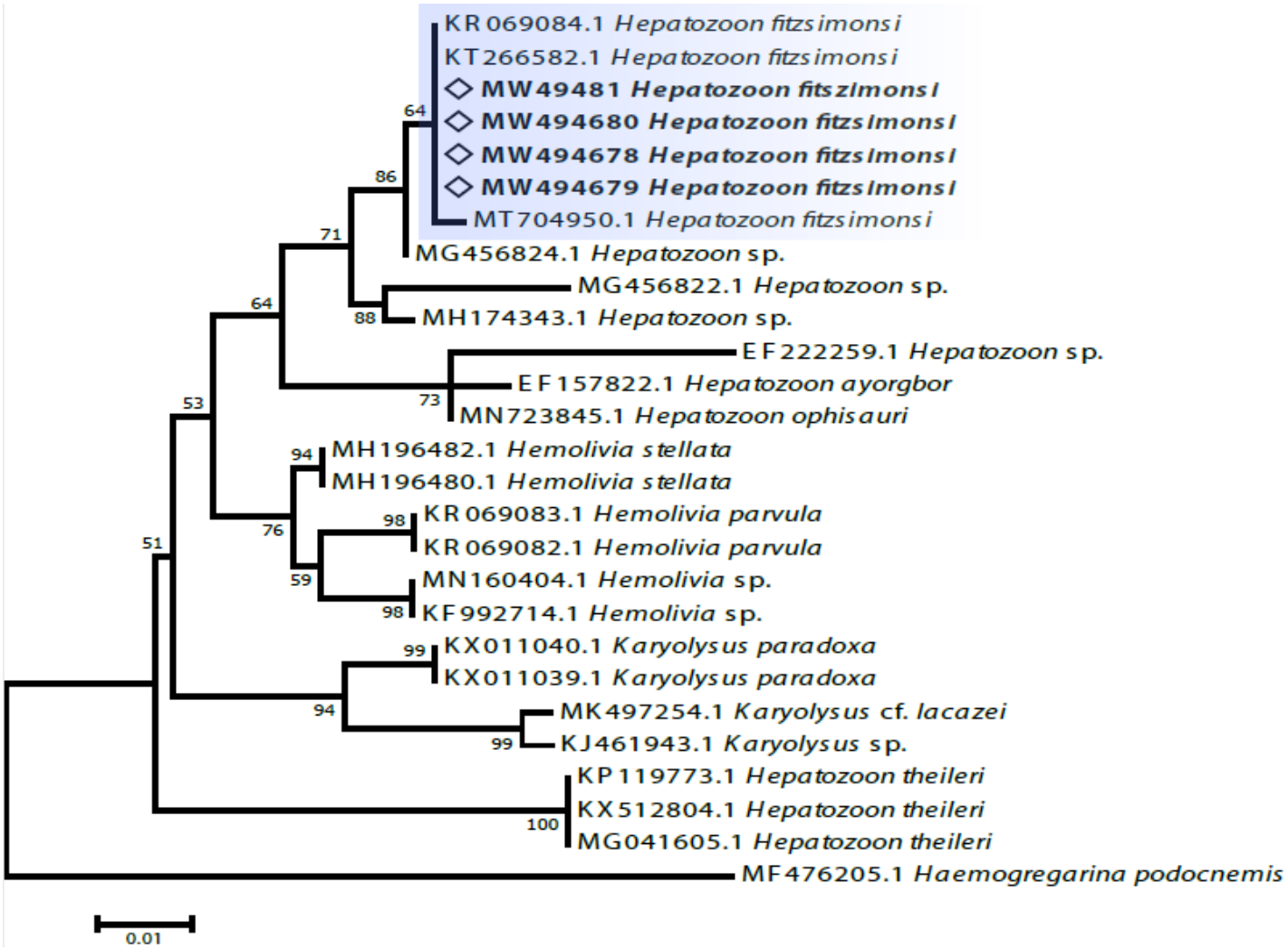


Figure 3-7: Maximum likelihood analysis of species of *Hepatozoon* based on the 410 bp of 18S rRNA sequences using Tamura 3-parameter + Gamma distribution model. Bootstrap values at the major nodes are of percentage agreement among 1000 replicates.

3.4. Discussion

Ticks represent a major source of growing economic and public health concern, especially in the tropics and sub-tropics, and proper identification of these acarines is essential as it represents the first step in the successful control of these ectoparasites, as well as their pathogens (Mathison and Pritt 2014; Senbill et al. 2022). Morphological identification is the backbone of tick systematics, however in certain cases it cannot be helpful, especially in differentiating closely related tick species, identifying immature stages, as well as in ticks with uncertain taxonomic status. These difficulties are recently being avoided by the intervention of molecular techniques. In this context, for adequate molecular identification of ticks, it was recommended to use multiple (mitochondrial and nuclear) genetic markers (Hornok et al. 2020). In the present study, a total of 253 *Amblyommma* ticks of four species (*Amblyomma marmoreum, A. sylvaticum, A. latum, A. exornatum*) were found infesting reptiles from two provinces of South Africa. One *V. albigularis* was found infested with four *A. marmoreum* and five *V. albigularis* were found infested with 37 *A. exornatum*. These results were not surprising because according to data encompassing several surveys, as well as studies focusing on the identification of *Amblyomma* ticks, *A. exornatum* is always associated with *Varanus* (Horak et al., 2006; Hornok et al. 2020; Ngoy et al. 2021), as observed here. Furthermore, *A. marmoreum* have been collected from different reptile species, avians, wild canivores, humans, domestic animals, as well as small mammals where reptiles harboured all stages of the tick, while other animals had only immature stages (Horak et al. 2006). These findings suggest that this tick species is a generalist and readily makes use of all these animals for the completion of its developmental stages. *Amblyomma latum* was found infesting different species of snakes in the current study; this tick species is known as the “snake tick”, but it has also shown its ability to infest amphibians, testudines and squamates that are phylogenetically distinct from its usual Afrotropical hosts (Burridge 2001). *Amblyomma sylvaticum* was found infesting only *Chersina angulata* from Western Cape and this was not a surprise, because to date, this tick species has been documented to occur largely in the western regions of the Northern and Western Cape, as well as in the south-western regions of the latter province and of the Eastern Cape Province (Horak et al. 2006). The *A. marmoreum* and *A. sylvaticum* are most frequently encountered on tortoises, as observed in this study (Theiler 1959; Horak et al. 2006). The quality images on morphological features of these tick species have been published (Horak et al. 2018).

Some members of genus *Amblyomma* use reptiles as a primary feeding source, with as many as six species being exclusive parasites of reptiles from Squamata (Guglielmone et al. 2014; Hornok et al. 2020). Most of these tick species are known as hosts and vectors of various pathogenic diseases that can cause considerable economic losses and may be of zoonotic

importance in tropical and sub-tropical regions of the world (Ogo et al. 2012; Andoh et al. 2015; Ogo et al. 2017; Vesco et al. 2013; Hornok et al. 2020; 2022; Mofokeng et al. 2022). However, molecular data of these species in South Africa is lacking, with only one detailed phylogenetic study of *A. exornatum* (Hornok et al. 2020). Moreover, even though the *A. sylvaticum* has been documented to harbour a relapsing fever *Rickettsia* (Halajian et al. 2016), the phylogenetic position of this tick species is not known and the current study provides insight on its position using both nuclear and mitochondrial markers.

While species identification and classification of *Amblyomma* ticks have historically been focused on morphological characteristics of the adult stages of these ticks, unless one is an expert in tick morphology and identification, it can be extremely difficult and there is the possibility of error. A DNA-based approach provides the possibility to examine the genetic variation and provides a much more straightforward route to quantify genetic variability between and within *Amblyomma* tick species, thus becoming increasingly important for studies in acarology (Black and Piesman 1994; Burger et al. 2012; Lopes et al. 2016). The present analysed tick species mainly infest reptiles, but can also infest a variety of vertebrate hosts showing different levels of specificity (Horak et al. 2006). Based on the analysis of the 16S rRNA gene in the current study, the phylogenetic tree showed that *A. exornatum* formed a sister group with all other members of Amblyomminae, and this is in agreement with the previous study based on the analysis of nuclear and mitochondrial markers (Hornok et al. 2020).

Considering the phylogenetic analysis of the highly conserved 18S rRNA gene, the overall topology of the tree reflected *A. sylvaticum* formed a sister group to all other members of Amblyomminae, while according to the fast evolving CO1 gene, this tick clustered withing the amblyomminae clade. This is the first insight on the phylogenetic position of this tick species.

Even though the 16S rRNA gene did not amplify *A. sylvaticum* DNA, both phenotypic characteristics and the CO1 and 18S rRNA confirmed this species as *A. sylvaticum*. It is not clear why the amplification of *A. sylvaticum* with the 16S rRNA was unsuccessful. We could speculate that the 16S rRNA gene in this tick species is difficult to amplify, given that fragments of this gene was able to amplify for the other 3 species of *Amblyomma* in the study . On the other hand, it may not be the species itself that could have influenced the amplification of this gene but the quality of the extracted DNA. It is worth noting that the above specimens were preserved in 70% ethanol for a long period of time (> 5 years), which could have resulted in the degradation of the tick's DNA (Hill and Gutierrez 2003). However, fresh tick specimens are known to be good for molecular analysis and they are needed to confirm these speculations, even though they are not easy to obtain (Mtambo et al. 2006). Sequence analyses of both

nuclear and mitochondrial genes have been previously used to distinguish and to assess the phylogenetic relationships of closely related tick species, and they appear to be informative molecular markers on several taxonomic scales (Black and Piesman 1994; Chao et al. 2009; Lopes et al. 2016; Hornok et al. 2020). Based on the analysis of both nuclear and mitochondrial markers in the current study, the phylogenetic trees showed a clustering of reptile associated *Amblyomma* tick sequences into different groups with high bootstrap values, indicating that the possibility of having a diverse population of reptilian *Amblyomma* ticks in Africa cannot be ruled out. However, a more detailed phylogenetic study of reptilian *Amblyomma* ticks needs to be undertaken to confirm any intraspecific variation among these ticks.

Morphological abnormalities are infrequently reported among species of the Ixodida and can occur in any developmental stage in natural populations, with a prevalence usually below 1% (Latif et al. 1988; Guglielmone et al. 2000; Dergousoff and Chilton 2007; Nowak-Chmura 2012; Kar et al. 2015; Munoz-Leal et al. 2018; Balinandi et al. 2019; Molaei et al. 2020; Shuaib et al. 2020; Laatamna et al. 2021). However, this report adds to others that have also reported anomalous ticks with a prevalence exceeding 1% (Labruna et al. 2000; Alekseev et al. 2007; Keskin et al. 2016; Chitimia-Dobler et al. 2017; Azzi et al. 2019; Salceda-Sanchez et al. 2020). In the current study, ectromely was recorded in two female *A. latum* ticks and was associated with body asymmetry. This abnormality has been frequently reported in many members of Ixodidae, and has been documented to follow an injury during the larval or nymphal stage of the tick, which leads to a complete loss of one or more legs as reported in numerous tick genera (Guglielmone et al. 2000; Dergousoff and Chilton 2007; Nowak-Chmura 2012; Keskin et al. 2016; Larson and Paskewitz 2016; Chitimia-Dobler et al. 2017; Balinandi et al. 2019; Molaei and Little 2018; Molaei et al. 2020; Shuaib et al. 2020). Other factors such as exposure to chemicals (Buczek et al. 2013), alterations in temperature and humidity (Buczek, 2000; Kar et al. 2015) during development can also be attributed as a possible cause. Additionally, atrophied legs and other exoskeleton abnormalities were observed from the same species of ticks. These abnormalities, as stated by Estrada-Pena (2001) and Shuaiab et al. (2020), could be attributed to a number of etiological factors, such as host resistance to tick infestation, which is known to change the morphology of tick developmental stages.

A species of *Hepatozoon*; *H. fitzsimonsi* was moleculary detected from one of four (25%) *A. marmoreum* infesting *V. albigularis*, from 10 of 96 (10.4%) *A. mamoreum* infesting tortoises and from three of sixty (5.0%) of *A. sylvaticum*. The detection of this *Hepatozoon* species has been documented from several species of *Amblyomma* infesting tortoises in Africa (Cook et al. 2014; Omondi et al. 2017). *Hepatozoon fitzsimonsi* was reassigned from the genus *Haemogregarina* (*sensu stricto*) to the genus *Hepatozoon* (Cook et al. 2014), and more recently to genus *Bartazoon* (Karadjian et al. 2015). The aforementioned authors suggested

ticks as probable vectors for *H. fitzsimonsi* due to the sporogonic stages (including sporocysts and sporozoites) observed in *A. marmoreum* and *A. sylvaticum* found infesting *H. fitzsimonsi* infected and seemingly uninfected tortoises. An attempt to molecularly identify and compare these stages to *H. fitzsimonsi* in the peripheral blood of infected tortoises was made with only bands of the targeted size obtained. Unfortunately, the sequences obtained in the Cook et al. (2014) study were unsatisfactory. To the best of our knowledge, there has been no molecular report to date showing the definite presence of *H. fitzsimonsi* in ticks from South Africa. As highlighted by Karadjian et al. (2015), there is a high potential for error when analysing vectors morphologically and molecularly for haemogregarine infections. It is possible to wrongly assume that the sporogonic stages observed in a vector such as the tick collected from a haemogregarine infected vertebrate, such as in the case of *H. fitzsimonsi* from tortoises, are the same parasite. This is particularly problematic if the ticks collected were engorged at the time of collection and molecular analysis.

In the present study, *H. fitzsimonsi* was detected from *A. marmoreum* infesting tortoises and monitor lizards. The presence of *H. fitzsimonsi* in ticks found infesting monitor lizards is noteworthy, as no infections of *H. fitzsimonsi* have been recorded in these reptiles (Cook et al. 2016). From southern African monitor lizards, two species of *Hepatozoon* have been described *Hepatozoon camari* (Dias 1954) and *Hepatozoon varani* (Laveran, 1905) from Mozambique and South Africa respectively (Dias 1954; Laveran 1905 respectively; Cook et al. 2016). However, morphologically, these species do not conform to *H. fitzsimonsi*. *Hepatozoon fitzsimonsi* displays a mature gamont measuring 17.2 $\times$ 3.8 μm, which is banana shaped with a rounded to square compact nucleus (Cook et al., 2014). In comparison, *H. camari* measures 11.8 $\times$ 5 μm (banana form) or 14.3 $\times$ 18.3 μm (curved form), with an irregular nucleus; whilst *H. varani* measures 14 $\times$ 3 μm, the gamont folded (suggesting a length of 24 μm), the nucleus occuring on the fold. *Hepatozoon fitzsimonsi* gamonts were never seen to fold (Cook et al. 2009; Cook et al. 2014). The occurrence of a tick harbouring *H. fitzsimonsi* collected from a monitor lizard could be considered as a good example for the potential errors highlighted by Karadjian et al. (2015), assuming the parasite sequenced in the ticks is related to that found in the vertebrate, when effectively the 'vectors' are still engorged with a previous blood meal from a different host. To avoid this scenario, this study only used ticks which had been given the opportunity to digest their blood meals. This would then suggest that the infection is long-lived in the tick, with the potential of ticks acting as hosts and potential vectors increasing in possibility (Cooper et al. 2012).

In Kenya, during a study aimed at the molecular detection of tick-borne pathogens (TBPs), *Amblyomma sparsum* and *Amblyomma falsomarmoreum*, collected from tortoises, were

documented to harbour *H. fitzsimonsi* (Omondi et al. 2017). Similarly, this study only used unengorged ticks for the same reasoning as mentioned above – that is to minimise contamination from the vertebrate hosts' DNA and effectively that of peripheral blood infections. Notably, *H. fitzsimonsi* was sequenced from ticks collected from only tortoises, the sequence data showing a high prevelance of *H. fitzsimonsi* in these ticks. Furthermore, *A. falsomarmoreum* was found exclusively on tortoises and is a tick species specifically associated with these vertebrates. Again, this leads to the question of whether or not *H. fitzsimonsi* is long-lived in ticks and is perhaps undergoing sporogonic development within these acarines. However, this will only be determined once more complete life cycle data is achieved for this haemogregarine, but simultaneously, the coincidence of the above findings is difficult to ignore.

Karadjian et al. (2015), in their systematic revision of adeleid haemogregarines, proposed that *H. fitzsimonsi* be reassigned to the newly erected genus *Bartazoon*, a genus to which all species of reptile *Hepatozoon* have been reassigned and which is vectored by biting insects. However, since the type species for *Bartazoon* (*Bartazoon breinli* Mackerras, 1960) is transmitted via the ingestion of infective stages, if *H. fitzsimonsi* belongs to *Bartazoon*, further research will need to take into account the ingestion of infected vectors such as mosquitoes by tortoises. However, this is a difficult scenario to envision with such slow-moving animals.

3.5. Conclusion

The current study was limited by the lack of *A. marmoreum, A. sylvaticum, A. exornatum,* and *A. latum* sequences in GenBank, as it did not afford us the opportunity to draw inferences. This highlights the importance of screening reptiles for different species of ticks in an effort to increase their known diversity, host preference, localities and haemoparasites they may harbour and transmit to their vertebrate hosts. Nevertheless, this study demonstrates the abundance and population diversity of *Amblyomma* ticks infesting some reptiles in South Africa, thereby raising the potential for the existence of various tick-borne zoonoses, possibly beyond those that are already documented in the country. In fact, all these four tick species from the current study are cited as known vectors of zoonotic pathogens (Peter et al. 2000; Omondi et al. 2017; Halajian 2018; Hornok et al. 2022; Mofokeng et al. 2022).

Morphological anomalies vary considerably among different populations of ticks across the world and their causes and ecological implications in nature are mostly unknown. Given these anomalies' importance for taxonomy and the possible implication of anomalous ticks being more efficient in the transmission of pathogens, it is of interest to intensify wide-scale investigation of morphological anomalies in tick populations across the country and to determine what drives these phenomena in different tick life stages in nature and on hosts.

Although a case of gynanders was reported in *Boophilus* and *Amblyomma* ticks from South Africa (Gothe 1967), this study presents the first report of anomalous ticks associated with reptiles in South Africa. With this study describing morphological abnormalities in hard ticks, the number of anomalous tick cases has been raised to two in South Africa. Further studies on the vectorial capacity of these tick species is needed to determine their medical and epidemiological significance in South Africa.

As *H. fitzsimonsi* has been observed infecting several species of tortoises from southern Africa, it would appear to be a generalist parasite with a potentially wide distribution (Dias, 1953; Cook et al., 2009; Cook et al., 2014). Taking into account the results of the present study and that of Omondi et al. (2017), as well as the findings of Cook et al. (2014), it cannot be ruled out that ticks may play a role as vector in the life cycle of *H. fitzsimonsi*. As such revisiting this and determining if there is a molecular link between the supposed stages of the parasite observed in the tick and that of the known stages of *H. fitzsimonsi* in the tortoise, would greatly assist in furthering knowledge on this specie's life cycle and its taxonomy. *Amblyomma marmoreum* ticks were found on tortoises imported into Florida and have become established in countries outside of Africa (Allan et al., 1998, Burridge, 2001). If these acarines are found to act as vectors for this haemogregarine, this may pose a conservation risk to naïve hosts, particularly those that are critically endangered. It is therefore important to elucidate the routes and modes of infection of this species of *Hepatozoon*, particularly in light of the translocation and illegal trade in many tortoise species (see Burridge, 2001). Furthermore, if this haemogregarine is found to have ticks as vectors, it will not fit within the present characteristic confines defining the genus *Bartazoon*, resulting in yet another paraphyletic genus.

CHAPTER 4: DEVELOPMENTAL STAGES AND MOLECULAR PHYLOGENY OF *HEPATOZOON FITZSIMONSI* (DIAS 1953) (ADELEORINA: HEPATOZOIDAE) IN TORTOISES *STIGMOCHELYS PARDALIS* (CRYPTODIRA: TESTUDINIDAE) AND TICKS OF THE GENUS *AMBLYOMMA* (ACARI: IXODIDAE) FROM SOUTH AFRICA

4.1. Introduction

Haemogregarines (Apicomplexa: Adeleorina) are commonly encountered protozoan parasites of erythrocytes or leukocytes and are frequently described from a wide variety of vertebrates including amphibians and reptiles (Davies and Johnston 2000; Rossow et al. 2013; Cook et al. 2014, 2018; Tomé et al. 2019). Even though this group of parasites contains several genera, such as *Hemolivia* Petit et al. 1990, *Haemogregarina* Danilewsky 1985, and *Karyolysus* Labbé 1894, the genus *Hepatozoon* Miller,1908 appears to be the most prevalent in reptiles (Telford 2009; Cook et al. 2014). However, even with the increase in research on these parasites in the last decade, knowledge on the diversity and systematics of these apicomplexans is still lacking and remains contentious (Borges-Nojosa et al. 2017; Cook et al. 2018). Based on relationships of these haemogregarine genera inferred using the 18S rRNA gene, *Hepatozoon* remains paraphyletic (Barta et al. 2012, Haklová-Kočíková et al., 2014; Kvičerová et al. 2014). This lead Karadjian et al. (2015) to erect a new genus *Bartazoon* Karadjian et al., 2015, to resolve this issue. Species of *Hepatozoon* display a heteroxenous life cycle, requiring a vertebrate host in which asexual reproduction occurs, as well as an invertebrate host in which sexual reproduction occurs (Smith 1996; Telford 2009; Karadjian et al. 2015). Invertebrate hosts range from haematophagous insects to acarine hosts such as ticks and mites (Karadjian et al. 2015). For transmission to occur between the parasitised vector and the vertebrate host, ingestion of this vector by the vertebrate is required or ingestion of tissue cysts in a vertebrate host is required (Davies and Johnston 2000; Karadjian et al. 2015). Karadjian et al. (2015) proposed that *Bartazoon* would comprise species vectored by haematophagous insects, whilst those species vectored by acarine hosts would remain as *Hepatozoon*. However, *Bartazoon* has up to now, not been widely accepted as the monophyly of this genus is not well supported (Maia et al. 2016; Borges-Nojosa et al. 2017).

Hepatozoon fitzsimonsi (Dias 1953), a haemogregarine of southern African tortoises, along with other *Hepatozoon* species of amphibians and reptiles, was one of the species proposed to be a member of *Bartazoon* (Karadjian et al. 2015). This, as such, suggested the potential vector of this parasite to be a biting insect. However, research done on this parasite persistently observed a close association with tortoises and ticks (Cook et al. 2009; 2014), the latter study observing what appeared to be sporogonic stages including sporocysts and sporozoites in tick haemocoel from infected and uninfected tortoises. Even though Cook et al. (2014) attempted to molecularly identify these tick stages, it was unsuccessful. Recently, two molecular screening studies (Omondi et al. 2017 and Mofokeng et al. 2021), both screening ticks collected from reptiles in Kenya and South Africa for tick-borne pathogens and *Hepatozoon* respectively, identified *H. fitzsimonsi* in ticks. In both studies unengorged ticks were used. These previous findings in mind, the present study aimed to revisit the potential

that ticks can act as vectors for *H. fitzsimonsi* in tortoises, by (i) collecting blood/tissue and ticks from tortoises, (ii) screening both microscopically for the presence of blood, merogonic, and sporogonic stages respectively, and (iii) molecularly characterising these stages to determine if they are that of *H. fitzsimonsi*. The present study thus provides further support for ticks acting as the vectors of *H. fitzsimonsi* based on observation of its life cycle stages in tortoises (*Stigmochelys pardalis*) as well as in the invertebrate host (*Amblyomma* spp.).

4.2. Materials and methods

For this part of the study, tortoises collected from Bonamanzi and Ndumo Game Reserve, KwaZulu-Natal (KZN) (2016–2017), were used. These were identified to species level using field guides (Branch 1998,2008; Boycott and Bourquin 2000). Blood was collected from the subcarapacial sinuses (McArthur et al., 2004) and thin blood smears were prepared so as to determine the presence and morphometrics of peripheral blood stages of haemogregarines, in particular *H. fitzsimonsi*. Additionally, a small volume of blood was stored in 70% ethanol in a -20°C freezer for molecular analysis. At the same time, ticks were collected in situ from the tortoises for the preparation of visceral impression slides, once the blood meal had been digested, so as to screen for and measure the sporogonic stages if present. The remains of these ticks were then preserved in 70% ethanol in a -20°C freezer for molecular analysis. In one case, a tortoise was found recently dead as a result of a roadkill incident. Samples from this individual included thin blood slides, a volume of whole blood stored in 70% ethanol in a -20°C freezer for molecular analysis, and impression slides of the kidney, heart, liver, lung, and spleen so as to be screened for gamont and merogonic stages. Ticks were also collected from this individual and impression slides prepared. The methods used in morphologically and molecularly describing the developmental stages of *H. fitzsimonsi* from tick haemocoel as well as tortoise liver and blood were determined as indicated in chapter 2.

4.3. Results

A total of 14 tortoises were collected, one *Kinixys natalensis* and *Kinixys spekii*, seven *Kinixys zombensis* and five *Stigmochelys pardalis*. Ten of the 14 (71%) tortoises were infested with ticks including larval, nymphal and adult stages. Larval and nymphal squashes yielded no identifiable haemogregarine stages and as such adult ticks formed the focus of this study. As species of *Kinixys* are often infected with both *H. fitzsimonsi* and *Hemolivia parvula*, three of the *S. pardalis* were selected for further analysis. Of these, one showed no evidence of haemogregarine peripheral blood stages, the second an extremely low parasitaemia infection and the third a relatively high parasitaemia, of what was morphologically comparable to gamonts of *H. fitzsimonsi* (Fig. 4-1A). Within the third tortoise, the roadkill individual,

merogonic stages were observed in the liver (Table 4-1). No observable stages were identified in the other organ smears.

Three species of *Amblyomma* ticks (Order Acari; Family Ixodidae) were collected from the three *S. pardalis Amblyomma herbraeum*, *Amblyomma marmoreum* and *Amblyomma nuttalli*. Impression slides from all three species showed sporogonic stages within the haemocoel. A single intact sporulated oocyst was observed in *A. marmoreum* (Table 4-1). No gametogenesis and subsequent fertilization were observed in any of the ticks.

Table 4-1. Summary of haemogregarine infection, tick species collected and screened for sporogonic stages, in and from three individual tortoises selected for analysis.

Stigmochelys pardalis individual	Peripheral blood infection	Liver stages	*Amblyomma* species (n)	Oocyst	Sporocyst	Sporozoite
One	-	-	*A. nuttalli* (1)	-	+	-
Two	+	-	*A. herbraeum* (2)	-	+	+
Three	+	+	*A. marmoreum* (1)	+	+	-

(-) negative
(+) positive
(n) number

Description of peripheral blood gamont and liver merogonic stages in S. pardalis

Gamonts: measuring 16.2±0.8 (15 – 17.3) × 2.7±0.4 (1.8 – 3.2), with one broad pole, opposite pole with a short, recurved tail, cytoplasm stained whitish-purple; nucleus 4.4±0.8 (3 – 5.9) × 1.7±0.3 (1.3 – 2.2) (n = 10), stained light purple, square or oval, lying closer to the pole with the recurved tail (Fig. 4-1A).

Liver meronts: measuring 22.3±2.1 (17.9 – 25.3) × 15.5±1.1 (13.7 – 17.1) (n = 9) (Fig. 4-1B-C), often observed with a non-staining thick capsule (Fig. 4-1B). Intrameront merozoites vaguely visible as elongated structures when not stained. Stained meronts, show elongate merozoites, staining purple with a dark blue-purple staining nucleus (Fig. 4-1C). Attempts to count merozoites resulted in <10 per meront (n = 9). No free merozoites were observed.

Remarks:

Gamont stages in the present study were morphologically comparable to those described by Dias (1953) (15.84 – 18.81 × 3.63 – 5.61) and Cook et al. (2014) (17 – 17.5 × 3.4 – 4.2). These

were the only peripheral blood stages found in the present study. Meront stages were observed only in the liver, unlike that of other species of *Hepatozoon*, which may be observed also infecting various other internal organs such as the lungs (Smith, 1996; Paperna et al., 2002; Telford, 2009; Sloboda et al., 2007). In the present study, the number of merozoites contained within meronts could not be conclusively determined, presumably due to the thick non-staining capsule. However, as numbers were low this may suggest that they represent macromeronts (see Telford, 2009). Even so, the number of merozoites contained in macromeronts is highly variable among species of *Hepatozoon*. For instance, in *Hepatozoon kisrae* Paperna, Kremer-Mecabell and Finkelman 2002 of the agamid *Laudakia stellio*, macromerozoites divided into four to 16 (Paperna et al., 2002), whilst in *Hepatozoon domerguei* Landau, Chabaud, Michel, and Brygoo 1970 of snakes, mature macromeronts could contain approximately 30 macromerozoites (Telford, 2009). No cyst or cyst-like stages were observed in the present study, these stages often observed in the liver and lung tissue of snakes and lizards infected with *Hepatozoon*, such as in the case of *H. domerguei* (Telford, 2009).

Description of sporogonic stages in unengorged Amblyomma *spp. Ticks*

Mature, sporulated oocyst: measuring 126.1 × 120.7 (n = 1), spherical in shape, with a thin dark blue membrane, containing countless, >100, oval sporocysts (Fig. 4-1D, arrow). Free in the haemocoel, surrounded by free sporocysts, some showing stained sporozoites (Fig. 4-1D, arrowhead). Oocyst contained sporocysts visible under 60× and 100× magnification, showing sporozoites, either unstained (Fig. 4-1E, arrow) or stained with light blue cytoplasm and visible nuclei stained dark blue (Fig. 4-1F, arrowhead) respectively.

Sporocysts: measuring 38.2±5.6 (29.2 – 49.5) × 19.6±2.7 (14 – 24.7) in *A. marmoreum* (n = 15), containing ~18 sporozoites (n = 7) (Fig. 4-1G-H), either unstained (Fig. 4-1G) or stained (Fig. 4-1G-H), the latter sporozoites with blue staining cytoplasm and blue to dark blue staining nuclei, some sporozoites seen exiting the sporocyst (Fig. 4-1H), variability in size of sporocyst, one larger than the other (Fig. 4-1H); 37.2±3.0 (29.9 – 43.4) × 17.8±1.9 (14.9 – 21.9) in *A. nuttalli* (n = 26), containing ~20 sporozoites (n = 4) (Fig. 4-2A), matured sporocyst showing stained individual sporozoites (Fig. 4-2A, arrowhead), maturing sporocyst showing basal mass or residual body at the centre (Fig. 4-2A, arrow); and 33.8±3.3 (29 – 40.1) × 17.5±2.5 (13.7 – 20.9) in *A. herbraeum* (n = 20), containing ~21 sporozoites (n = 3) (Fig. 4-2B-C), showing smaller, more spherical than narrowly to broadly oval sporocysts (Fig. 4-2B, arrowheads) and potentially degrading sporocysts or those prior to division (Fig. 4-2B, arrow), in some cases mature sporocysts with well differentiated sporozoites (Fig. 4-2C, arrowhead), being much

larger than a maturing sporocyst and a potentially undeveloped sporocyst to the right. All sporocysts with a thin, often white, or blue stained capsule.

Sporozoites: measuring 10.2±1.0 (8.7 – 11.8) × 1.7±0.3 (1.3 – 2.1) (n = 12), nucleus 1.4±0.3 (0.8 – 1.7) × 1.0±0.2 (0.7 – 1.4) (n = 10) in *A. herbraeum* (Fig. 4-2D), elongate and slender, slightly curved with blue staining cytoplasm and darker blue staining central nucleus, rounded in shape (Fig. 4-2D, arrow). Denser elongated oval structure on one side of the nucleus, potentially representing a crystalline body (Fig. 4-2D, arrowhead).

Remarks:

In the present study, sporogonic stages were observed free within the tick haemocoel, typical of species of *Hepatozoon* and unlike that of *Hemolivia*, which is found within the intestinal cells (Davies and Johnston, 2000; Telford, 2009). The single mature sporulated oocyst, containing numerous sporocysts was spherical in shape, similar to that found with oocysts of the tick hosted *Hepatozoon tuatarae* (Laird 1950) of tuatara. Compared to *H. tuatarae* and another tick hosted *Hepatozoon* (*H. kisrae*), it was half the size 126.1 × 120.7 as compared to 236 × 228 and 200 – 230 × 230 respectively (Paperna et al., 2002; Telford, 2009; Herbert et al., 2010). Difference in size was also noted in sporocyst stages, those in this study being longer (mean 33 – 38) than those of *H. tuatarae* (22.4), but containing similar numbers of sporozoites, this study 18 – 21 and those of *H. tuatarae* 18. In comparison to *H. kisrae* sporocysts (34 × 24 – 61 × 23), this study's sporocysts were on average smaller (mean 33 – 38 × 18 – 20) and differed in shape, ovoid compared to the ellipsoidal sporocysts of *H. kisrae* (Telford, 2009), with fewer sporozoites (*H. kisrae* 16 – 35). Sporozoite dimensions were not provided for *H. tuatarae* as none were found free of the sporocysts (Herbert et al. 2010), nor for *H. kisrae* (Paperna et al. 2002; Telford 2009). Interestingly, sporocyst stages in this study were longer and much wider than those described by Cook et al. (2014), measuring 33 – 38 × 18 – 20 as compared to 26 – 30 × 9 –13 respectively. Sporozoite numbers contained in sporocysts, however, were similar, 18 – 21 in this study as compared to 16 – 18 in Cook et al. (2014). Sporozoites in both studies were similar in size, measuring 10.2 × 1.7 in the present study as compared to 13.6 × 1.9 in Cook et al. (2014). In both studies, sporozoites were slightly curved or banana shaped as was seen with *H. tuatarae*. In Cook et al. (2014), sporozoites appeared to have two purple stained structures on either side of the nucleus, suggested at the time to be crystalline bodies. Only one of these structures were observed in the sporozoites in this study.

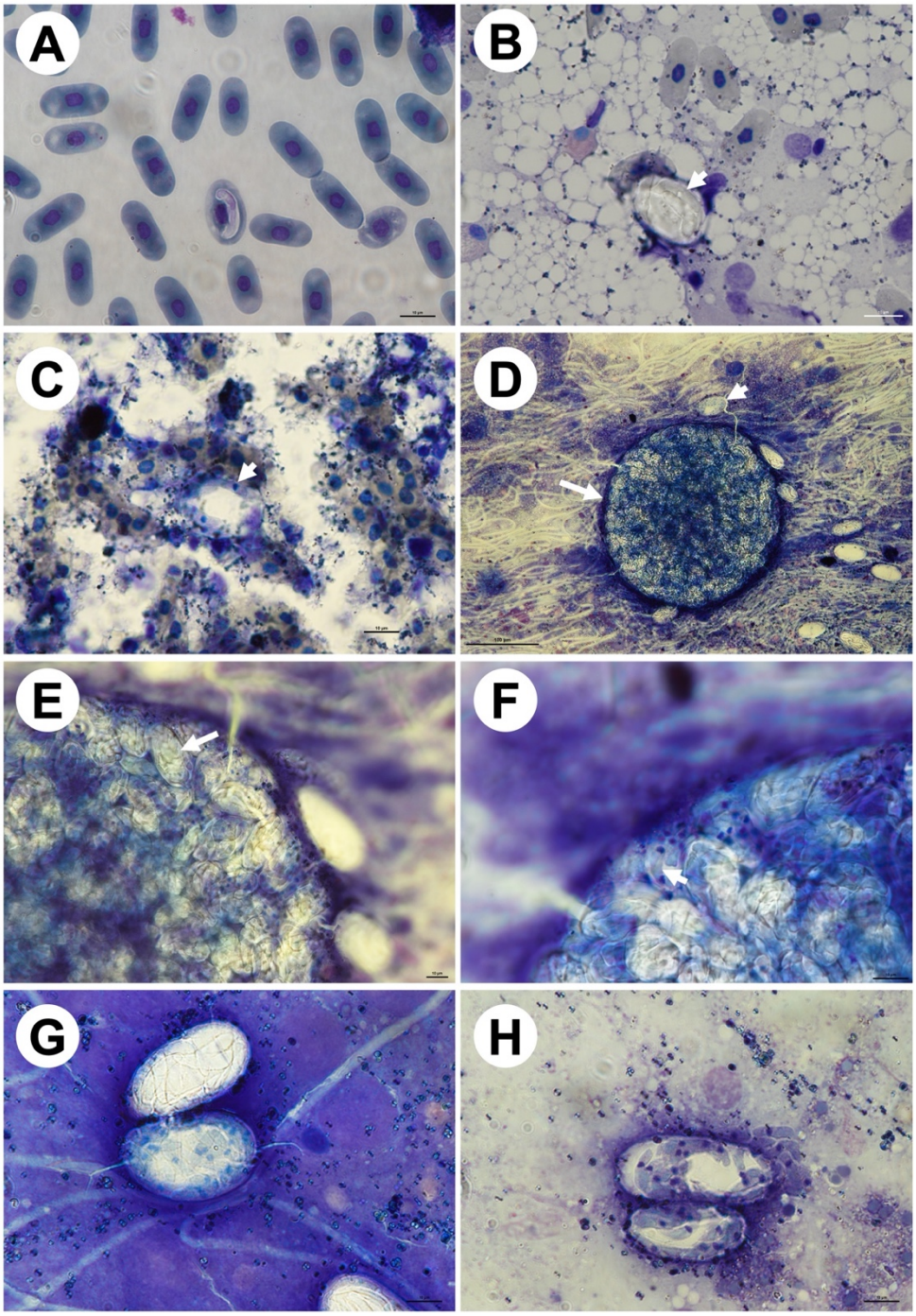


Figure 4-1: Peripheral blood, liver and tick stages. (A) intraerythrocytic gamont in the peripheral blood of *Stigmocheylys pardalis* (3); (B-C) meronts, unstained and stained respectively, (C) showing stained merozoites (arrowhead); (D-F) oocyst in the haemocoel of *Amblyomma marmoreum*, (D) 200× magnification of mature sporulated oocyst containing numerous sporocysts (arrow), with surrounding free sporocysts (arrowhead), (E-F) 600, 1000× magnification of oocyst respectively showing sporocyst (arrow), (F) showing stained sporozoites within a sporocyst (arrowhead); (G-H) free sporocysts in haemocoel, one unstained, others stained showing intrasporocystic sporozoites. Scale bar (A-C, E-H) = 10 µm, (D) = 100 µm.

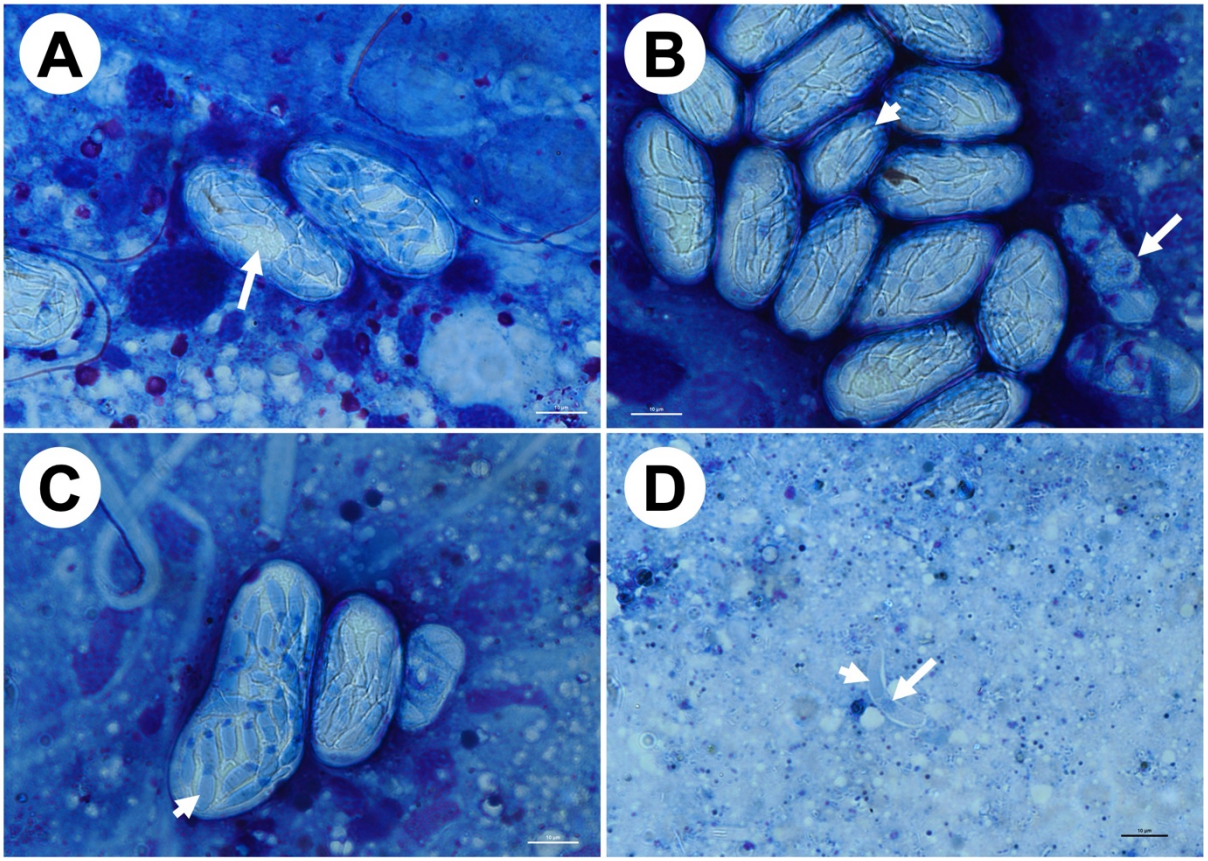


Figure 4-2: Sporogonic stages in the haemocoel of *Amblyomma nuttalli* and *Amblyomma herbraeum* collected from *Stigmochelys pardalis* (1) and (2) respectively. (A) sporocyts in *A. nuttalli*, one showing developed sporozoites, the other a central residual body (arrow); (B-D) sporocysts and sporozoite in *A. herbraem*, (B) showing larger and smaller more ovoid sporocyst (arrowhead), and either sporocysts prior to division or in the process of degrading (arrow), (C) showing a mature sporocyst with developed sporozoites (arrowhead), a developing/maturing and undeveloped sporocyst; (D) sporozoite showing a nucleus (arrow) and potential crystalline body (arrowhead). Scale bar = 10 µm.

Phylogenetic analysis of blood and tick stages

A 1733 bp and 1688 bp sequence obtained from the 18S rRNA was achieved for the *Hepatozoon* infecting *S. pardalis* (2) and *S. pardalis* (3). A 626 bp sequence was obtained from *A. nuttalli* collected from *S. pardalis* (1), along with a 620 and 626 bp sequence for *A. herbraeum* collected from *S. pardalis* (2). From *A. marmoreum* collected from *S. pardalis* (3), a 994 bp sequence was obtained. The genetic divergences between all sequences obtained from ticks and the blood of tortoises in the current study were 0% (see table 4.2), strongly

suggesting that all the obtained gene sequences represent the same haemogregarine species.

Comparisons of those sequences from the current blood stage material (*S. pardalis* (2) and (3)) to previous molecular descriptions of *H. fitzsimonsi* (KJ702453, KR069084), showed a genetic divergence of 0 – 0.2% (Table 4-2). This was the same for the sequences from the ticks in the current study; the blood and tick sequences clustering in a monophyletic clade comprising known *H. fitzsimonsi* (Fig. 4-3). Genetic divergence was 0% for that sequenced from both tortoise blood and ticks with *A. sparsum* collected off tortoises in Kenya; grouping within the same *H. fitzsimonsi* clade. *Hepatozoon cuorae* (Chai and Chen 1990) showed the closest relationship with the sequences from this study, with a divergence of 0.2 – 0.6% (Table 4-2). The *Hepatozoon* sp. sequenced from *Kinosternon scorpioides* showed variable divergence (0.2 – 1.1%) between sequences of this study and known *H. fitzsimonsi* (Table 4-2), which was dependent on length of sequence and coverage. Both *H. cuorae* and the latter *Hepatozoon* sp. claded as sister taxa to the *H. fitzsimonsi* clade. Divergence between *Hepatozoon simidi* Gutiérrez-Liberato et al. 2021 and the current material was 3%, clustering separately, basal to a clade containing *Hepatozoon* of squamata (Fig. 4-3).

Table 4-2. Genetic distances between species of *Hepatozoon* infecting chelonians with species of *Hemolivia* as reference for genetic distance between genera. Gene sequences in bold are those of the current study

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. KF992699 *Hemolivia mauritanica* ex *Testudo marginata*													
2. KR069083 *Hemolivia parvula* ex *Kinixys zombensis*	0.006												
3. MT754271 *Hepatozoon simidi* ex *Rhinoclemmys melanosterna*	0.035	0.035											
4. KY684006 *Hepatozoon* sp. Ex *Kinosternon scorpioides*	0.048	0.021	0.016										
5. KJ702453 *Hepatozoon fitzsimonsi* ex *Chersina angulate*	0.035	0.036	0.023	0.003									
6. MW514213 *Hepatozoon cuorae* ex *Cuora galbinitrons*	0.031	0.020	0.028	0.009	0.004								
7. KT266582 *Hepatozoon fitzsimonsi* ex *Amblyomma sparsum*	0.033	0.031	0.045	0.007	0.003	0.002							
8. KR069084 *Hepatozoon fitzsimonsi* ex *Kinixys zombensis*	0.024	0.021	0.030	0.009	0.002	0.002	0.000						

	1	2	3	4	5	6	7	8	9	10	11	12	13				
9. ***Hepatozoon fitzsimonsi* ex *Stigmochelys pardalis* (2)**	0.029	0.020		0.030	0.011	0.002	0.006		0.000	0.000							
10. ***Hepatozoon fitzsimonsi* ex *Amblyomma herbraeum* ex *Stigmochelys pardalis* (2)**	0.028	0.028		0.030	0.002	0.002	0.002		0.000	0.000	0.000						
11. ***Hepatozoon fitzsimonsi* ex *Amblyomma nuttalli* ex *Stigmochelys pardalis* (1)**	0.028	0.028		0.030	0.002	0.002	0.002		0.000	0.000	0.000		0.000				
12. ***Hepatozoon fitzsimonsi* ex *Stigmochelys pardalis* (3)**	0.029	0.020		0.030	0.011	0.002	0.005		0.000	0.000	0.000		0.000	0.000			
13. ***Hepatozoon fitzsimonsi* ex *Amblyomma herbraeum* (2)**	0.028	0.028		0.030	0.002	0.002	0.002		0.000	0.000	0.000		0.000	0.000	0.000		
14. ***Hepatozoon fitzsimonsi* ex *Amblyomma marmoreum* ex *Stigmochelys pardalis* (3)**	0.025	0.022		0.030	0.009	0.002	0.002		0.000	0.000	0.000		0.000	0.000	0.000	0.000	

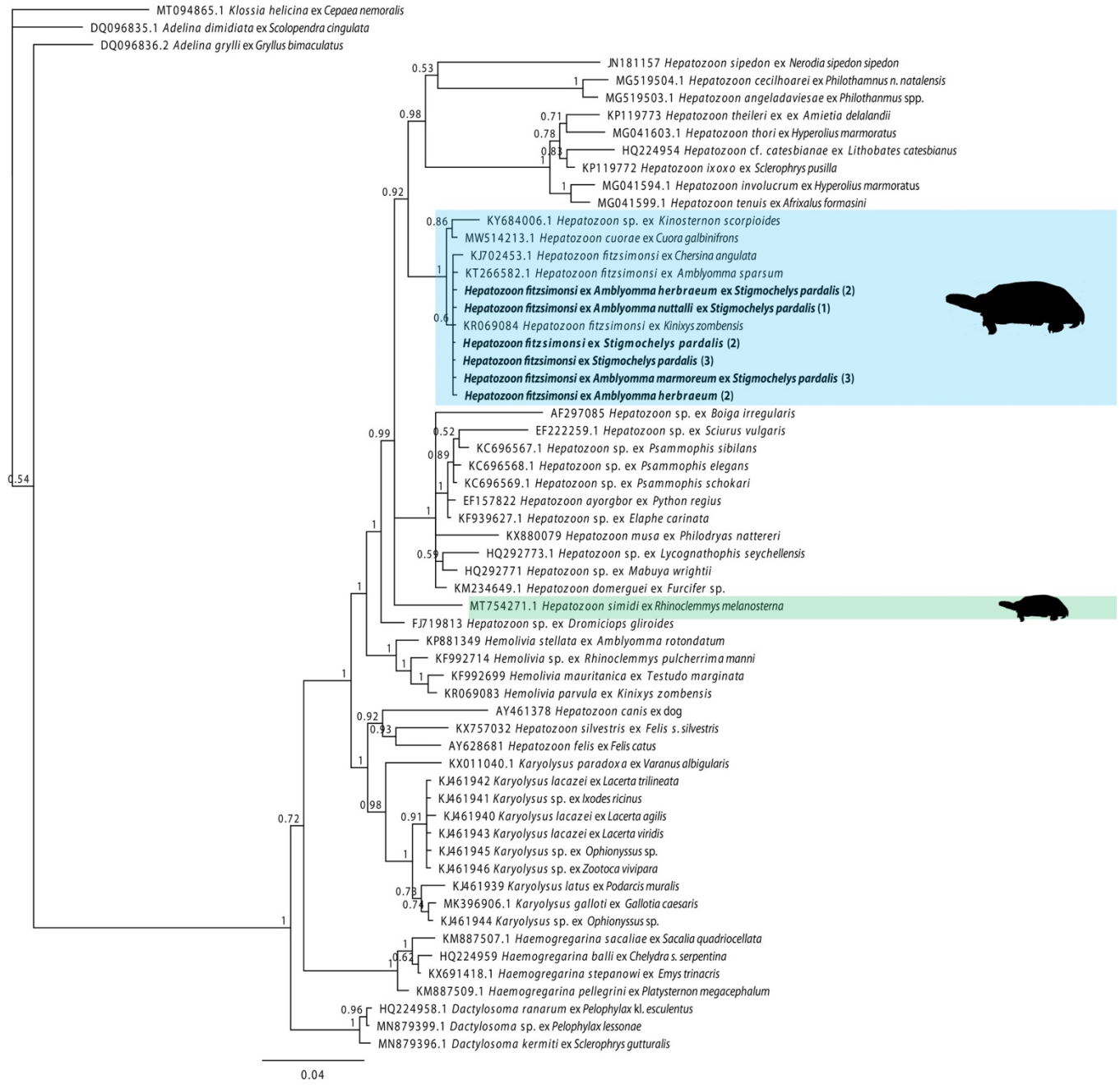


Figure 4-3: Phylogenetic tree of haemogregarine 18S rDNA sequences using Bayesian Inference (BI) analysis with the General Time Reversible model with estimates of invariable sites and a discrete Gamma distribution (GTR + I + Γ). Nodal supports shown as Bayesian posterior probabilities. Blue clade showing species of *Hepatozoon* of chelonians, including *Hepatozoon fitzsimonsi* from this (bolded) and previous studies. Green sequence is that of *Hepatozoon simidi* sequenced from a terrapin species from Brazil. It is the only *Hepatozoon* not included in the main chelonian *Hepatozoon* clade. Outgroup comprised *Adelina dimidiata*, *Adelina grylli* and *Klossia helcina* (GenBank: DQ096835, DQ096836, MT094865). The scale bar represents 0.04 nucleotide substitutions per site.

Remarks

As the isolates of the present study clade primarily with known *H. fitzsimonsi* and within a larger clade containing *Heptozoon* of other chelonians, those isolates from unengorged ticks containing sporogonic stages are considered representative of *H. fitzsimonsi*. Where a divergence of 0.2% was noted between gene sequences of *H. fitzsimonsi*, this would be because of comparisons between shorter and longer sequences. Sequences from ticks were shorter than desired as the primer set HepF300 and HepR900 was used instead of the primer sets and protocol used for the tortoise blood (which produced longer sequences). However, this primer set does amplify a fragment of a highly variable region in the 18SrRNA gene and is therefore adequate for phylogenetic analysis (Zechmeisterová et al., 2022). Alignment of the present study's sequences and those of known *H. fitzsimonsi* were identical but for one base pair in one sequence, when comparing this region, confirming the sequenced DNA within the ticks to be that of *H. fitzsimonsi*. Comparisons of *H. fitzsimonsi* from tortoise blood to *H. cuorae* showed a divergence of 0.5 – 0.6% for comparatively long sequences (*H. cuorae*: 1715 bp; H. *fitzsimonsi* tortoise 2 and 3: 1733 bp and 1688 bp respectively), strongly suggesting that they are separate species whilst taking into consideration the conservative nature of the 18SrRNA gene. Regarding the placement of *H. simidi* with *Hepatozoon* spp. of squamata, this too was observed by Zechmeisterová et al. (2022), wherein these authors' phylogenetic analysis clustered this species with a *Hepatoon* sp. from a snake.

4.4. Discussion

In the present study, a total of 14 tortoises of three genera along with *Amblyomma* ticks of three genera were collected and screened for the developmental stages of *H. fitzsimonsi*. Based on southern African *Kinixys* spp. often being co-infected with two species of haemogregarine (Cook et al. 2009), one being *H. fitzsimonsi* and the other *Hemolivia parvula* (Dias 1953), it was decided to focus on individuals of *S. pardalis* as co-infection with these two haemogregarines has been documented only once (Cook et al. 2009). Considering that both members of the genus *Hepatozoon* and *Hemolivia* may be vectored by ticks, particularly in the case of the latter where ticks are typically considered the sole vector (Davies and Johnston, 2000), this did need to be taken into account. To date the vector for *Hm. parvula* has not been determined and it could be just as likely that the sporogonic stages observed in the ticks could represent those of *Hm. parvula*. Compounding this is that this study was based on wild tortoises and ticks, and as such we could not control for ticks having fed on multiple tortoise hosts before collection.

As the four species of tortoises collected in this study occur sympatrically in the region of NGR (Bates et al. 2014), and the three tick species *A. herbraeum*, *A. marmoreum* and *A. nuttalli* collected are known to infest various species of tortoises (Horack et al. 2006), it is possible for host switching to occur and that the sporogonic stages in the tick impression slides to be that of *Hm. parvula* instead of *H. fitzsimonsi*. However, the impression slides were made with the intention of being as gentle as possible so as not to burst the oocyst. As such the tick gut remained largely intact, suggesting that the observed sporogonic stages were in the haemocoel. This is typical of sporogonic stages of *Hepatozoon*, whilst those of *Hemolivia* develop within the intestinal cells of the tick (Davies and Johnston 2000). The above in mind, it was taken that the sporogonic stages seen in these ticks were probably that of *H. fitzsimonsi*.

Hepatozoon fitzsimonsi was the first chelonian haemogregarine to be reassigned to the genus *Hepatozoon* based on morphological and molecular findings (Cook et al., 2014). In the years that followed, two other species of *Hepatozoon* were described and reassigned from chelonians, *Hepatozoon simidi* Gutiérrez-Liberato et al. 2021 and *Hepatozoon cuorae* (Chai and Chen 1990) respectively (Gutiérrez-Liberato et al. 2021; Zechmeisterová et al. 2022). Further reports for *Hepatozoon* in chelonians include an unpublished speculative sequence of *H. fitzsimonsi* from an unnamed *Kinixys* sp. from Nigeria, a sequence of *H. fitzsimonsi* from the tick *Amblyomma sparsum* from tortoises from Kenya (Omondi et al., 2017; Zechmeisterová et al. 2022), and the *Hepatozoon* sp. reported from *Gopherus polyphemus* (Cooney et al. 2019) and *Sternotherus odoratus* (Davis and Sterrett 2011) in the USA (Zechmeisterová et al. 2022). As mentioned by Zechmeisterová et al. (2022), two sequences, named as *Hepatozoon* on GenBank, from *Mauremys leprosa* by Marzal et al. (2017) are in fact representatives of *Haemogregarina*.

For the three morphologically described and molecularly supported species of chelonian *Hepatozoon* above, the only species for which a potential definitive host has been provided is *H. fitzsimonsi* (Cook et al. 2014; Gutiérrez-Liberato et al. 2021; Zechmeisterová et al. 2022). The present study found sporogonic stages in three tick species of the genus *Amblyomma*, which were morphologically comparable to those found in ticks by Cook et al. (2014), linking these molecularly to *H. fitzsimonsi*. Sporocyst stages in the present study were on average longer and much wider than those described in Cook et al. (2014), this particularly evident when observing the range in length and width of sporocysts. Simultaneously, 61 sporocysts were measured in the present study as compared to the 10 in Cook et al. (2014). Furthermore, sporocysts were observed in the present study in what appeared to be various stages of development, some mature and containing developed sporozoites, with others in which a residual body was still present and others which were smaller and more spherical. At times sporocysts were seen which

appeared to have no sporozoite development within, and equally sporozoites were seen that appeared to be degrading or prior to division, the former potentially representing an older phase of infection. The variability in size and shape of sporocysts, besides being potentially a result of phase of development, appears to be typical in *Hepatozoon* between species (Smith 1996), and is also evident within a species – *Hepatozoon rarefaciens* (Sambon and Seligmann 1907) (21 × 21 – 59 × 59), *Hepatozoon fasciatae* Telford, Wozniak and Butler 2001 (15 – 45 × 14 – 30), *Hepatozoon seminatrici* Telford, Wozniak and Butler 2001 (30 – 56 × 23 – 34), *Hepatozoon sistruri* Telford, Butler and Telford 2002 (25 – 50 × 20 – 50), and *Hepatozoon ayorgbor* Sloboda, Kamler, Bulantová, Votýtpka and Modrý 2007 (20 – 53 × 18 – 42) (see Telford 2009). Two purple stained structures were seen on either side of the nucleus of sporozoites in the material of Cook et al. (2014), which were not seen in the present study's sporozoites, however this may be as a result of staining bias. Regardless, as sequencing of the 18S rRNA gene from tick stages in Cook et al. (2014) was unsuccessful, it cannot be ascertained that the infection seen in this previous study was representative of *H. fitzsimonsi*. It is not clear why the sequences in Cook et al. (2014) were unsatisfactory. However, we could speculate that it could have been as a result of the potential co-founding effect from the tortoise blood, as the authors did not mention if the ticks were allowed to digest their blood meal; or the unsastisfactory sequences may represent a different *Hepatozoon* species or a distinct genotype from the documented *H. fitzsimonsi*.

Only a single intact oocyst was observed in the material of the present study, regardless of the care taken to be as gentle as possible when preparing the impression slides. This, and the thin oocyst wall, suggest that these stages rupture readily, as in *Hepatozoon clamatae* (Stebbins 1905) of frogs. Similarly, as with sporocysts, oocysts too show a high variability in dimensions between species (Smith 1996), however, as only one oocyst was found, it cannot be determined if this may be intraspecies too.

Meronts observed in the liver of the one tortoise were tentatively identified as macromeronts. No larger meronts containing higher numbers of merozoites, which may have represented micromeronts, were observed in the current material. Macromeronts are considered the first stage of merogonic development in species of *Hepatozoon*, which eventually give rise to micromeronts, the micromerozoites of which will in time enter erythrocytes to form gamonts (Smith, 1996). As only mature gamonts were observed in the peripheral blood of the tortoise, with no younger stages such as trophozoites, along with the lack of probable micromeronts in the liver, it may be suggested that these indicate two separate infections. Both macro- and micromeronts have not been observed for any species of *Hemolivia* so far (Široký et al. 2007), this including for *Hemolivia mauritanica*, a species infecting terrestrial tortoises of the western palearctic realm (Široký et al.

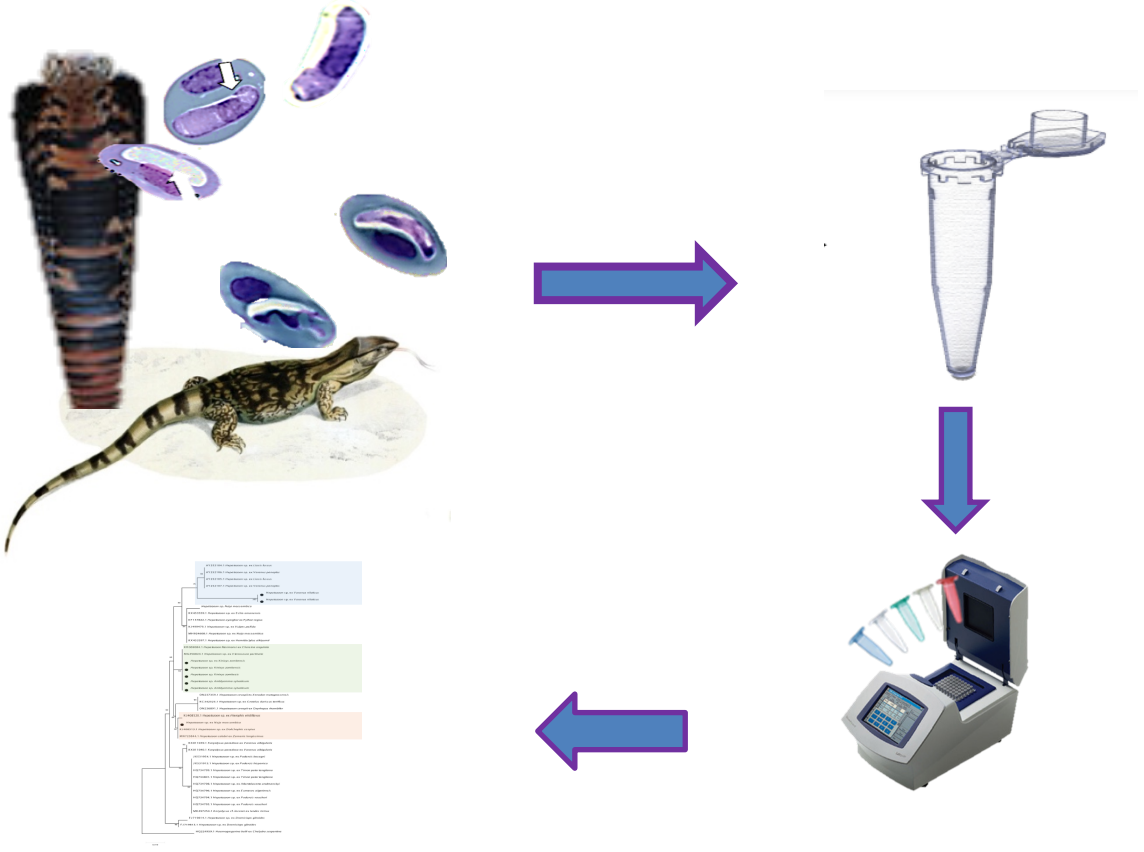
2007; Javanbakht et al. 2015). *Hemolivia parvula* (Dias 1953) has been found infecting terrestrial tortoises of eastern southern Africa. However, naturally it appears to infect only species of *Kinixys*, with only one finding in a captive *S. pardalis* (Cook et al. 2009; 2014; 2015). This along with the molecular findings, supports that the merogonic stage seen in the liver of the present material is most likely that of *H. fitzsimonsi* and not *H. parvula*. Compared to other species of *Hepatozoon* and species of *Hemolivia*, in which cystic stages are often present (Smith 1996; Široký et al. 2007; Telford 2009), no cystic stages were observed in the current material. It has been suggested that these stages aid in the persistence of infection (Široký et al. 2007; Telford 2009), and may play a role in transmission of infection from one vertebrate to another, particularly in three host life cycles (Telford 2009). However, these scenarios require further investigation at a more individual level and cannot be considered a general rule.

Karadjian et al. (2015) suggested that *H. fitzsimonsi* be reassigned as a species of the then newly erected genus *Bartazoon*, which is transmitted by biting insects. Even though infection of more than one group of arthropod hosts cannot be ruled out at this stage, particularly given the findings on *Hepatozoon fusifex* Ball et al.(1969), which showed infection in its natural tick host along with experimentally infected mosquitoes of widely different origin (Telford 2009), it appears rare. For instance, dissection of 100 ticks feeding on *H. ayorgbor* infected snakes, only the mosquitoes were found to contain sporogonic stages (Sloboda et al. 2007; Telford 2009). Equally, attempts by Morsy et al. (2013) to detect sporogony of the snake *Hepatozoon seurati* (Laveran and Pettit 1911) in mites, ticks, sand flies and *Aedes aegypti* fed on infected snakes were unsuccessful, stages were only found in epidemic *Culex pipiens molestus* (see Telford, 2009; Morsy et al. 2013). This was equally true for *Hepatozoon tupinambis* (Laveran and Salimbeni 1909), where attempts to infect leeches and triatomid bugs on infected teiid lizards was unsuccessful (Telford 2009). Given that sporogonic stages, with the final infective developmental stage, the sporozoite, has been identified in unengorged *Amblyomma* ticks and linked molecularly to *H. fitzsimonsi* in this study, it is assumed that these or at least three species of this genera can act as definitive hosts and vectors for *H. fitzsimonsi.* However, the transmission capability of these ticks will need further investigation, as well as to how tortoises become infected. Tortoises do display aggressive behaviour during courtship or competing for a mate, often biting the other tortoise. This can be observed in Desert tortoises in the USA, as well as in tortoise species in South Africa (Black 1976; Cook, personal observation). It may be in such cases where ingestion of infected ticks occurs. If species of *Amblyomma* are vectors for *H. fitzsimonsi*, which these findings highly suggest that they are, *H. fitzsimonsi* does not fit within the taxonomic description of *Bartazoon* (transmitted by biting insects only), making the genus paraphyletic.

4.5. Conclusion

As mentioned previously, *H. fitzsimonsi* was the first haemogregarine of chelonians to be reassigned from *Haemogregarina* to *Hepatozoon* based on morphological and molecular findings. This was particularly significant as it broadened the range of potential vectors for this species and was therefore no longer limited to leeches. Even though Cook et al. (2014) did find sporogonic stages in ticks associated with tortoises infected with *H. fitzsimonsi*, these parasites could not be confirmed as definitive hosts. In the present chapter, following on from the findings of Chapter 3 in which *H. fitzsimonsi* was molecularly detected in both unengorged *A. marmoreum* and *A. sylvaticum* ticks collected from tortoises, the potential of ticks to act as definitive hosts was revisted. Here, sporogonic stages, particularly sporocyst and sporozoite stages (the final development stages in vectors of species of *Hepatozoon*) in three species of *Amblyomma* ticks *A. herbraeum*, *A. marmoreum* and *A. nuttalli* were molecularly confirmed with the use of fragments of 18S rDNA sequences to be that of *H. fitzsimonsi*. In one tortoise, gamont stages in the peripheral blood and sporogonic stages in an *A. marmoreum* collected off this same tortoise was molecularly identified as *H. fitzsimonsi*; this substantiating that these parasites do act as definitive hosts and strongly suggesting that they are a vector. Furthermore, this study provided detailed descriptions of the sporogonic stages of *H. fitzsimonsi*, along with its potential merogonic stages in the tortoise liver. Although the liver stages will still need to be confirmed to be that of *H. fitzsimonsi*, through the separation and molecular characterisation of meronts from the liver tissue or through infection experiments, there is a good possibility that they are stages of *H. fitzsimonsi* as the tortoise showed no gamont stages of *Hm. parvula*. Even though further research into the transmission of *H. fitzsimonsi* from ticks to tortoises may be required, this would be the second haemogregarine of tortoises for which ticks are the vector, *Hemolivia mauritanica* being the first. Importantly, this is the first species of *Hepatozoon* infecting chelonians for which ticks have been identified as a definitive host. This will hopefully encourage further research, identifying definitive hosts and vectors for other species of chelonian *Hepatozoon*, a research area that remains largely neglected at present.

CHAPTER 5: TAXONOMIC AND MOLECULAR CHARACTERISATION OF *HEPATOZOON* SPECIES (APICOMPLEXA: HEPATOZOIDAE) PARASITIZING SPECIES OF *VARANUS* (REPTILIA: VARANIDAE) AND *NAJA MOSSAMBICA* (REPTILIA: ELAPIDAE) FROM SOUTH AFRICA



5.1. Introduction

The genus *Hepatozoon* (Adeleorina), first proposed by Miller (1908) to support the description of *Hepatozoon perniciosum* Miller (1908) is an apicomplexan parasite infecting erythrocytes and leucocytes. Members of this genus are commonly found parasitising a vast range of invertebrate definitive hosts (various dipteran insects, ticks, mites, fleas, lice) and vertebrate intermediate hosts (mammals, amphibians, reptiles, birds) (Smith 1996; Barta 2000; Telford 2009; Harris et al. 2011). Given the breadth of hosts associated with *Hepatozoon* spp., the monophyly of the genus has been put into question (Smith and Desser 1997; Karadjian et al. 2015). Over the past decade several phylogenetic studies have provided useful insight into the evolutionary relationships of adeleorinid parasites, as well as better capability to distinguish between species, using 18S rRNA sequences. Recent estimated relationships using the 18S rRNA gene sequences have indicated that *Hepatozoon* is paraphyletic, with genus *Karyolysus* as well as genus *Hemolivia* forming a lineage within *Hepatozoon* (Barta et al. 2012; Haklova-Kocikova et al. 2014; Kvicerova et al. 2014; Cook et al. 2016; Netherlands et al. 2017). Recently, a new genus, *Bartazoon* Karadijian et al. 2015, was proposed as a part of taxonomic revision in an effort to resolve the polytomies between predicted phylogeny and the current taxonomy of these haemogragarines (Karadijian et al. 2015; Borges-Nojosa et al. 2017; Netherlands et al. 2017; Cook et al. 2018). This new erected genus was proposed for *Hepatozoon* species previously regarded to parasitize amphibians, marsupials, avian, rodents and reptiles, and was proposed to be solely transmitted by biting insects. However, it has been documented that the life history of some species such as *Hepatozoon fitzsimonsi* (Dias 1953) within the new genus do not correlate with the defining characteristics of *Bartazoon* (Cook et al. 2014; Karadijian et al. 2015; Netherlands et al. 2017). As Maia et al. (2016b) has also pointed out, it is possible that *H. perniciosum*, the type species of *Hepatozoon,* may in fact form part of the newly erected genus *Bartazoon*, as do most other rodent haemogregarine species. Furthermore, several analyses using a single gene continues to identify new genetic lineages, indicating that *Bartazoon* is not a well-supported group (Maia et al. 2016a; Tome et al. 2016). Thus, according to Leveille et al. (2014) and Netherlands et al. (2017), the deeper taxonomic revision of haemogregarines based on their phylogenetic affinities and life histories requires more studies. However, as suggested and recommended by Maia et al. (2016) and Borges-Nojosa et al. (2017), we will conservatively continue to refer to species parasitizing reptilian hosts as species of *Hepatozoon* and not *Bartazoon*.

To date, more than 30 species of *Hepatozoon* have been documented from reptiles throughout Africa (Van As et al. 2015; Cook et al. 2016, 2018). In South Africa, these include H*epatozoon langii, (*Van As et al. 2013*), Hepatozoon vacuolatus (*Van As et al. 2013*), Hepatozoon affluomaloti*

*(*Van As et al. 2015*),* and *Hepatozoon varani* (Laveran 1905) described from two genera and four species of lizards; *Hepatozoon bitis* (Fantham 1925), *Hepatozoon refrigens* (Sambon and Seligmann 1907), *Hepatozoon angeladaviesae* (Cook et al. 2018) and *Hepatozoon cecilhoarei* (Hoare 1920; Cook et al. 2018), *Hepatozoon* spp. (Harries et al.2018) from six species of snakes, and *H. fitzsimonsi* (Dias 1953) from five species of tortoise (Cook et al. 2014).

Within the family Elapidae, snakes of the genus *Naja* recently formed part of a haemoparasite survey in South Africa, this reporting infections of potentially two different species of *Hepatozoon*. However, conclusive taxonomic inferences regarding these two *Hepatozoon* species were not providedand none were named (Harris et al. 2018). To the authors' knowledge, to date, only one *Hepatozoon* species has been described from South African varanids (Laveran 1905). Hence, this study presents the first formal description of *Hepatozoon* species parasitizing *Naja mossambica,* and one more *Hepatozoon* species from *Varanus albigularis* and *Varanus niloticus* in South Africa based on morphological description and molecular characterization.

5.2. Materials and methods

Of the 25 tortoises, 30 snakes and 16 monitor lizards collected during this study, all were screened for the presence of haemoparasites. Blood was collected from the caudal vein for snake and lizard hosts and the subcarapacial sinuses in tortoises, and thin blood smears prepared with the remaining volume of blood preserved in 70% ethanol and stored in a -20°C freezer for subsequent molecular analysis. If haemoparasites were observed in the blood smears (this particular chapter focusing on haemogregarines observed in snake and lizard hosts), these were micrographed and the morphometrics determined as detailed in Chapter 2. Parasitemia was calculated per 100 erythrocytes, with ~10^4 erythcytes examined per blood smear as indicated by Cook et al. (2016). Molecular analysis included amplification of fragements of the 18S rRNA gene as detailed in Chapter 2. The sequences for this chapter will be provided once the manuscript has been accepted to publication.

5.3. Results

Prevalence of Hepatozoon *species*

Hepatozoon species were observed in 2/21 (9.52%) of snakes from Ndumo Game Reserve. Two (20%) *N. mossambica* (with a parasitaemia of 2% and 4%, respectively) were parasitised by two morphologically, morphometrically and molecularly different species of *Hepatozoon* (*Hepatozoon* sp. A and *Hepatozoon* sp. B). One of the three (33.3%) (with parasitemia 0.2%) *V. niloticus* and one of the 13 (7.7%) (with parasitemia 0f 0.5%) *V. albigularis* were infected with a

haemogregarine alike to *Hepatozoon camarai* described by Dias 1954. No meront stages were found in the present study, while a single trophozoite stage was observed in *V. niloticus* and gamont stages were observed for all the *Hepatozoon* species. The *Hepatozoon* species in *Naja* will be described and discussed first followed by the description of *Hepatozoon* in *Varanus* spp. below*:*

Hepatozoon identified from Naja mossambica

Description: *Hepatozoon* sp. A

Taxonomic summary:

Vertebrate host: *Naja mossambica* Peters 1854 (Elapidae: Squamata)

Type locality: Ndumo Game Reserve (26°54′18.5″S, 32° 19′24.7″E), Kwa-Zulu Natal, South Africa (Present study)

Site of infection: Peripheral blood

Vector and definitive host: unknown

Description of peripheral blood stages

Gamonts: Immature and mature gamont stages (or possible microgamonts and macrogamonts) observed in the peripheral blood. Immature (or microgamonts) are rare (n=4), and curved, measuring 9.06±1.60 × 2.58±0.17 (7.30–10.44 × 2.38–2.66, n= 4) µm, with LW 63.35±9.51 (37.48–74.98) µm, L/W 3.01±0.37 (2.23–3.78) µm, area 55.29±7.59 (30.91–63.38) μm^2 with cytoplasm staining whitish. Nucleus is either less compact or not visible (Fig. 5-1A–C).

Mature (or macrogamonts) are broadly elongate, measuring 13.75±1.04 × 4.61±0.54 (11.5–15.38 × 3.74-5.94, n=26) µm, with LW 63.51±9.51 (37.48–74.98) um, L/W 3.53±0.71 (2.71–3.96), area 19.77±1.12 (18.49–20.61) μm^2 with thin transparent parasitophorous vacuole (PV); however, in some cases, the PV is not evident. Gamonts are straighter than curved, one pole faintly pointed (Fig. 5-1D, E), opposite pole rounded, sometimes a short, recurved tail is visible (Fig. 5-1D), cytoplasm staining purple. Nucleus of mature gamont is elongated 4.22±0.52 × 2.91±0.59 (3.41–5.24 × 1.87–4.03, n = 26), slightly displaced to one side of the parasite with dense chromatin sometimes, loosely arranged and situated in the posterior half of the parasite (Fig. 5-1H). It is located almost centrally with mid-nucleus to the second quarter of the gamont measuring 6.66 µm, extending slightly into the first quarter measuring 6.93 µm. Gamonts were infrequently observed free from erythrocytes (exoerythrocytic) (Figure 5-1I).

Effects on the host cell

Non-parasitized erythrocytes measuring 16.55±0.88 × 10.09±0.96 (14.91–17.99 × 7.53–11.61) µm, with LW 167.40±21.46 (121.38–200.27) µm, L/W 1.65±0.17 (1.49–2.14) µm, area 136.45±15.84 (104.31–167.36) μm^2 compared to the slightly wider infected erythrocytes measuring 17.80±1.54 × 11.72±1.48 (15.16–19.98 × 8.04–13.25) µm, with LW 209.19±35.97 (163.17–245.69) µm, L/W 1.54±0.22 (1.21–4.28) µm, area 167.43±29.01 (101.95–207.49) μm^2. Gamonts affect the dimensions of the host cell, widening both the length and width of the erythrocytes and causing displacement of the host cell nucleus. Nuclei of infected erythrocytes 9.39±1.66 × 4.17±1.10 (6.92–11.90 × 2.53–7.27) µm, with LW 38.72±10.38 (23.46–59.40) µm, L/W 2.42±0.85 (1.12–4.28) µm, area 32.22±9.04 (18.19–57.9) μm^2, usually forced to one side of host cell, showing a slight degree of pyknosis, sometimes central and parallel to gamont, or displaced to the apical pole (Fig. 5-1D–F). Nuclei of uninfected erythrocytes measuring 7.17±0.74 × 4.30±0.51 (6.02–8.11 × 3.42–5.30) µm, with LW 30.64±3.98 (23.80-37.95) µm, L/W 1.70±0.31 (1.24–2.20) µm, area 25.84±2.56 (20.21–29.97) μm^2. Destruction of a host cell was also observed (Fig. 5-1G)

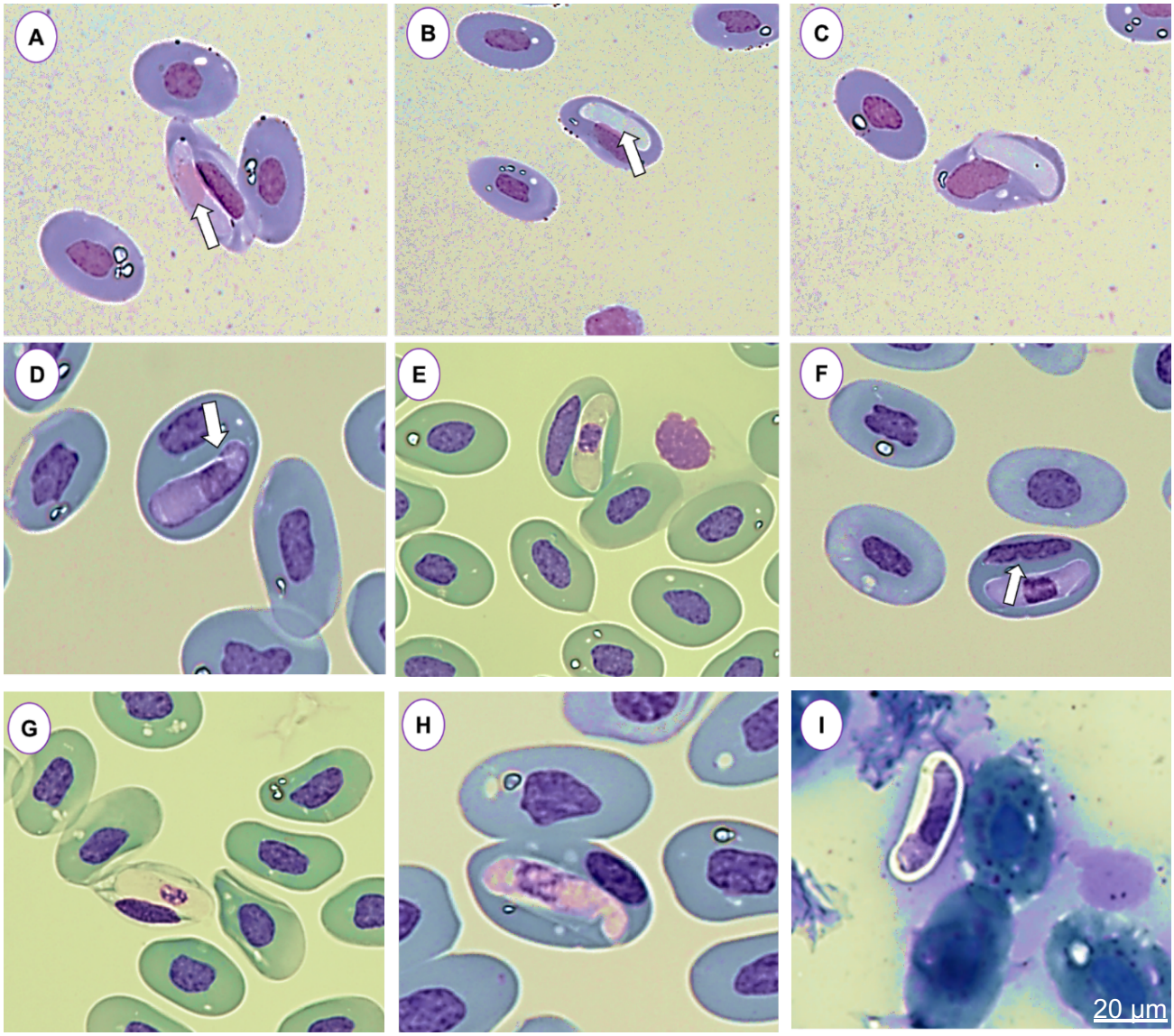


Figure 5-1: *Hepatozoon* sp. A gamonts parasitizing the erythrocytes in the *Naja mossambica*. (A-C) immature or microgamonts with no visible or less compact nucleus (arrows); (D-F) mature or macrogamonts, (D) short curved tail (arrow), (F) pyknosis of the host cell's nucleus (arrow); (G) mature or macrogamont destructing the host cell; (H) gamont displaying a short recurved tail; (I) extracellular or free gamont. Scale bar: 20 µm .

Description: *Hepatozoon* sp. B

Taxonomic summary:

Vertebrate host: *Naja mossambica* Peters 1854 (Elapidae: Squamata)

Type locality: Ndumo Game Reserve (26°54′18.5″S, 32° 19′24.7″E), Kwa-Zulu Natal, South Africa (present study)

Site of infection: Peripheral blood

Vector and definitive host: unknown

Description of peripheral blood stages

Gamonts: Immature and mature gamont stages (or possible microgamonts and macrogamonts) observed. Immature (or microgamonts) are rare, elongate without a visible PV 12.15±1.22 × 3.17±0.91 (10.91–13.35 × 2.02-3.81, n=4) µm, with LW 63.35±9.51 (37.48–74.98) µm, L/W 4.26±2.04 (2.81–6.61) µm, area 46.26±2.98 (43.13–49.07) μm^2 with cytoplasm staining whitish-purple. The nucleus is either less compact or not visible. The staining properties were not sufficient to recognise the structure of the nucleus. When developing into mature gamonts, these become concave with the PV beginning to form (Fig. 5-2A–D).

Mature (or macrogamonts) observed in both developing and mature erythrocytes, are long and curved, measuring 16.52±1.38 × 3.36±0.94 (15.15–20.11 × 2.39-5.26, n=15) µm, with LW 55.31±14.71 (33.48–74.80) um, L/W 5.27±1.46 (3.06–7.11), area 48.79±10.34 (34.26–55.71) μm^2, with thin transparent PV; however, in some cases, the PV is not evident. Gamonts are more curved than straight, (Fig. 5-2E–L). Nucleus stains crimson to dark purple, being compact, sometimes long, covering almost the entire gamont with an irregular shape. In developing erythrocytes, the nucleus is roundish and situated either at the end of one pole or in the middle of the gamont (Fig. 5-2E, F)

Effects on the host cell

Non-parasitized erythrocytes measuring 19.06±1.54 × 12.06±0.84 (17.62–22.46 × 10.20–13.69 n=15) µm, with LW 229.94±26.68 (193.64–257.65) µm, L/W 1.59±0.16 (1.35–1.89) µm, area 185.09±20.48 (162.48–220.85) μm^2, compared to the slightly wider infected erythrocytes measuring 20.33±1.08 × 11.21±2.07 (18.33–22.28 × 8.36–14.53) µm, with LW 228.66±47.01 (151.27–294.58) µm, L/W 1.87±0.36 (1.42–2.67) µm, area 186.21±37.28 (122.55–246.09) μm^2. Gamonts affect the dimensions of the host cell, widening both the length and width of the erythrocytes, causing displacement of the host cell nucleus. Nuclei of infected erythrocytes not visible, but when visible, imeasures 9.61±1.10 × 2.85±0.70 (8.93–11.36 × 1.81–2.96) µm, with

LW 27.09±6.14 (18.14–34.86) µm, L/W 3.61±1.17 (2.30–5.54) µm, area 21.82±5.12 (13.46–23.99) μm^2, usually forced to one side of host cell. Nuclei of uninfected erythrocytes measuring 7.27±1.01 × 3.60±0.45 (6.01–8.87 × 2.94–4.32) µm, with LW 26.21±5.13 (17.19–37.97) µm, L/W 2.04±0.40 (1.59–2.76) µm, area 22.78±4.28 (13.41–29.58) μm^2. Destruction of a host cell was also observed (Fig. 5-2L)

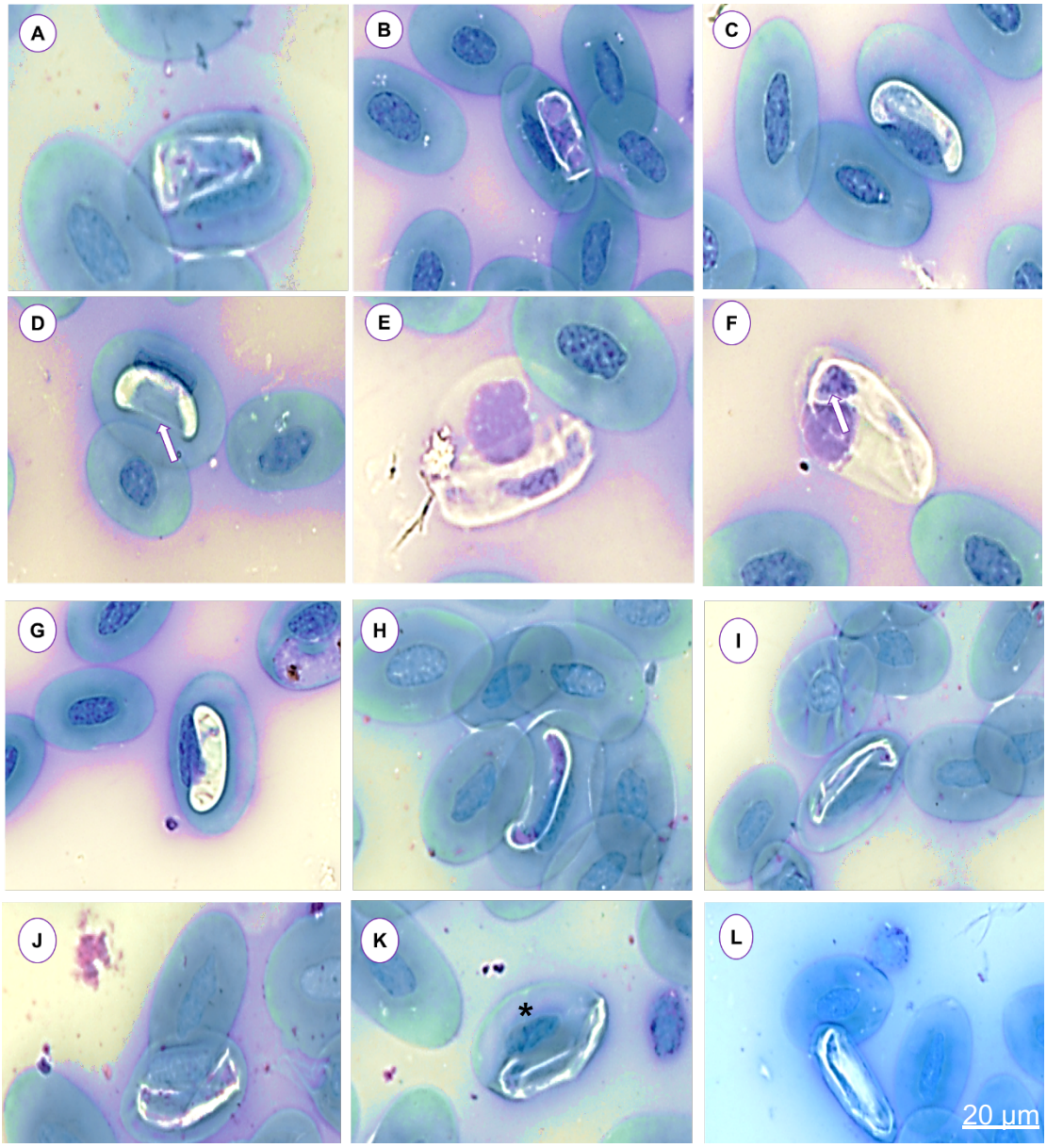


Figure 5-2: *Hepatozoon* sp. B gamonts parasitizing the erythrocytes of *Naja mossambica*. (A-C) immature or microgamonts with no visible or less compact nucleus; (D-F) mature or macrogamonts, (D) short curved tail (arrow), (E & F) mature gamonts in developing erythrocytes; (G) pyknosis of the host cell nucleus (H) gamont displaying a short recurved tail (L) destruction of a host cell. Scale bar: 20 µm.

Remarks:

The mature gamonts of *Hepatozoon* sp. A are smaller and wider (13.75 × 4.61) than that of *Hepatozoon* sp. B (16.52 × 3.36). Although, both *Hepatozoon* species are from *N. mossambica* from the same region, their gamonts are morphologically and morphometrically distinct, particularly with the translucent or non-staining nature of the PV of *Hepatozoon* sp. B in comparison to *Hepatozoon* sp. A.

According to the available literature from the early 1900s to date, *Hepatozoon* species have been reported parasitizing species of snakes in Africa (Sambon and Seligmann, 1909; Hoare, 1932, Smith, 1996). However, most of these studies are based solely on peripheral gamont stages. Within the family Elapidae, snakes of the genus *Naja* in Africa, to knowledge, have three species described *Hepatozoon weissi* (Conor, 1912), *Hepatozoon enswerae* (Hoare, 1932) and *Haemogregarina answanensis* (likely Hepatozoon) from *Naja haje* (Egyptian cobra) in North Africa, *Naja melanoleuca* (Forest cobra) in Uganda, and *N. haje* from Egypt respectively (Smith, 1996; Hoare, 1932, Mohammad et al., 1996) (Table S2). However, these have not been reported on since and no molecular data have been provided for them. Recently a haemoparasite survey of wildlife in South Africa, reported infections of potentially two different species of *Hepatozoon* in *Naja* species hosts. However, conclusive taxonomic inferences regarding these two *Hepatozoon* species were not provided and none were named (Harris et al. 2018). Sloboda et al. (2007) reported *Hepatozoon ayorgbor* from *Python regius* in Ghana, and *Hepatozoon* sp. A from the current study closely resembles *H. ayorgbor*. The parasite was described to be 11–13 µm × 2–3.5 µm in size, closely conforming to the length of the *Hepatozoon* from the present study, but the width was described as slightly narrower compared to the present material. Regarding *Hepatozoon* sp. B, this parasite closely conforms in shape and size with *H. enswerae*, this latter parasite measuring 15 × 3.8 µm (bean shaped form, correlating to the immature gamont in this study) and 19 × 3 µm (slender form, correlating to the mature gamont in this study).

Considering the effects on the host cell when comparing the description of Sloboda et al. (2007) to that of the present study (*Hepatozoon* sp. A), both haemogregarines cause slight host cell alteration, narrowing the width of the erythrocytes and displacing the host cell nucleus. Furthermore, the gamont lay parallel to the host cell nucleus and in some instances showed some degree of pyknosis.

However, besides Sloboda et al. (2007) mentioning that the infected erythrocytes may be slightly hypertrophied, the authors also mentioned that the gamont does not curve, the curve was evident in the present study and this could be due to the host species and geographical distribution or that this is a different species from *H. ayorgbor*. In contrast to the observations of Sloboda et al.

(2007), the *Hepatozoon* species from *N. mossambica* was characterized by the presence of only a single gamont in infected erythrocytes, with destruction of the host cell observed in the present study. Moreover, extracellular or free moving gamonts were also observed from the present material.

Comparisons of the effects on the host cell when comparing *Hepatozoon* sp. B and *H. enswerae*, show both parasites' slender forms (or mature gamonts) to cause dehaemoglobinisation, with the host cell nucleus somewhat flattened and deformed. Unfortunately, the description of *H. enswerae* was fairly concise and as such no further inferences can be made.

Hepatozoon identified from Varanus albigularis *and* Varanus niloticus

Taxonomic summary:

Vertebrate host(s): *Varanus albigularis* Daudin 1802 and *Varanus niloticus* Linnaeus in Hasselquist 1762 (Squamata: Varanidae)

Type locality: Ndumo Game Reserve, Kwa-Zulu Natal, South Africa (present study)

Site of infection: Peripheral blood

Vector and definitive host: unknown

Description of peripheral blood stages

Trophozoite: Rare, ovoid, with vacuolated cytoplasm (n=1); nucleus with loose chromatin, staining pink (Fig 5-3 A).

Gamonts: two forms of gamonts are observed, both elongated and oval in shape with irregular nuclei. The PV is evident in some cases, but often appears highly translucent as if the inner parasite is not occupying the entire space. The first form is fatly banana shaped, which measures 10.38 µm –13.58 µm × 2.02 µm – 3.77 µm in *V. niloticus* and 14.44 µm –16.86 µm ×1.59 µm – 6.01 µm in *V. albigularis* (Fig 5-3 B), while the second form is long, curved and shows an increase in length with a corresponding decrease in width as compared to the fatly banana shaped gamonts (Fig 5-3 C-F), measuring 10.44 µm – 15.45 µm × 1.65 µm – 4.09 µm. The second form was not observed in *V. albigularis*. The nucleus of both forms stain crimson to dark purple, being either short and compact, sometimes long, covering almost the entire gamont and in some cases it is shown appearing as if it is outside of the gamont. As this cannot be the case, perhaps this is

some section of the PV taking on the stain. The cytoplasm displays a whitish colouration with some streaks of blue-violet hue.

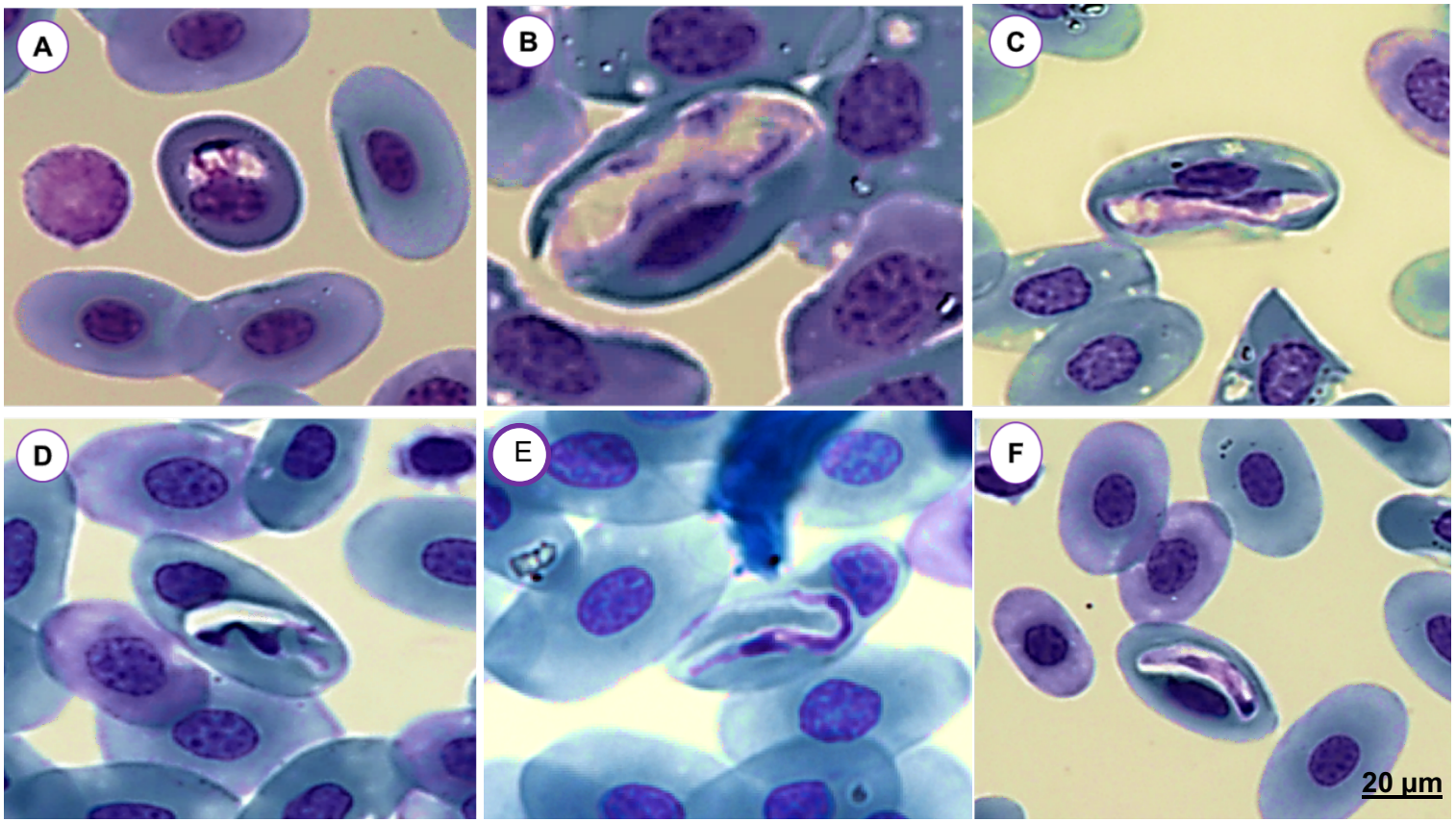


Figure 5-3: *Hepatozoon* species gamonts parasitizing the erythrocytes in *Varanus albigularis and V. niloticus.* (A) trophozoite stage; (B) fatly banana shaped gamonts; (C–F) long curved gamonts; (D) nucleus appearing to be on the outside of the gamont, which most likely is a section of the parasitophorous vacuole taking on stain. Scale bar: 20 µm

Remarks:

With regards to species of *Hepatozoon* described from South African lizards, there are currently four recognised species, of which one (*H. varani*) has been described parasitizing species of the Varanidae.

When comparing the *Hepatozoon* from this study with other described species of *Hepatozoon* infecting African varanids, the *Hepatozoon* described in the present study from South African *V. niloticus* and *V. albigularis* was found to be morphologically similar to *Hepatozoon camarai* described by Dias (1954) from a specimen of *V. a. albigularis* in neighbouring Mozambique and *H. varani* originally described from *V. niloticus* in South Africa by Laveran in 1905, and later in other countries (see Table S4). However, in the case of *H. varani*, Lavern described that the mature gamont folded over on itself, almost resembling a hairpin shape. This was not observed in the current study. As the haemogregarine observed in this study shared a number of unique characteristics with *H. camari,* including having two forms (the banana shaped gamont and the long, curve shaped gamont) and low parasitaemia, it is suggested that it likely is *H. camari*. Furthermore, the cytoplasm of both the present study's haemogregarines and *H. camarai* display a whitish colouration, with some jagged streaks of bluish hue. Even though these parasites share the above characteristics, they differ to a degree in their morphometric measurements (Table S4). From the current study, it is worth noting that the two forms of *Hepatozoon* were only observed in *V. niloticus*, whilst in the *V. albigularis,* only one form was observed.

Molecular analysis

Sequence analysis of Hepatozoon *from* Naja mossambica *and* Varanus *hosts*

Comparison between 18S rRNA gene sequences of *Hepatozoon* species and related haemogregarines confirm that the sequences from the present study belong to genus *Hepatozoon* (Fig. 5-4).

The BLAST analysis of *Hepatozoon* sp. A and *Hepatozoon* sp. B from *N. mossambica* showed the highest sequence identity of >98% with *Hepatozoon ayorgbor* (EF157822.1) and *Hepatozoon ophisauri* (MN723845.1). However, from the phylogenetic analysis, the parasitic lineage of *Hepatozoon* sp. A found in *N. mossambica* was most closely related to *H. ayorgbor* Sloboda et al. (2007) described from *Python regius* in Ghana, while the lineage of *Hepatozoon* sp. B from the second *N. mossambica* was most closely related to *Hepatozoon colubri* Börner (1901) that Zechmeisterová et al. (2021) recovered from *Zamenis langissumus* in Iran.

The BLAST analysis of each sequence from *V. Albigularis* and *V. niloticus* varied, however both were closely related to *Hepatozoon* gene sequences obtained from reptiles (>95%). The

sequence from *V. albigularis* was most closely related to a *Hepatozoon* species from *Macrovipera lebetina obtusa* (MZ208827.1) (>95%) while the sequence from *V. niloticus* was >96% related to *H. ayorgbor* (EF157822.1). Molecular results, however, show that the parasites from *V. niloticus* and *V. albigularis* in the present study are the same species, as can be seen from the phylogenetic analysis and the evolutionary divergence (100% identical, p=0.00).

According to the evolutionary divergence estimates, the *Hepatozoon* sequences from *Varanus* in this study were most closely related to the *Hepatozoon* sp. sequence (GenBank: EU430234.1) from *Amblyomma fimbriatum* collected off *Varanus panoptes* from Australia (P=0.03). The *Hepatozoon* sp. A from *N. mossambica* was most closely related to *H. ayorgbor* and *Hepatozoon* spp. sequences (GenBank: EF157822.1; KX453597.1; MH924608.1) from *Python regius, Hemidactylus alkiyumii*, and *N. mossambica* from Ghana, Oman, and South Africa, respectively. These sequences clustered together with a high bootstrap value (85%) (Fig. 5-4). Furthermore, *Hepatozoon* sp. B from *N. mossambica* was most closely related to *Hepatozoon colubri* and *Hepatozoon* spp. sequences (MN723844.1; KJ408513.1; KJ408520) amplified from *Hierophis viridiflavus* and *Dolicophis caspius*, respectively. These four sequences clustered together with a bootstrap of 79% in a clade separate from the *Hepatozoon ayorgobor* clade (Fig 5-4).

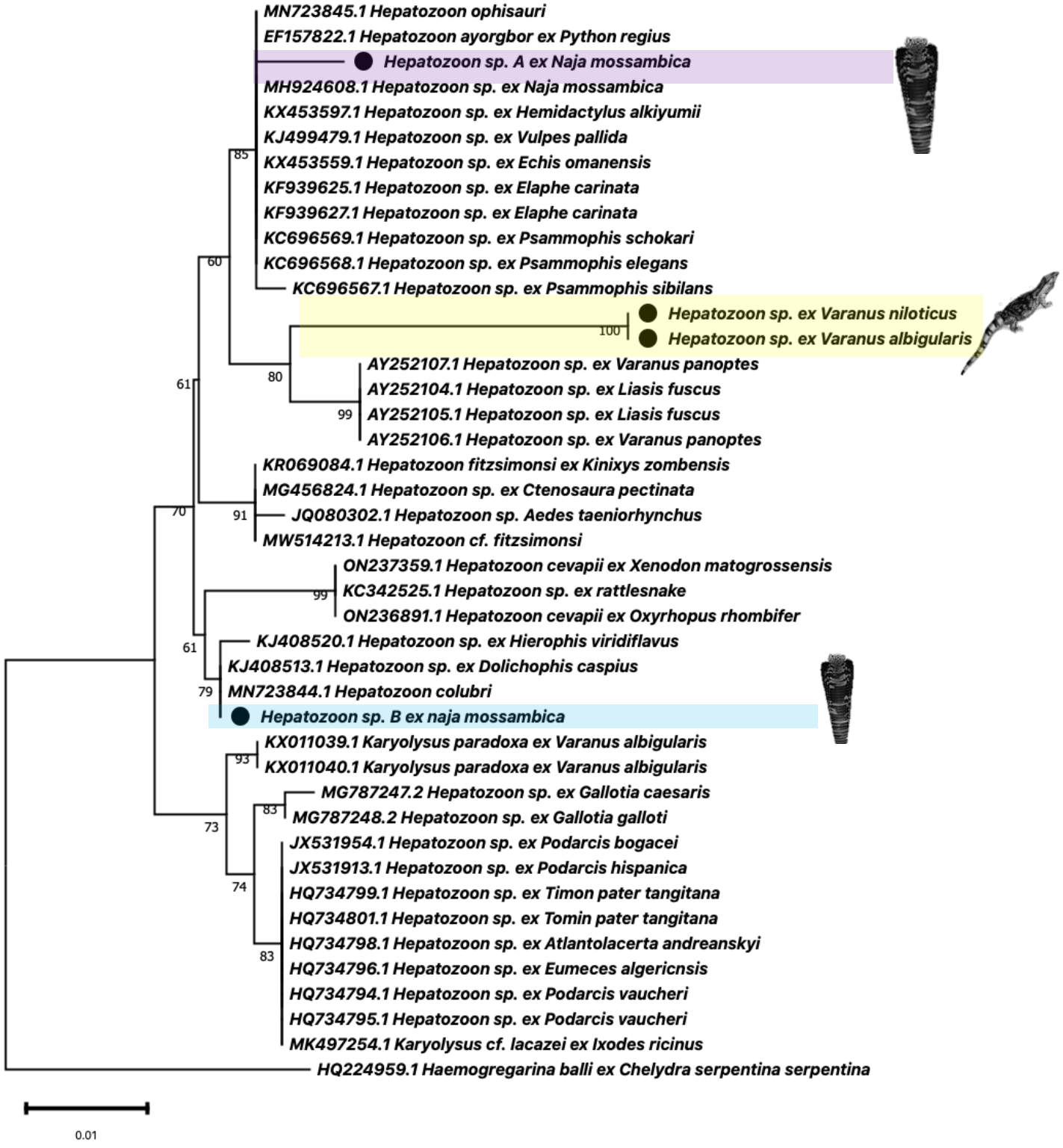


Figure 5-4: Phylogenetic tree of *Hepatozoon* 18S rDNA sequences of reptiles based on maximum likelihood analysis. Bootstrap values at the major nodes are of percentage agreement among 1000 replicates. The branch scale represents 0.01 substitutions per site.

5.4. Discussion

A total of 30 snakes and 16 monitor lizards were collected and screened for the presence of haemogregarines. Both species of lizard, *V. niloticus* and *V. albigularis* were found infected. Only one species of snakes (*N. mossambica*) was found infected with two morphologically and molecularly different species of *Hepatozoon*. Morphological and molecular data indicate that the hemogregarine species parasitizing *N. mossambica* in the present study represent two distinct species of *Hepatozoon,* herein described as *Hepatozoon* sp. A and *Hepatozoon* sp. B, whilst both *Varanus* species were parasitized by a single species of *Hepatozoon. Hepatozoon* species are considered the most commonly reported haemoparasites found in squamates and studies overtime have shown their complexity (Telford 2009). In South Africa, species of *Hepatozoon* from snake and lizard hosts are also commonly reported. This genus is the most widely reported and the most speciose, with over 300 species documented (Telford 2009). Predation might present an important route of transmission for members of family Hepatozoidae. For example, Maia et al. (2014) suggested that wild carnivores can become infected by *Hepatozoon* species through predation of rodents.

Both morphological and molecular data revealed two different *Hepatozoon* species infecting *N. mossambica* screened in the present study. The finding of these two very different lineages of *Hepatozoon* in two individuals of *N. mossambica* is not unexpected. The finding of *Hepatozoon* species in only *N. mossambica* in the present study could be attributed to their diet and the fact that some snakes can be resistant to infection and parasitic prevalence is a reflection of their basic ecology (Tomé et al. 2013). Past studies have shown habitat and diet of snakes important, particularly where more than one species of intermediate host is involved (Telford et al. 2001; Telford 2009; Tomé et al. 2013). For instance, *Hepatozoon ayorgbor* is known to naturally infect snakes through ingestion of rodents, and Sloboda et al. (2008) also experimentally transmitted the latter to snakes by feeding these reptiles infected rodent tissue. Furthermore, snakes are naturally infected with *Hepatozoon sipedon* through ingestion of anurans (Smith et al. 1994; Netherlands et al. 2014). It is interesting that snake species such as *Dendroaspis* spp., and *Bitis arientans* of South Africa showed no presence of *Hepatozoon* even though they have recently been documented to be infected by members of this genus (Smit 2022).

The epidemiology of species of *Hepatozoon* in snakes is still not fully elucidated. The role of prey items (including amphibians, birds, mammals, and reptiles) in the epidemiology of *Hepatozoon* spp. should be further investigated to evaluate if genotypes circulating in such prey items are specific of these groups or might also circulate in a wide range of predatory reptiles such as

snakes; in which these would then play a role as intermediate or paratenic hosts in the epidemiological cycles. In the case of species of *Naja*, these snakes often feed on a variety of prey, including other snakes. As such it would be beneficial to widen the *Hepatozoon* survey to include more genera and species of snakes, particularly those that often form part of *Naja* spp. diets, so that these parasites may be compared in morphological and phylogenetic analyses.

Although the gamonts of *Hepatozoon* sp. A, from the the current study morphologically resembles *H. ayorgbor*, the morphometrics were slightly different (11.5-15.4 × 3.7-5.9) with gamonts longer and thicker, but the cell area could not be compared as it was not provided in the original description. Regarding *Hepatozoon* sp. B, characteristics were comparable to those of *H. enswerae*, however, with slight differences in size. These differences among *Hepatozoon* species may be as a result of phenotypic plasticity that usually occurs in various haemograrines based on the infected host and their behaviour (Ball 1967).

The phylogram of 18S rRNA gene sequences revealed that the *Hepatozoon* sp. A of *N. mossambica* from this study clustered with *H. ayorgbor* and other closely related sequences. However, it was worth noting that its long branch length revealed that *Hepatozoon* sp. A has an increased degree of genetic divergence in relation to the other selected sequences, which may suggest that geographical distribution, climate change, different host species and dietary preferences could be the reason for this difference in divergence. Furthermore, and the fact that other closely related sequences in the clade were not named or confirmed molecularly, could be another reason for this genetic divergence with the possibility that they may be different species of *Hepatozoon* or variants of the same species. This increased degree of genetic divergence of *Hepatozoon* spp. in *N. mossambica* from South Africa and their clustering in separate clades is quite expected, and shows how complex and difficult it is to identify co-evolutionary patterns between these parasites and their reptilian hosts.

Varanus albigularis and *V. niloticus* are known to occupy terrestrial and aquatic environments, respectively (Bates et al. 2014; Cook et al. 2016). Both species examined in this study can be found throughout South Africa and they are geographically sympatric in Ndumo Game Reserve, demonstrating that the finding of the same *Hepatozoon* species in both these reptiles is not completely surprising. Furthermore, the *Hepatozoon* species from the present study is most closely related to a *Hepatozoon* species documented from other monitor lizards and distinct from the *Karyolysus* clade.

Co-infection of this *Hepatozoon* species with a *Karyolysus* sp. resembling *Karyolysus paradoxa* was microscopically observed in *V. niloticus (*see Fig S1*).* However, the amplification and

sequencing of the 18S rRNA gene of *Karyolysus* species was not successful, most likely owing to the low parasitemia observed. As a result, the generic diagnosis was made solely on the morphology of the gamonts. This *Karyolysus* sp. had the same characteristics as those reported by Cook et al. (2016), including the shrinkage of the host cell and destruction of host cell nucleus resulting in a vacuolated appearance. The co-infection of *Karyolysus* and *Hepatozoon* in these monitor lizards have been reported before (see Cook et al. 2016), suggesting that these two haemogregarines may have the same vector. Even though the ticks from both Cook et al. (2016) and this study were not morphologically and molecularly positive for any *Karyolysus* species, future studies should focus on identifying the possible vectors and attempt to elucidate complete life cycles of these organisms.

Until recently, both molecular diagnosis and phylogenetic analyses of *Hepatozoon* species relied solely on nuclear 18S rDNA data. Extending the range of available molecular markers to plastid or mitochondrial genes will greatly improve the understanding of diversity, phylogeny and taxonomy of the genus *Hepatozoon* and intergeneric relationships between individual clades of hemogregarines. Although the 18S rDNA gene has been commonly used as a molecular marker for phylogenetic analyses, problems in separating closely related species might occur due to its high degree of conservation (Morrison et al. 2004; Cook et al. 2018). However, it does emphasize the need to combine morphological descriptions with molecular data (Cook et al. 2016; Tomé et al. 2016; Borges-Nojosa et al. 2017).

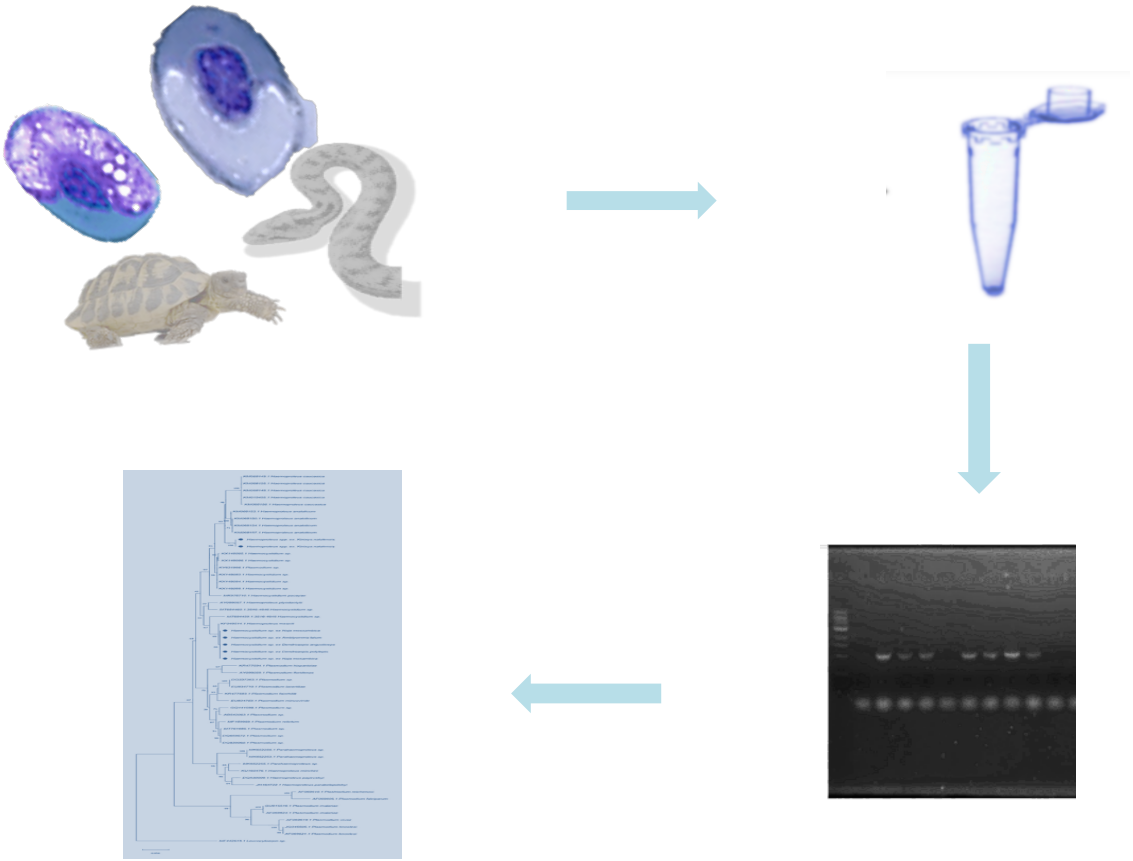
In addition, Schreeg et al. (2016) used mitochondrial genomes to help resolve the complex phylogenetic relationship of piroplasmids. Furthermore, Léveillé et al. (2019) described mitochondrial genome sequences of *Hepatozoon canis* using next-generation sequence approaches. Moreover, it is anticipated that mitochondrial genomes will prove useful in the future as a barcode for differentiating *Hepatozoon* species that are shown to be closely related in phylogenetic inferences based on 18S rRNA (Perles et al. 2019). Additionally, the complete genome of *H. canis* by Léveillé et al. (2019) will open opportunities to identifying novel target genes, allowing better differentiation of *Hepatozoon* species circulating in both vertebrate and invertebrate animals. However, it is also important to focus on morphological descriptions and life-cycles of these organisms in order to resolve their complex taxonomy. Such approaches would contribute to a better understanding on the description, diversity and phylogeny of *Hepatozoon* species, thus allowing us to make well-informed decisions.

5.5. Conclusion

Besides the present study representing the first morphological and molecular report of *Hepatozoon* within the South African varanids and elapids, it also highlights the possibility of *H. camari* being present in South African varanids. However, in order to accurately ascertain this, one would need to collect this parasite from the type host and locality in Mozambique. The host and distribution range of *H. ayorgbor* might be extended from a single specimen of *Python regius* and two experimental hosts (*Boa constrictor* and *Lamprophis fuliginosus*) in Ghana to an additional one specimen of *N. mossambica* in South Africa as we observed a *Hepatozoon* sp. that was closely related to *H. ayorgbor*, whilst *H. camarai* may be extended from a single specimen of *V. albigularis* in Mozambique to an additional two specimens in South Africa, as well as including *V. niloticus* as an additional host in South Africa. Furthermore, the current study provides the sequence data of *Hepatozoon* species infecting varanid lizards, which were not provided by other authors describing this haemogregarine from varanids. Future research should include the use of faster evolving genetic markers to increase the molecular resolution among different species of *Hepatozoon* infecting squamate reptiles.

CHAPTER 6: HAEMOSPORIDIAN DIVERSITY OF TORTOISES AND SNAKES IN KWAZULU-NATAL AND WESTERN CAPE, SOUTH AFRICA

The content from this chapter was presented at the below conference proceedings- 4th International Congress on Parasites of Wildlife and 50TH Annual PARSA Conference, Kruger National Park, 11–15 September 2022.



6.1. Introduction

The order Haemosporida (Phylum Apicomplexa) (Davies and Johnston, 2000; Duc et al. 2021) consists of several genera of single-celled eukaryotic parasites that are transmitted by haematophagous insects and are frequently described from a vast range of vertebrate hosts, although most of our understanding of their phylogeny and diversity is based on avian and mammalian hosts (Valkiunes 2005; Nourani et al. 2018; Van Hemert et al. 2019; Taioe et al. 2020). There are currently four recognised families in the order Haemosporida*,* and this includes Haemoprotidae, characterized by the presence of haemozoin pigment with no erythrocytic merogony; Plasmodiidae, characterized by the presence of both haemozoin pigment and erythrocytic merogony; Garniidae, characterized by the presence of erythrocytic merogony but the haemozoin pigment is absent; and Leucocytoidae, characterized by the absence of these two characteristics (Perkins 2014; Maia 2015). Most available information about haemosporidians is with regard to the genera that include species of medical importance, such as genus *Plasmodium,* which includes the causal agents of human malaria (WHO 2018), while information for other taxonomic groups remains lacking, particularly those infecting reptiles. This lack of knowledge has led to an increase in phylogenetic studies over the past few years (Javanbakht et al. 2015; Maia et al. 2016; Boudenga et al. 2017; Pacheco et al. 2018; Gonzalez et al. 2019).

The haemosporidians found in reptiles have been mainly classified as *Plasmodium,* as well as the species rich genus *Haemoproteus.* However, based on both morphology and molecular descriptions, Pineda-Catalan et al. (2013) proposed that reptilian *Haemoproteus* belong to genus *Haemocystidium*. To date, the classification of the majority of haemosporidian parasites solely relied on their morphology, their life-history traits, and the taxonomy of the infected vertebrate hosts and insect vectors (Laison and Naiff 1998; Valkinas et al., 2008a; Telford 2009; Cook et al. 2010; Perkins, 2014).

Despite South Africa's extraordinary reptilian diversity, data on South African reptile Haemoproteidae are particularly scarce, and little is known about the prevalence, diversity and taxonomy of haemosporidian parasites infecting these ectotherms. Only a few species of haemosporidians (*Plasmodium bitis* Fantham and Porter 1950 *, Plasmodium pythonis* Fantham and Porter 1950*, Plasmodium causi* Fantham and Porter 1950*, Plasmodium melanoleuca* Fantham and Porter 1950, *Plasmodium zonuriae* Pienaar 1962; *Plasmodium cordyli* Telford 1987; *Plasmodium intabazwe* van As et al. 2016), *Haemoproteus testudinalis* (Laveran 1905) Wenyon

1915; *Haemoproteus natalensis* Cook et al. 2010) have been documented from South African reptiles, including four species from ophidian hosts, three from saurians and two from chelonians (Fantham and Porter 1950; Cook et al. 2010; van As et al. 2016). Hence, the aim of this study was to investigate the diversity of haemosporidians infecting reptiles from two of South Africa's biodiversity rich provinces, KwaZulu-Natal and the Western Cape.

6.2. Materials and Methods

Of the 25 tortoises, 30 snakes and 16 monitor lizards collected during this study, all were screended for the presence of haemoparasites. Blood was collected from the caudal vein from snake and lizard hosts and from the subcarapacial sinuses for tortoises, and thin blood smears prepared with the remaining volume of blood preserved in 70% ethanol and stored in a -20°C freezer for subsequent molecular analysis. If haemoparasites were observed in the blood smears (this particular chapter focusing on haemosporidians), these were micrographed and the morphometrics determined as detailed in Chapter 2. Parasitemia was calculated per 100 erythrocytes, with ~10^4 erythcytes examined per blood smear as indicated by Cook et al. (2016). Molecular analysis included amplification of fragments of the *cyt-b* gene as detailed in Chapter 2. Besides screening the reptiles for haemosporidians, 253 *Amblyomma* ticks (43 *Amblyomma latum*, 60 *Amblyomma sylvaticum*, 37 *Amblyomma exornatum*, 113 *A. marmoreum*) were molecularly screened. These ticks were identified as described in Chapter 2. The sequences for this chapter will be provided once the manuscript has been accepted to publication.

6.3. Results

Resulting haemosporidian sequences were compared with each other and to those on GenBank. Of the 25 tortoises, two (8.0%) (with parasitemia 2–4%) were parasitized by a species of *Haemoproteus* (*Haemocystidium)*. Of the 30 snakes, four (13.3%) (with parasitemia 12.4%) were found infected with a species comparable to *Haemoproteus mesnili*, and of the 43 *A. latum* ticks, only one (2.3%) was found with a species comparable to the latter. The morphological stages of this snake *Haemoproteus* was only observed in *N. mossambica*, while the molecular sequences of the same *Haemoproteus* species were also obtained from two species of genus *Dendroaspis*. None of the monitor lizards', *A. exornatum, A. marmoreum*, and *A. sylvaticum* were positive for any haemosporidian species. Based on morphological data, a co-infection of haemoproteids and haemogregarines was only observed in snakes, and not in tortoises, despite haemogregarines being commonly found in tortoises (Cook et al. 2009; Cook et al. 2014).

Description:

Haemoproteus (Haemocystidium) sp. *ex Kinixys natalensis*

Taxonomic summary:

Vertebrate host: *Kinixys natalensis* Hewitt, 1935 (Testunidae, Cryptodira)

Type locality: Ndumo Game Reserve, Kwa-Zulu Natal, South Africa

Site of infection: Peripheral blood

Vector and definitive host: unknown

The parasite did not induce hypertrophy of the erythrocytes.

Young gametocytes:

The outline of the gametocyte is amoeboid to rounded, found rarely (7.36–8.42 × 3.82–6.74) µm, usually occurring singly in mature erythrocytes. They are more frequently observed in a polar to sub-polar position relative to erythrocyte nucleus, in some cases slightly touching the nucleus and envelope of the erythrocytes (Fig. 6-1 A-C). When observed, small vacuoles are clustered at one end of the gametocyte (Fig. 6-1 C). No nucleus/ parasitophorous vacuole observed and lacking granules.

Microgametocytes:

Microgametocytes vary in shape from rounded (Fig. 6-1 D), terminally or laterally microhalteridial to halteridial (Fig. 6-1 E–G), occurring singly or in pairs, within erythrocytes, often laterally (Fig. 6.1F) 11.49±2.53 x 5.38±1.12 (8.98–16.43 × 3.56–7.77, n=17) µm with LW 60.46±11.66 (41.54–79.52) µm, L/W 2.27±0.80 (1.21–3.65), area 43.05±7.42 (32.05–57.46) μm^2. Cytoplasm is foamy, or sponge-like and whitish to pale-purple, with perimeter staining purple, 1–4 vacuoles, in some cases one big vacuole is evident on each end of the gametocytes (Fig. 6-1 G). No nucleus, haemozoin granules or parasitophorous vacuole (PV) evident.

Macrogametocytes:

Gametocytes are round, elongate, kidney-shaped, terminally or laterally halteridial (Fig. 6-1 H-L),16.04±6.19 × 6.55±1.11 (8.78–32.09 × 4.76–8.79, n=13) µm with LW 102.78±34.07 (72.67–171.36) µm, L/W 2.57±1.27 (1.24–6.00), area 68.45±18.61 (38.45–111.14) μm^2. Purple, foamy cytoplasm with periphery tending to stain dark-purple, 1 to ~11 vacuoles either scattered or clustered. No haemozoin granules, nucleus nor PV were evident.

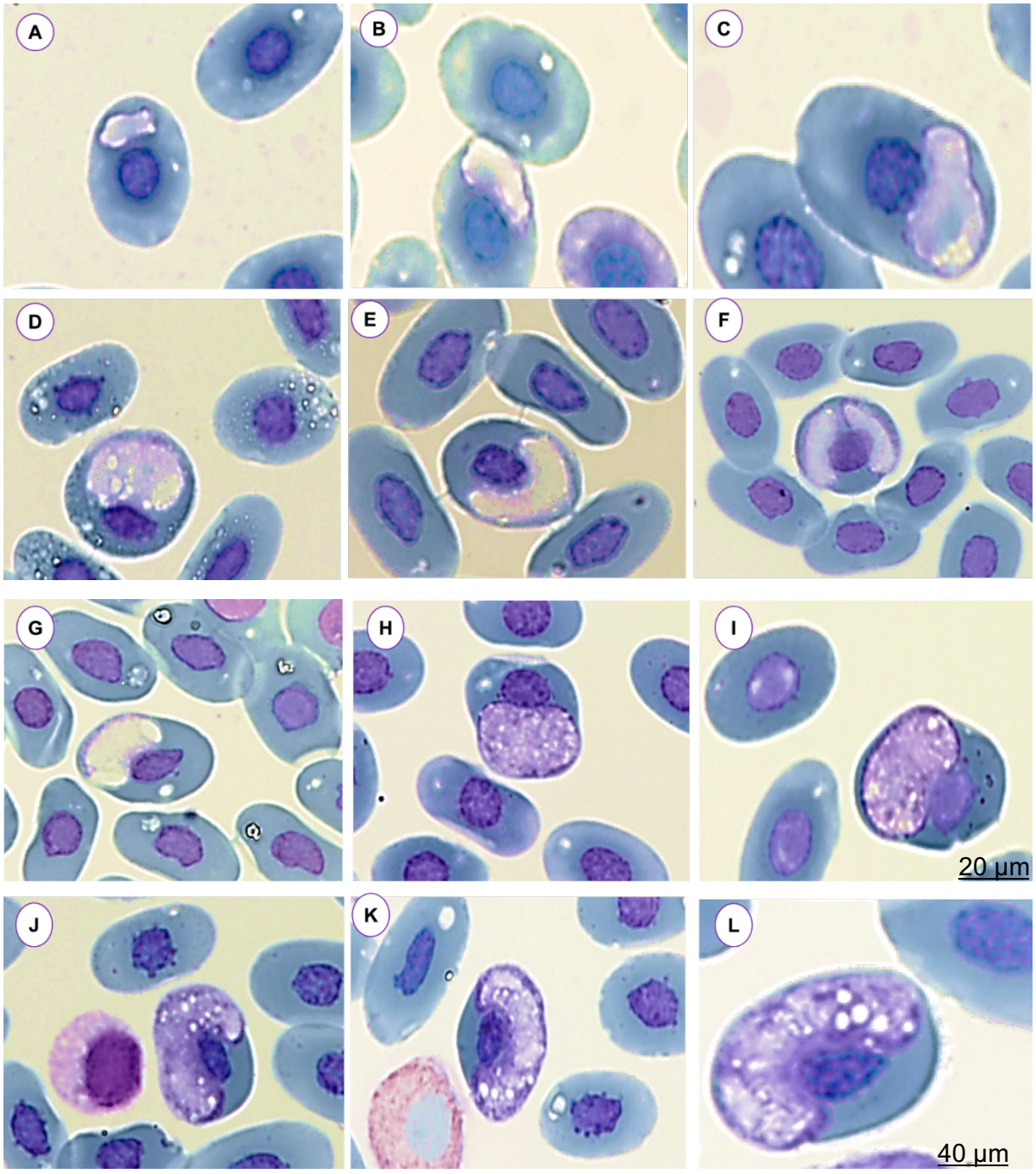


Figure 6-1: Haemosporidian species gametocytes parasitizing the erythrocytes in the *Kinixys natalensis*. (A-C) rounded and amoeboid young gametocytes; (D-G) rounded, microhalteridial and halteridial microgametocytes; (H-L) kidney shaped, elongate macrogametocytes. Scale bar: 20 µm, 40 µm for C & L.

Effects on the host cell

The normal erythrocytes measured 14.82–17.38 (16.44±0.94) × 8.47–10.09 (9.24±0.62), LW 151.90±13.92 (125.97–161.97), L/W 1.78±0.15 (1.53–2.05), area 128.69±13.22 (102.66–142.84) compared to the slightly wider infected erythrocytes measuring 14.28–19.20 (16.12±1.29) × 7.98–13.39 (10.92±1.62), LW 175.79±29.91 (137.27–257.09), L/W 1.51±0.29 (1.18–2.15), Area 140.26±22.56 (104.9–210.3). Host cell infected with both micro- or macrogametocytes resembled mature erythrocytes and did not appear to be greatly altered, both in shape and size, except some slight lengthening and widening of the host cell. Moreover, erythrocytes infected with two fully developed gametocytes are neither enlarged nor deformed. The normal erythrocyte nucleus measured 4.94–7.33 (6.48±0.57) × 3.35–5.64 (4.44±0.65), LW 28.95±5.95 (17.33–41.34), L/W 1.48±0.19 (1.27–1.81), area 24.32±4.98 (14.3–32.34) compared to the diminished and slightly narrowed infected erythrocyte nucleus measuring 4.21–7.75 (5.50±1.04) × 2.68–5.57 (3.98±0.78), LW 21.48±4.03 (16.08–29.07), L/W 1.46±0.49 (0.81–2.26), area 18.46±3.65 (12.64–26.03). The infected host cell nucleus shows no distortion but some degree of displacement in some rare cases.

Remarks:

Data regarding *Haemoproteus* (*Haemocystidium)* species in South African terrestrial tortoises is lacking. Prior to this study, only two species of *Haemoproteus* (*Haemocystidium*) had been described parasitizing chelonians in South Africa, *Hp. testudinalis* from *Stigmochelys pardalis* and *Kinixys* spp., and *Hp. natalensis* from *K. natalensis* (Laveran, 1905; Wenyon, 1915; Cook et al. 2010). Observation of no divisional stages within the peripheral blood erythrocytes indicated that this haemosporidian belongs to genus *Haemoproteus* (*Haemocystidium)*. This parasite has unique morphological and morphometric features when compared to the other two *Haemoproteus* (*Haemocystidium)* species from African terrestrial chelonians. It is morphologically smaller than *Hp. natalensis, Hp. testudinalis*, and *Hp. roumei*. *Haemoproteus natalensis* has an area of 142.5 and 58.9–87.3 µm for macro- and microgametocytes respectively; *H. testudinalis* has an area of 155.8 and 84.4 µm for macro- and microgametocytes respectively; and *H. roumei* measures 12.6 in diameter for spherical forms, or 12.6–16.2 × 10.8 for elongate and halteridial forms, and 9 µm in diameter for spherical forms, or 14.4 × 3.6 for elongate forms, for macro- and microgametocytes respectively (no area measurements were provided in the original description). Furthermore, both microgametocytes and macrogametocytes showed the absence of haemozoin granules which is not a characteristic of any *Haemoproteus* spp. of any other terrestrial chelonian haemoproteids described across the world (Wenyon 1915; Lainson and Naiff 1998; Telford 2009; Cook et al. 2010; Orkun and Guven 2013).

The *Hp. natalensis* was observed from a captive *K. natalensis* from the same province as the *Haemoproteus* (*Haemocystidium)* in the current study (Cook et al. 2010). However, the kidney shape, widening of the host cell, and the absence of the pigment granules distinguishes the current study's parasite from *Hp. natalensis*. Furthermore, no molecular data was provided for *Hp. natalensis*. As a result of these comparisons, *Haemoproteus* sp. in wild *K. natalensis* from the current study is different from *Hp. natalensis* despite from being from the same host and wider region. The newly detected lineage of *Haemoproteus* (*Haemocystidium)* in *Kinixys* tortoises from South Africa may represent a possible new species that needs to be further sampled, ideally with multigene data analysis combined with data on the life cycle stages and identification of the vectors to increase understanding of this new species and for their best taxonomic classification (Martinsen et al. 2006; Cook et al. 2010; Pineda-Catalan et al. 2017). From a molecular point of view (see section below on molecular results), the two obtained sequences confirmed that the parasites from the two tortoises are a single species with no genetic differentiation between the sequences. It is worth mentioning that these tortoises were in the wild, suggesting the possibility that this lineage is not introduced to the area, and these tortoises are naturally infected.

Haemocystidium sp. ex. *Naja mossambica*

Taxonomic summary:

Type host: *Naja nigricollis* Reinhardt (Serpentes: Elapidae)

Other hosts: *Naja haje, Hemachatus haemachatus*

Vertebrate host from this study: Naja mossambica Peters 1854 (Serpentes: Elapidae)

Type locality: Odienne, Ivory Coast

Additional localities: North slope of Uluguru Mountains at edge of Sokoine University campus, Morogoro, Tanzania (06°51′S, 37°38′E); Gaoua, Upper Volta; Bamako, Mali; Accra, Ghana; Deleib Hill and Nasser on River Sobat, Sudan; region surrounding Durban, KwaZulu-Natal, South Africa (this study)

Site of infection: Peripheral blood

Vector and definitive host: unknown

Young gametocytes:

Trophozoites (Fig. 6-2 A-B) 3.69–5.98 × 2.3–3.37, with one vacuole visible at one end of the trophozoite, with no pigment granules. With growth into young gametocytes (Fig. 6-2 C) 6.17–7.38 × 3.15–3.70, the trophozoites becomes elongate lying laterally.

Microgametocytes:

Microgametocytes are rounded to elongate (Fig. 6-2 D-F) 14.51±3.47 × 6.50±1.02 (10.7–24.63 × 4.5–8.18, n=25) µm, with LW 93.23±20.96 (70–148.52) µm and L/W 2.32±0.86 (1.31-5.44) µm, area 76.11±13.86 (49.88–106.58) μm^2 heavily pigmented with black granules clumped or concentrated near ends of the gametocytes. In some cases, they are microhalteridial most commonly lateral touching the nucleus of the erythrocyte. Vacuole mostly not visible but range from 1–2 when present.

Macrogametocytes:

The gametocytes are elongate, laterally microhalteridial to halteridial, sometimes covering the entire erythrocyte and surrounding its nucleus (Fig. 6-2 G-I), 16.62±2.76 × 7.71±1.06 (11.2–24.98 x 5.67–10.54, n=32) µm, with LW 129.65±36.37 (73.65–210.56) µm, and L/W 2.17±0.34 (1.65–3.28) µm. area 108.59±32.44 (61.65–185.46) μm^2. Haemozoin granules 35–51 are dispersed throughout the gametocyte. Gametocytes were at some stage observed inside a developing erythrocyte (Fig. 6-2 K). or free from the erythrocyte (Fig. 6-2 P). In some cases, double infections of the gametocytes in a single erythrocyte are observed (Fig. 6-2 J & 6-2 N).

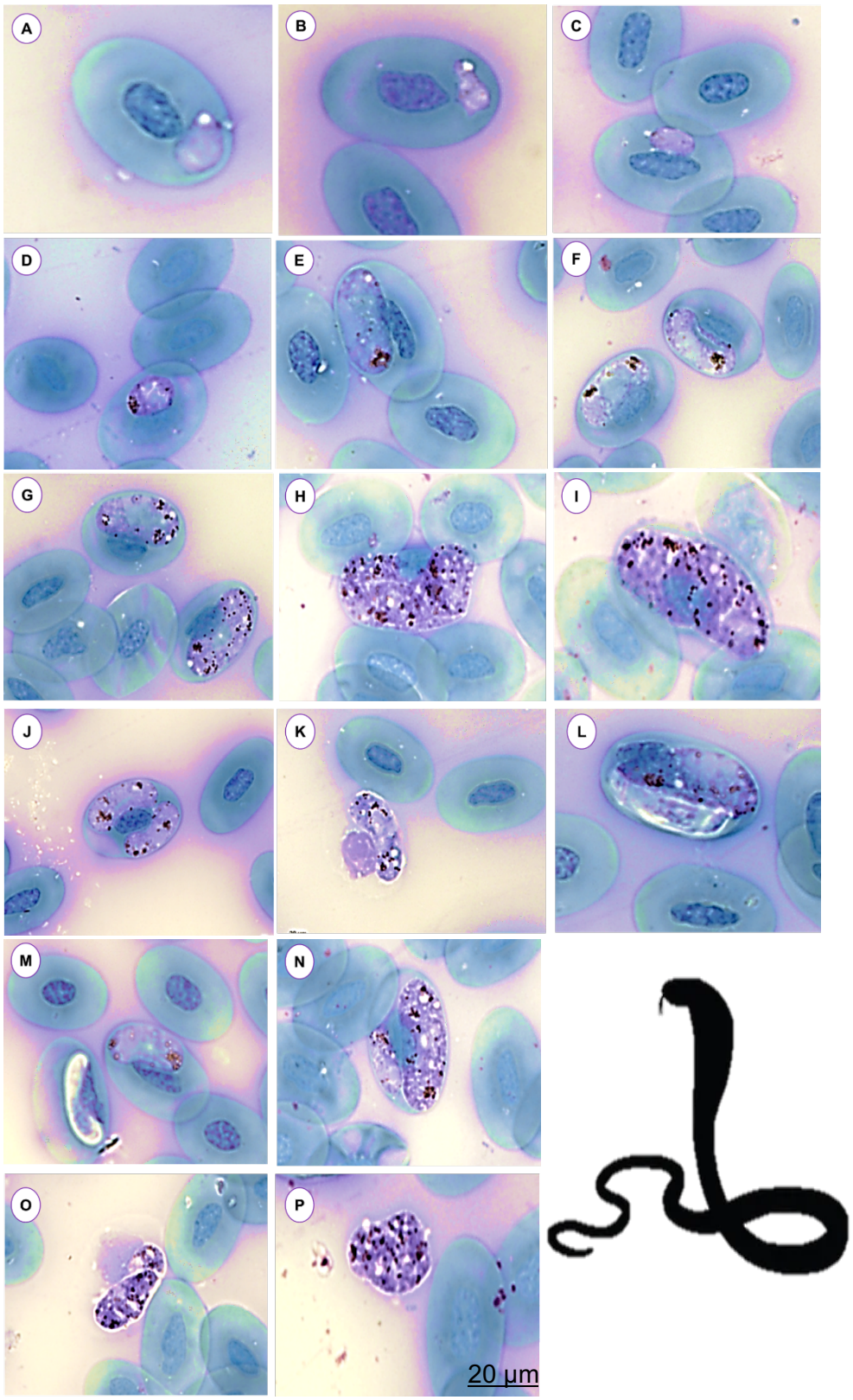


Figure 6-2: Haemosporidian species gamonts parasitizing the erythrocytes in the *Naja mossambica*. (A-C) young gametocytes; (D-F) microgametocytes. (G-I) macrogametocytes; (K) macrogametocyte in developing red blood cell; (P) exoerythrocytic gametocytes;Scale bar: 20 µm.

Effects on the host cell

Non-parasitised erythrocytes (n=25) measured 15.81–23.12 (19.12±1.49) × 10.12–13.87 (12.42±0.91), LW 240.50±28.93 (160–307.5), L/W 1.56±0.14 (1.32–1.76), area 194.91±22.85 (141.98–247.72), compared to elongated and wider parasitized host cells (n=25) measuring 19.39–21.53 (21±1.29) × 11.49–15 (13.58±1.10), LW 285.33±32.05 (241.52–338.55), L/W 1.56±0.15 (1.34–1.90), area 230.46±24.46 (187.97-268). Although the infected cells were longer amd wider than uninfected cells, infected erythrocytes were similar in L/W to uninfected cells. Uninfected erythrocyte nucleus measures 4.93–9.64 (7.80±1.22) × 2.93–5.92 (3.92±0.63), LW 30.59±7.44 (22.38–55.59), L/W 2.04±0.46 (1.09–3), area 27.52±7.36 (20.06–47.03), compared to neither enlarged nor narrowed, but sometimes displaced infected erythrocyte nuclei measuring 5.01–9.55 (7.96±1.09) × 2.2–5 (3.51±0.82), LW 28.07±8.20 (19.01–40.69), L/W 2.39±0.66 (1.23–3.56), area 25.20±6.88 (16.44–37.07). Although infected cell nuclei and uninfected cell nuclei are similar in length and width, the area and LW of uninfected erythrocyte nuclei are greater than that of infected erythrocyte nuclei. Furthermore, the L/W of infected erythrocyte nuclei is greater than that of uninfected erythrocyte nuclei. The infected snake also harboured a haemogregarine developmental stages of which were sometimes found in the same host cell as the *Haemocystidium* gametocytes. In such infections the erythrocyte nucleus is markedly displaced and deformed either by the haemogregarine or the haemosporidian.

Remarks:

To date, only two *Haemocystidium* spp., have been reported in African snakes, *Haemocystidium mesnili* (Bouet 1909) and *Haemoproteus balli* Telford 2007 (potentially *Haemocystidium*) originally described from cobras of the Ivory Coast and Kenya, respectively (Bouet 1909; Ball 1967, Telford 2009). The haemosporidian in this study most closely resembles *Hp. mesnili* (or *Hc. mesnili*) described by Bouet (1909) and redescribed by Telford (2007), the latter parasite diagnosticly having elongate stages measuring 11–29 × 5–10 µm, LW 72–247 µm , L/W 1.1–4.4 µm. As such the present study's parasite size ranges for both micro- and macrogametocytes fall well within the range of *Hp. mesnili*. Furthermore, the halteridial forms, pigment granules scattered in the macrogametocytes or clumped at the ends in the microgametocytes, described in Telford (2007; 2009), were observed from the current study, as was also observed by all the other authors who have described/reported on this parasite (Bouet 1909; Leger and Leger 1914; Macfie 1919). As this study did not involve lethal sampling of the vertebrate host, no tissue samples in the current study were examined for meront comparison with *Hp. mesnili*.

Molecular identification and phylogenetic analysis

In the phylogenetic analysis, the two *Haemoproteus* (*Haemocystidium*) *cyt-b* gene from two individuals of *K. natalensis* were shown to be 100% identical over a 497 bp of the gene, forming a monophyletic clade with 100% bootstrap support. Furthermore, these sequences clustered in a larger clade with *Haemocystidium* of both terrestrial and aquatic chelonians. With regard to the sequences of *Haemocystidium* from snakes and a single tick species, they clustered together in a monophyletic clade. With high bootstrap support (99%), this clade formed a sister clade to *Haemoproteus mesnili* (KF049514.1), which in turn clustered within a larger monophyletic clade consisting of *Haemocystidium* of snakes and lizards. Important to note, however, is that the sequence of *Hc. mesnili*, was provided without a morphological comparison to the original and Telford's (2007) description. Furthermore, the sequence was isolated from *N. annulifera* from the Kruger National Park, South Africa. As such the sequence for this species was provided from neither the type host or locality (Pineda-Catalan et al., 2013). All reptilian *Haemocystidium* species formed a monophyletic group distinct from *Haemoproteus* of birds, sharing a common ancestor with the genus *Plasmodium* (Figure 6.3).

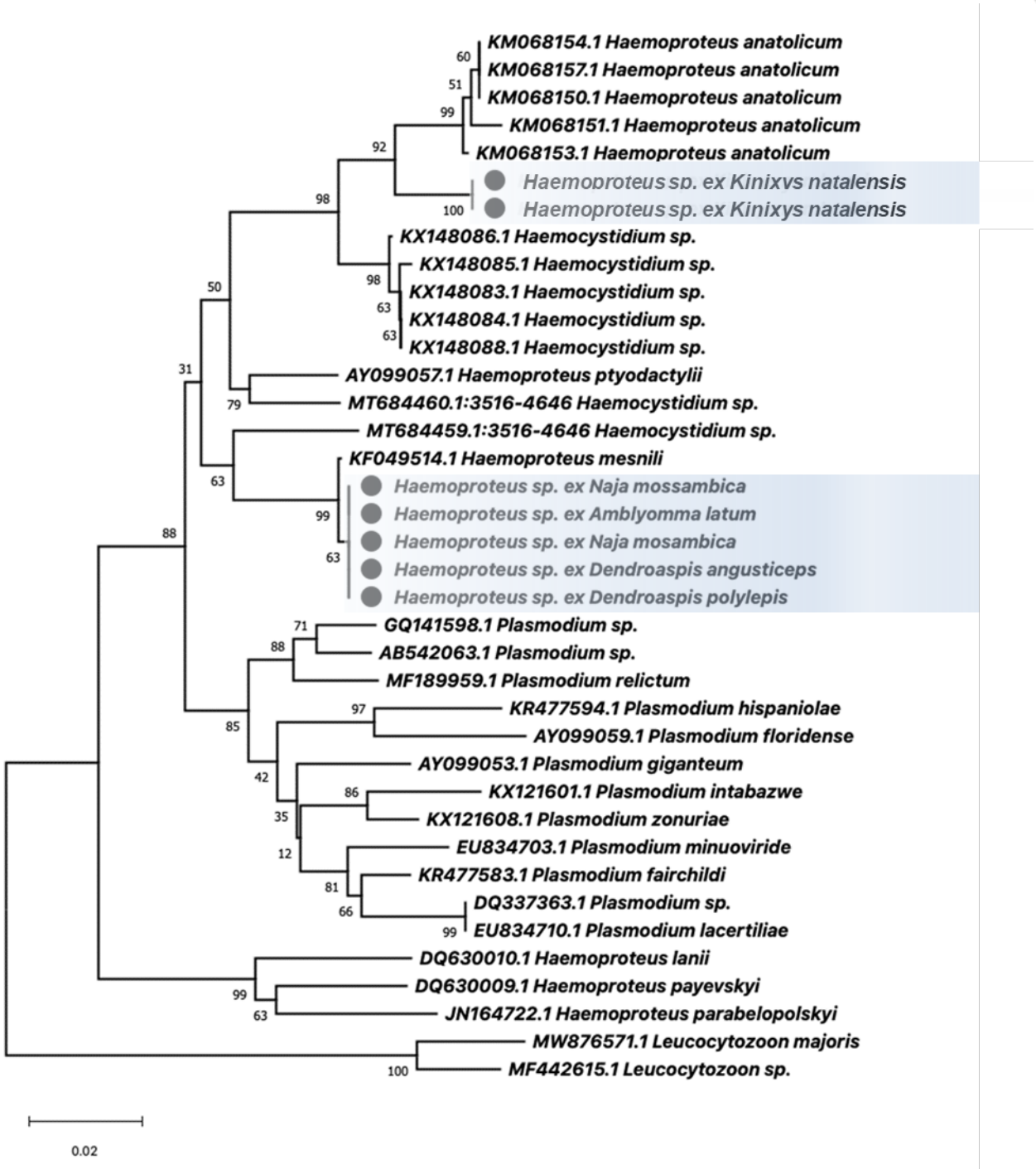


Figure 6-3: Phylogenetic tree of 497 bp haemosporidian *cyt-b* gene sequences based on maximum llikelihood analysis (Tamura-Nei + Gamma distribution model). Bootstrap values at the major nodes are of percentage agreement among 1000 replicates. The branch scale represents substitutions per site.

5.6. Discussion

A number of haemosporidian species belonging to the family Plasmodiidae have been detected in reptiles, particularly in squamates (Telford 1987, 2009; van As et al. 2016). However, reports of *Haemocystidium* spp. Worldwide are limited. Thus this study represents the third report of species likely belonging to this genus in South Africa and the first in *Naja mossambica* and *Dendroapis* spp. It is noteworthy to highlight that no parasitemia was observed in *Dendroaspis* species but the use of PCR confirmed positivity with haemosporidia. This again shows that estimating prevalence through the sole use of microscopy can underestimate the true infection rates (Jarvi et al. 2002; González et al. 2019).

The reptilian haemoproteid parasites have undergone many taxonomic changes though the years. *Haemocystidium* has been a controversial genus with species having been described as *Haemoproteus* in the past (Lainson and Naiff 1998; Jakes et al. 2001; Javanbakht et al. 2015). Even though the number of studies on these parasites is still limited, both in terms of hosts and parasite species, recent phylogenetic studies using a fragment of cyt-b gene suggested that it is a distinct clade from avian *Plasmodium* and *Haemoproteus* species (Pineda-Catalan et al. 2013; Javanbakht et al. 2015; Boundenga et al. 2017; González et al. 2019; Austen et al. 2020). Based on such molecular evidence, these parasites have been reclassified as members of the genus *Haemocystidium*, following the original description by Castellani and Willey (1904) (Pineda-Catalan et al. 2013; González et al. 2019). *Haemocystidium* is characterized by sharing some morphological traits with both *Plasmodium* and *Haemoproteus*, such as the presence of haemozoin granules. However, the absence of erythrocytic meronts in the peripheral blood, characteristic of the genus *Haemoproteus* and *Haemocystidium* set these apart from the genus *Plasmodium* (González et al. 2019). The morphological and developmental stage similarities in the peripheral stages of both *Haemoproteus* and *Haemocystidium* make these two genera impossible to differentiate (Davies and Johnston 2000). As such reptilian *Haemocystidium* have been described as *Haemoproteus* in the past (Lainson and Naiff, 1998; Jakes et al. 2001; Cook et al. 2010; Javanbakht et al. 2015). However, recent studies on the phylogenetic analysis of the cyt-b gene (Pineda-Catalan et al. 2013; González et al. 2019) and the results of this study, showed that avian *Haemoproteus* species and reptilian *Haemocystidium* are two distinct monophyletic groups.

The vectors of these *Haemocystidium* species in South Africa are unknown, but haemaphagous insects are potentially responsible for their transmission. The presence of *Haemocystidium* in *A. latum* likely was as a result of the tick not having fully digested its blood meal at the time of fixation, as ticks, have to date, not been implicated in the transmission or life cycle of these parasites. At

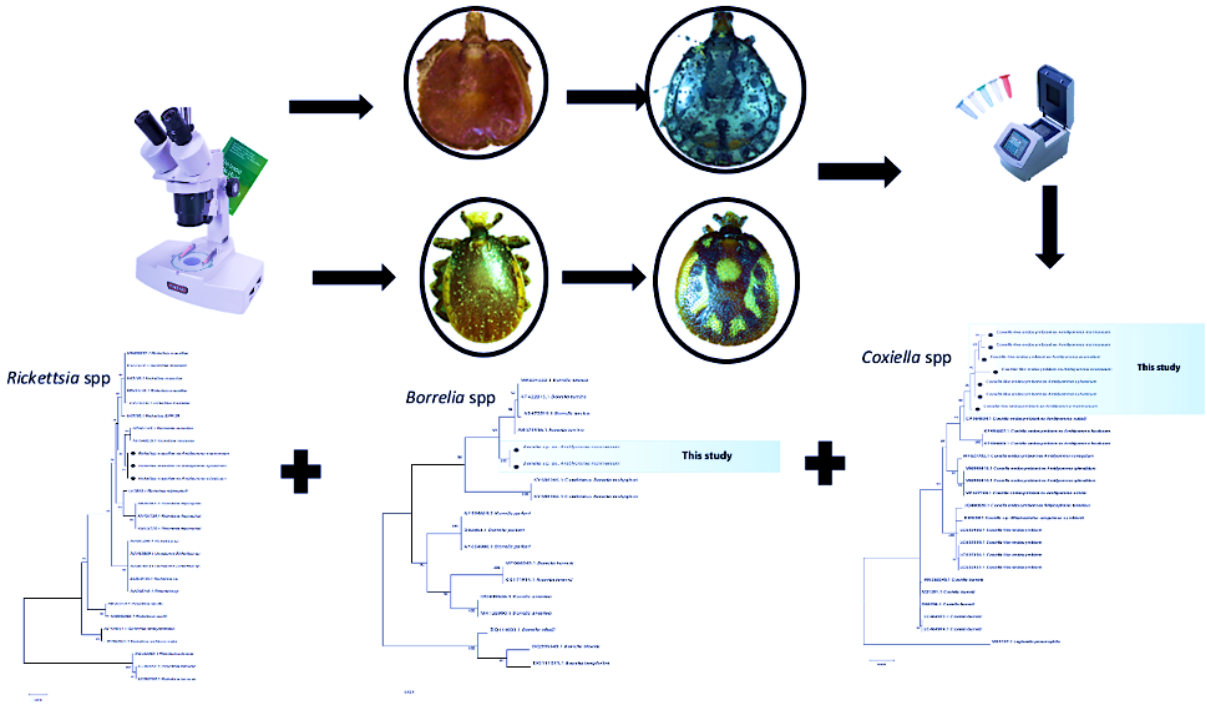
the same time however, ticks may present a less invasive method in identifying the prevalence of blood parasites such as the haemosporidians. Further studies are required to establish the host range and vector competence of *A. latum* tick for *Haemocystidium* as these haemosporidians could represent an infectious disease threat to wildlife.

6.4. Conclusion

This study provides the first available molecular data for a species of *Haemocystidium* (*Simondia*) of tortoises in South Africa, and the first combined descriptive approach of *Haemocystidium* species for chelonians in Africa. As the present species of *Haemocystidium* (*Simondia*) does not compare to *Hp. Natalensis* or *Hp. Testudinalis*, it likely represents an unknown and new species, however, it would be beneficial to find fresh samples of these to compare molecularly. Furthermore, it provides both morphological and molecular data on *Haemocystidium* infecting snakes. Future research should include the description of sporogonic stages in the possible vectors such as Acari and Dipterans in order to understand the host-parasite relationship between *Haemocystidium* and reptiles, as well as to compare these vector stages to those in *Haemoproteus*. However, this study contributes to the biodiversity knowledge of haemosporidians circulating in reptiles as well as highlighting the need for the use of both morphology and molecular approaches in identifying parasites, given their zoonotic potential and role in conservation of endangered species.

CHAPTER 7: MOLECULAR DETECTION OF TICK-BORNE BACTERIA FROM *AMBLYOMMA* (ACARI: IXODIDAE) TICKS COLLECTED FROM REPTILES IN SOUTH AFRICA

The content from this chapter was published in the below conference proceedings- “British Society of Parasitology (BSP), (Parasites online 2021), 21–25 June 2021” and the below full publications- Mofokeng, L.S., Smit, N.J. and Cook, C.A., 2022. Molecular Detection of Tick-Borne Bacteria from Amblyomma (Acari: Ixodidae) Ticks Collected from Reptiles in South Africa. *Microorganisms*, *10*(10), 1923. https://doi.org/10.3390/microorganisms10101923



7.1. Introduction

Tick-borne pathogens (TBPs) are responsible for some of the most severe emerging infectious diseases across the world (Parola et al. 2005; Omondi et al. 2017). The wildlife-domestic-human interaction has the potential to accelerate the spread of zoonotic tick-borne pathogens that cause significant diseases, and eventually death (Uilenberg 1995; Omondi et al. 2017). To date, roughly 61% of human diseases have been documented to have a zoonotic origin, and wildlife is associated with approximately 75% of emerging zoonotic infections globally (Warwick and Corning 2013). In South Africa, four tick-borne spotted fever group (SFG) *Rickettsia* spp. (*Rickettsia africae, Rickettsia conorii conorii, Rickettsia aeschlimannii* and *Rickettsia sibirica mongolitimonae)* have been associated with human diseases (Pretorius and Birtles 2002, 2004; Parola et al. 2013). Additionally, members of family Ixodidae are involved in the epidemiology of other pathogens such as *Borrelia, Anaplasma* and *Coxiella* spp. Increasing numbers of studies have implicated reptiles and their associated ticks as potential reservoirs involved in the transmission cycles of TBPs (Halajian et al. 2016; Omondi et al. 2017; Santodomingo et al. 2018). This fact is of great concern in tick-borne disease control strategies, since these ectotherms are usually asymptomatic carriers that may serve as reservoirs of the infection. Although diverse tick-borne pathogens are emerging across the world, with documented impact on the health of humans and livestock, studies into their diversity and specific interactions with ticks and their reptilian host species in South Africa are lacking. In particular, the role of these ectotherms in the epidemiology of TBPs remains unknown, despite the fact that exported ticks and reptiles (*Amblyomma marmoreum*, tortoises and lizards) have been implicated with TBPs of livestock (Peter 2000; Omondi et al. 2017). This lead therefore to a lack of knowledge on the diversity of these pathogens in reptiles and whether or not reptiles can act as reservoir hosts for TBPs infecting domestic animals and humans.

In addition to pathogens, ticks also harbour various nonpathogenic microbes which include symbiotic and commensal bacteria (Daveu et al. 2021). Tick endosymbionts have gained interest over the last decade and are now being investigated due to their physiological significance in ticks (Kobayashi et al. 2021). These endosymbionts are involved in the reproduction and growth of ticks by providing these arthropods with necessary compounds that are absent in their haematophagous diet (Rio et al. 2016; Nardi et al. 2021). Furthermore, they are also involved in the maintenance of pathogens in arthropods (Moreira et al 2009). To date, three genera of symbionts (*Coxiella*-like endosymbiont, *Francisella*-like endosymbionts, and *Midichloria*) have been documented to be exclusive to ticks, and some of these symbionts are mutualistic, whereas others may be facultative (Sánchez-Montes et al. 2019; Daveu et al. 2021).

Despite the above, the presence and diversity of bacteria associated with ticks of reptiles, particularly those species that can also infest livestock and are close to human settlements, have not been studied in South Africa. This lack of information on tick-borne bacteria of reptilian ticks in South Africa warrants novel research in this area. Furthermore, since the literature related to tick-borne bacteria in reptiles and their associated ectoparasites is very recent, and most of these studies have been conducted in South America (Andoh et al. 2015; Muñoz-Leal et al. 2016; Santodomingo et al. 2018, the aim of this study was to investigate the presence of bacterial microorganisms from reptile associated ticks in South Africa.

7.2. Materials and methods

A total of 36 reptiles (23 tortoises, seven snakes, six monitor lizards) and 253 *Amblyomma* ticks (43 *Amblyomma latum*, 60 *Amblyomma sylvaticum*, 37 *Amblyomma exornatum*, 113 *A. marmoreum*) were collected from Ndumo Game Reserve, KwaZulu-Natal (KZN) and a region near the village of Paternoster, Western Cape (WC). Reptiles were restricted by hand whilst ticks were collected in situ. Blood was collected from the caudal vein of the squamate reptiles and from the subcarapacial sinuses of the toroises. The preparation of the blood smears, identification of the ticks, as well as the molecular analyses of all the specimens are detailed in Chapter 2. The sequences derived from this study were submitted to GenBank database under the accession numbers: *Coxiella* spp. (16S rRNA gene: OP070964–OP070972; 23S rRNA gene: OP071239–OP071243); *Borrelia* spp. (16S rRNA gene: OP081024–OP081246; *flagellin* gene: OP082442–OP082443); *Francisella* spp. (16S rRNA gene: OP068354 & OP068355); *Rickettsia* spp. (16S rRNA gene: OP068356; ompA: OP329173-OP329175).

7.3. Results

Overall prevalence of tick-borne bacterial microorganisms

Two hundred and fifty-three ticks (113 *A. marmoreum*, 43 *A. latum*, 37 *A. exornatum*, and 60 *A. sylvaticum*) and 36 reptiles were collected and screened for tick-borne bacterial microorganisms (142 ticks in KZN; 111 ticks in WC). Based on the PCR results, all four tick species showed evidence of infection with at least one bacterial microorganism (see Table 7-1). A spotted fever group *Rickettsia* species was detected from 21 out of 253 samples (8.3%) (95%CI= ±3.39), reptile associated *Borrelia* species was detected from 16 out of 253 samples (6.3%) (95%CI= ±2.99), *Francisella*-like endosymbiont from 2 out of 253 samples (0.8%) (95%CI= ±1.10), *Brevibacterium* spp. was detected from 2 out of 253 samples (0.8%) (95%CI= ±1.10) and *Coxiella*-like endosymbiont (CLE) from 104 out of 253 samples (41.1%) (95%CI= ±6.06) (Table 7-1). Mixed infections of CLE and *Borrelia* spp. were observed from 9 out of 253 samples (3.6%) (95%CI=

±2.21). *Anaplasma* species and *Coxiella burnetii* were not found in the ticks examined. None of the reptilians tested positive for any of the tick-borne bacterial microorganisms.

Bacterial prevalence in Amblyomma *ticks*

In KZN, three genera of bacteria were observed in *Amblyomma* ticks. *Borrelia* spp. was observed at a prevalence of 5.6% (95%CI= ±3.78), *Francisella*-like endosymbiont at 1.4% (95%CI= ±1.93), *Coxiella*-like endosymbiont at 21.1% (95%CI= ±6.71), and *Brevibacterium* sp. at 1.4% (95%CI= ±1.93) while in WC, *Borrelia* sp. was observed at 7.2% (95%CI= ±4.81), *Coxiella*-like endosymbiont at 66.7% (95%CI= ±8.77), and *Rickettsia* sp., at 18.9% (95%CI= ±7.28).

Francisella-like endosymbiont was observed only in *A. latum* at a prevalence of 4.7% (95%CI= ±6.33), while *A. sylvaticum* harboured *Rickettsia* sp. at a prevalence of 23.3% (95%CI= ±10.61), *Coxiella*-like endosymbiont at a prevalence of 45.9% (95%CI= ±12.61), and *Borrelia* sp. at a prevalence of 6.7% (95%CI= ±6.33). Four bacterial genera were observed in *A. marmoreum*. *Borrelia* sp. was observed at a prevalence of 10.6% (95%CI= ±5.68), *Coxiella*-like endosymbiont at 35.4%, (95%CI= ±8.82), *Rickettsia* spp. at 6.2% (95%CI= ±1.16), and *Brevibacterium* was observed at a prevalence of 1.8%. *Coxiella*-like endosymbiont was observed at a prevalence of 32.4% (95%CI= ±15.08) in *A. exornatum*. Mixed infections of *Coxiella*-like endosymbiont and *Borrelia* sp. were observed at a prevalence of 6.19% (95%CI= ±4.44) in *A. marmoreum*, and 3.33% (95%CI= ±4.52) in *A. sylvaticum.*

Table 7-1. Occurence of bacterial micro-organisms from reptilian *Amblyomma* ticks

Province	Tick species	N	Host (n)	16S rRNA Bacteria	*Rickettsia* spp.		*Borrelia* spp.		*Coxiella* spp.	
					gltA gene	*OmpA gene*	*Fla gene*	16S rRNA	16S rRNA	23S rRNA
KZN	*A. marmoreum*	4	*Varanus albigularis* (1)	-	-	-	-	-	50% (2/4)	-
	A. marmoreum	13	*Kinixys* sp. (1)	-	-	-	46.2%(6/13)	46.2 (6/13)	46.2% (6/13)	30.8% (4/13)
	A. marmoreum	11	*Kinixys zombensis* (1)	-	-	-	18.2% (2/11)	18.2% (2/11)	45.5% (5/11)	45.45% (5/11)
	A. marmoreum	12	*Stigmochelys pardalis* (7)	16.7% (2/12) *Brevibacterium* spp.	-	-	-	-	41.7% (5/12)	16.7% (2/12)
	A. latum	43	*Naja mossambica* (3), *Dendroapis* spp. (4)	4.7% (2/43) *Francisella* spp.	-	-	-	-	-	-
	A. exornatum	37	*Varanus albigularis* (5)	-	-	-	-	-	32.4% (12/37)	24.3% (9/37)
WC	*A. marmoreum*	21	*Kinixys zombesis* (3)	-	-	-	4.8% (1/21)	4.8% (1/21)	53.4% (11/21)	38.1% (8/21)
	A. marmoreum	37	*Stigmochelys. pardalis* (2)	-	-	-	-	-	56.8% (21/37)	40.5% (15/37)
	A. marmoreum	15	*Chersina angulata* (9)	-	46.7% (7/15)	33.3% (5/15)	20% (3/15)	20% (3/15)	53.3% (8/15)	53.3% (8/15)
	A. sylvaticum	60	*Chersina angulata* (9)	1.7% (1/60) *Rickettsia* spp.	20.0% (12/60)	23.3% (14/60)	-	6.7%(4/60)	56.7% (34/60)	23.3% (14/60)

(-) = negative for specific pathogen; KZN = KwaZulu-Natal; WC = Western Cape; n = number of ticks collected. Number of vertebrate hosts collected is indicted in parentheses next to the host's name

Phylogenetic identity of bacterial micro-organisms in Amblyomma *ticks*

According to the BLASTn analysis, the ~478 bp fragment of 16S rRNA for the universal bacterial micro-organisms of *A. latum* yielded 98%–99% with uncultured *Francisella* species (MN998636-MN998638) from France and these sequences further clustered within the monophyletic clade of previously described FLEs rather than with pathogenic *F. tularensis* (Fig. S2). The same gene from *A. sylvaticum* yielded (449/457, 98.25%) with uncultured *Rickettsia spp*. isolated from *Amblyomma helvolum* and *Aponomma varanensis* (KF318168.1 and JQ412124.1) in Thailand, and this sequence was submitted to GenBank as uncultured *Rickettsia* sp. under accession number (OP068356). Furthermore, two sequences from *A. marmoreum* had an identity score of 95.15%–97.22% with *Brevibacterium* spp. (JF905607.1, MK205175.1, & KJ081490.1) (see supplementary files for sequences of *Brevibacterium*).

The 482 bp fragment of the flagellin gene (*Fla*) from *A. marmoreum* yielded an identity of 95% with *Borrelia turcica* (MK604332.1) from Poland, while the 1205bp fragment of the 16S rRNA from both *A. marmoreum* and *A. sylvaticum* yielded an identity of 99.3% with *Borrelia* sp. Tortoise (AB473532.1) and *Borrelia turcica* (CP028884.1). The sequences from this study form a monophyletic group with other sequences of the reptile-associated *Borrelia* group that is distinct but close with relapsing fever borreliae (Fig. 7-1 and 7-2).

On the other hand, the 396bp fragment of the citrate synthase gene (gltA) for *Rickettsia* amplified from *A. marmoreum* and *A. sylvaticum* yielded an identity of 97.9% with spotted fever group *Rickettsia massiliae* (KY640405.1) and *R. rhipicephali* (KX018048.1). However, all the sequences of 631bp fragment of ompA gene for *Rickettsia* amplified from both *A. marmoreum* and *A. sylvaticum* yielded 99.1% with only *R. massiliae* (MH549236.1 and KR401145.1). The phylogenetic analysis of the fragment of the ompA clusters the current study's *Rickettsia* sequences recovered from *Amblyomma* ticks in a monophyletic group with a support value of 75%. With high support (86%), these sequences form a sister clade to *Rickettsia massiliae*; this group in turn clusters within a larger monophyletic clade containing unnamed *Rickettsia* species and *Rickettsia massiliae* (Fig. 7-3). See the amino acid translated phylogenetic tree (Fig. S4) on the supplementary material section of the thesis.

The fragment of the 16S rRNA and 23S rRNA yielded an identity of 99% with the complete genome of *Coxiella*-like endosymbiont (CP064834.1) from *Amblyomma nutalli* available in GenBank. Furthermore, these sequences also yielded an identity of 95.9% with the complete genome of *Coxiella burnetii* strain RSA439 (CP040059.1) and *C. burnetti* gene fragment from

Hyalomma asiaticum (MN880312.1) available in GenBank. The phylogenetic analysis based on the 16S rRNA gene and 23S rRNA showed that *Coxiella*-like endosymbiont from reptilian *Amblyomma* ticks are grouped into an independent and distinct clade, it further clustered in a large monophyletic clade of *Coxiella*-like endosymbionts of *Amblyomma* tick species previously reported (Fig. 7-4 and 7-5).

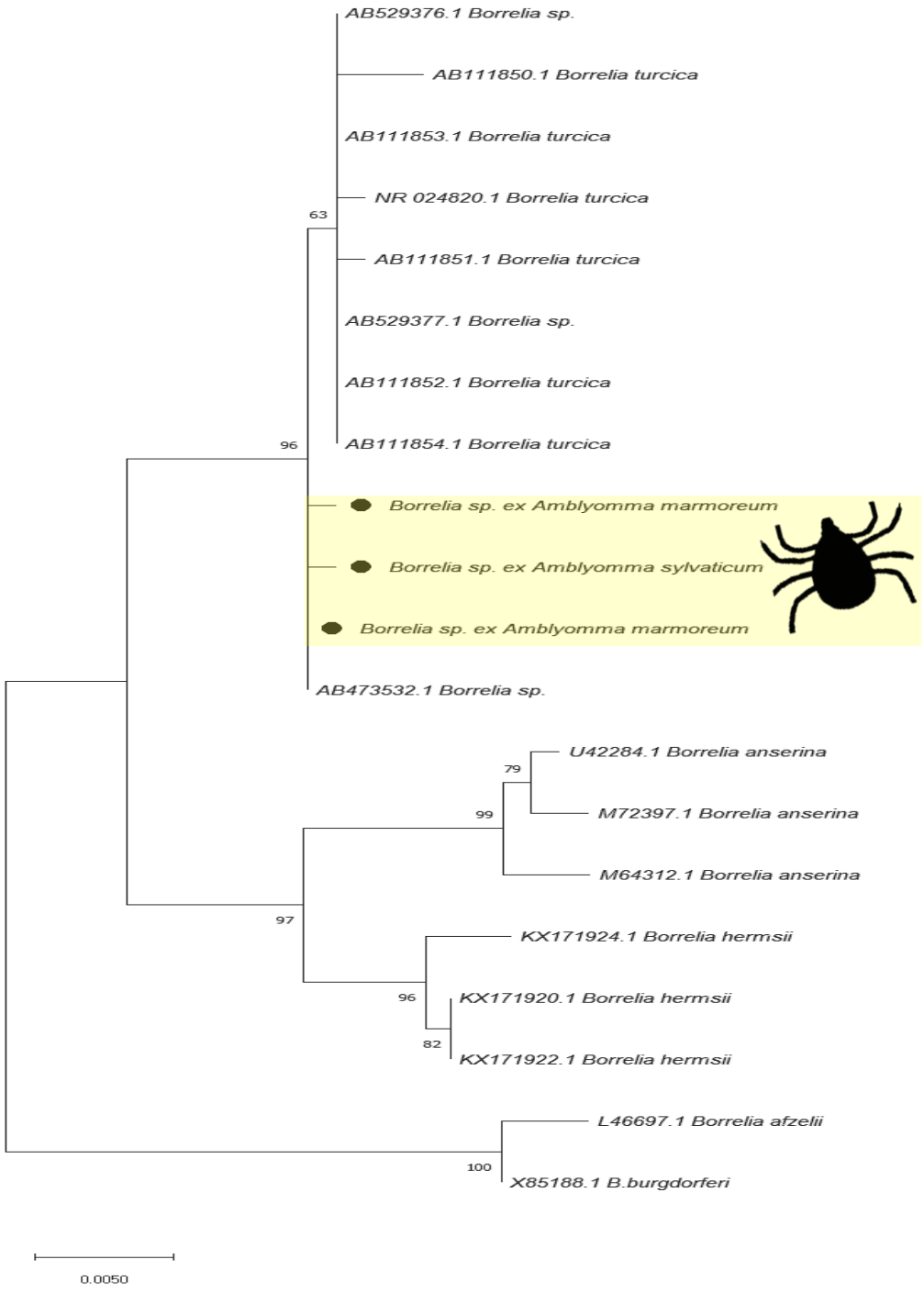


Figure 7-1: Phylogenetic tree based on the 998 bp sequences of the *Borrelia* 16S rRNA gene. The tree was constructed using MEGA 11 based on the maximum likelihood, using the Hasegawa-Kishino-Yano Model + Gamma distribution model. The sequences obtained in this study are indicated with bullets.

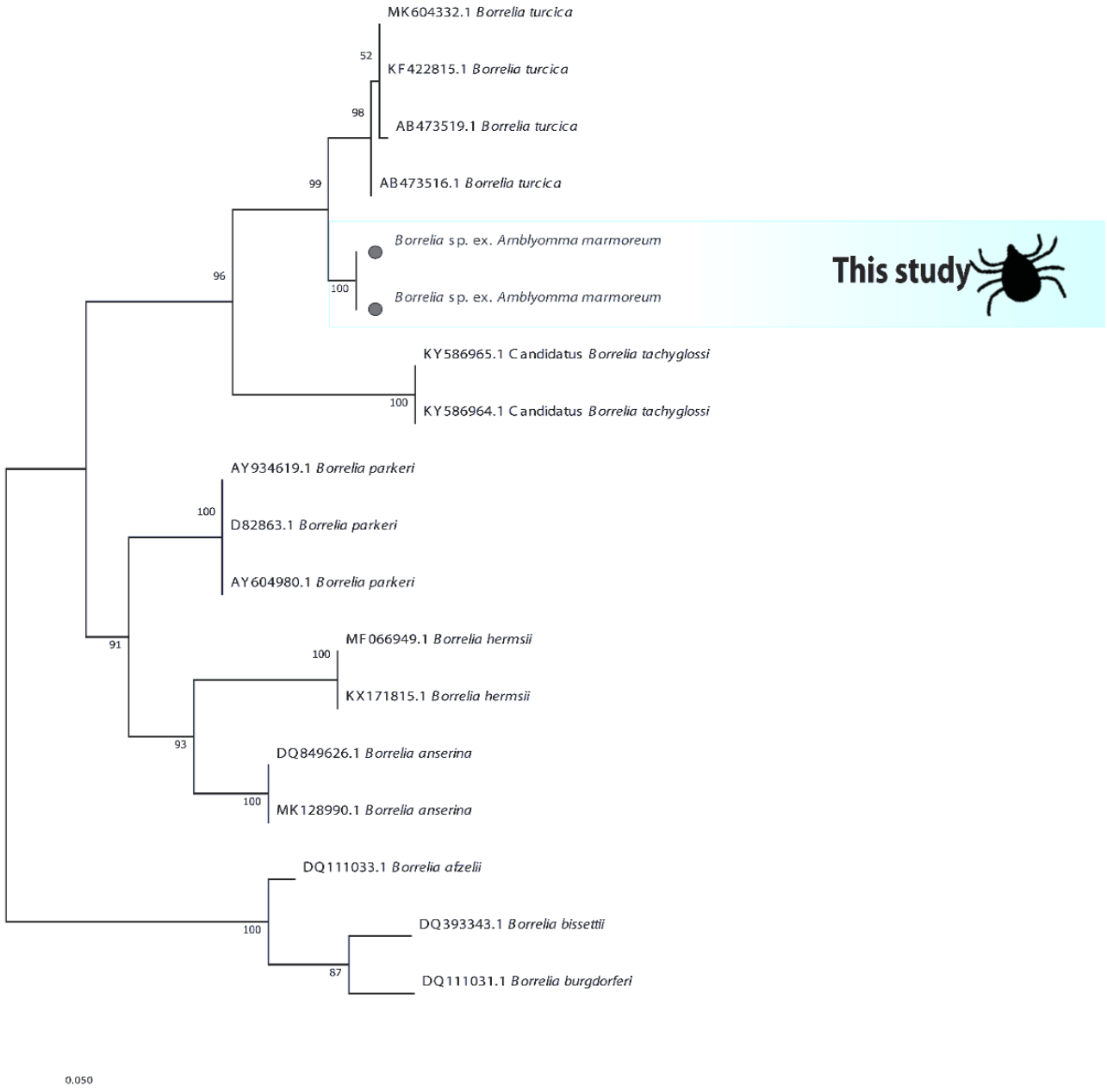


Figure 7-2: Phylogenetic tree based on the 304 bp sequences of the *Borrelia* FLA gene. The tree was constructed using MEGA 11 based on the maximum likelihood, using the Tamura 3-parameter Model. The sequences obtained in this study are indicated with bullets.

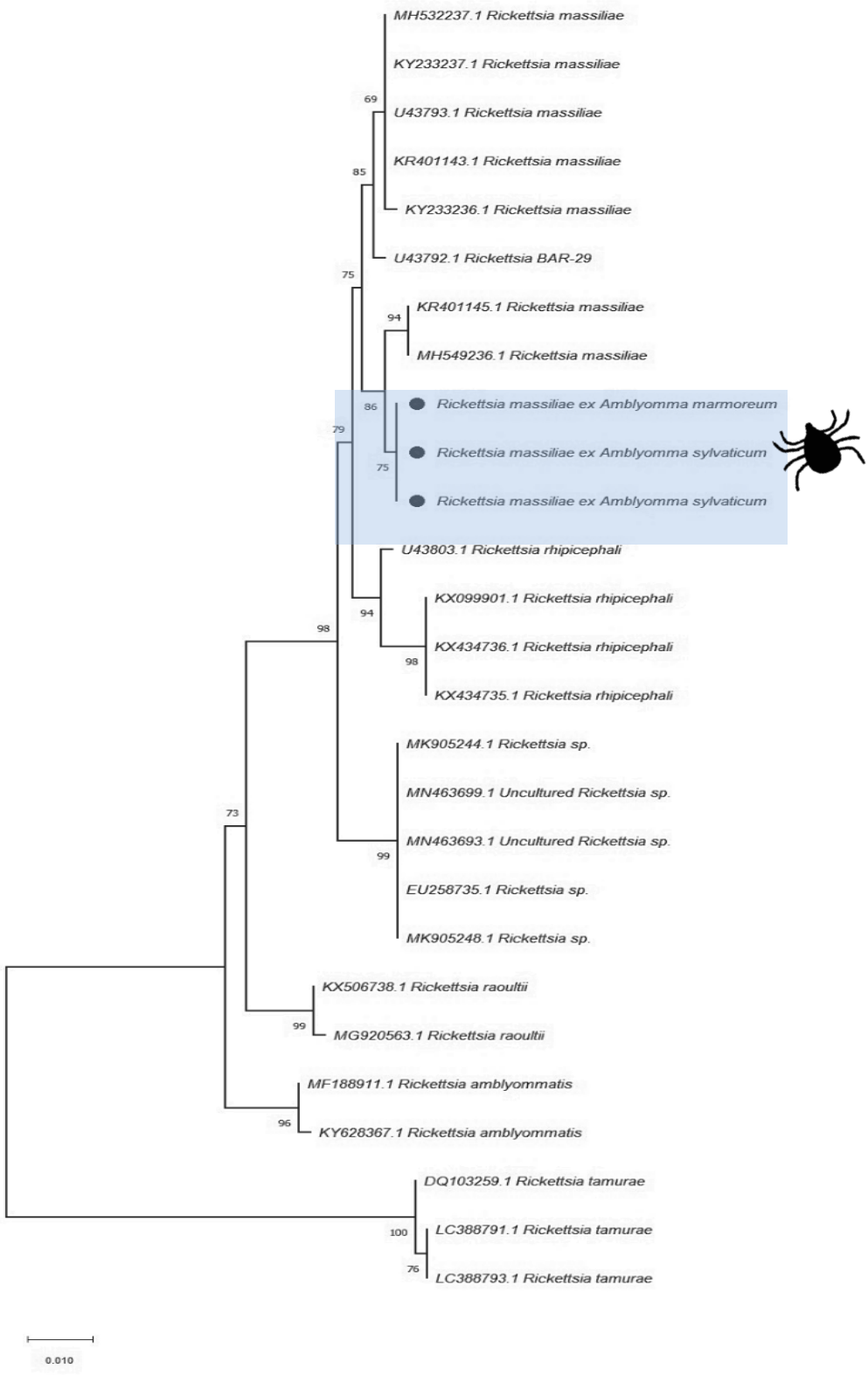


Figure 7-3: Phylogenetic tree based on the 574 bp sequences of the *Rickettsia* OmpA gene. The tree was constructed using MEGA 11 based on the maximum likelihood, using the Tamura 3-parameter Model. The sequences obtained in this study are indicated with bullets.

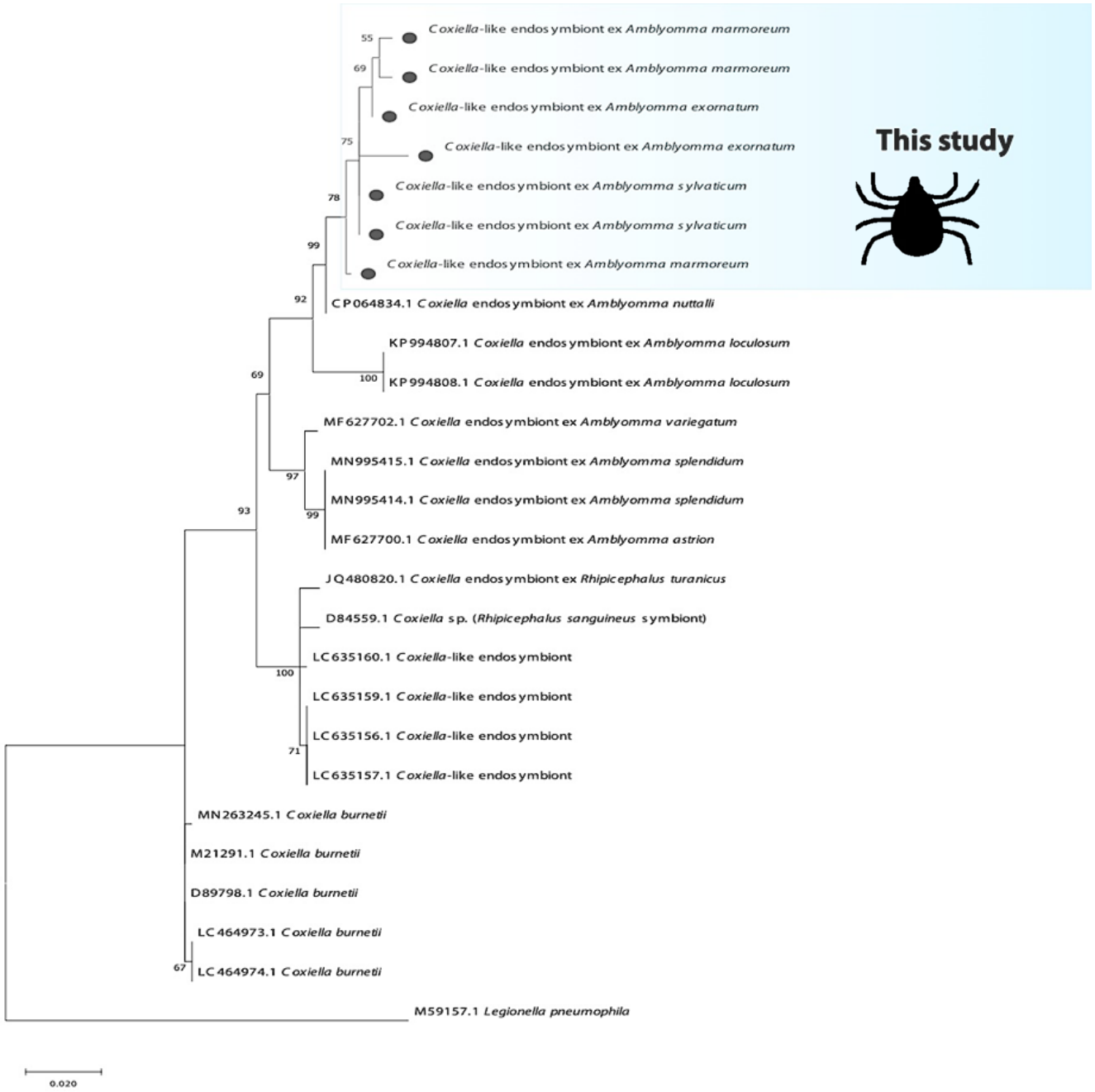


Figure 7-4: Phylogenetic tree based on the 573 bp sequences of the *Coxiella* 16S rRNA gene. The tree was constructed using MEGA 11 based on the maximum likelihood, using the Kimura 2-parameter + Gamma distribution Model. The sequences obtained in this study are indicated with bullets.

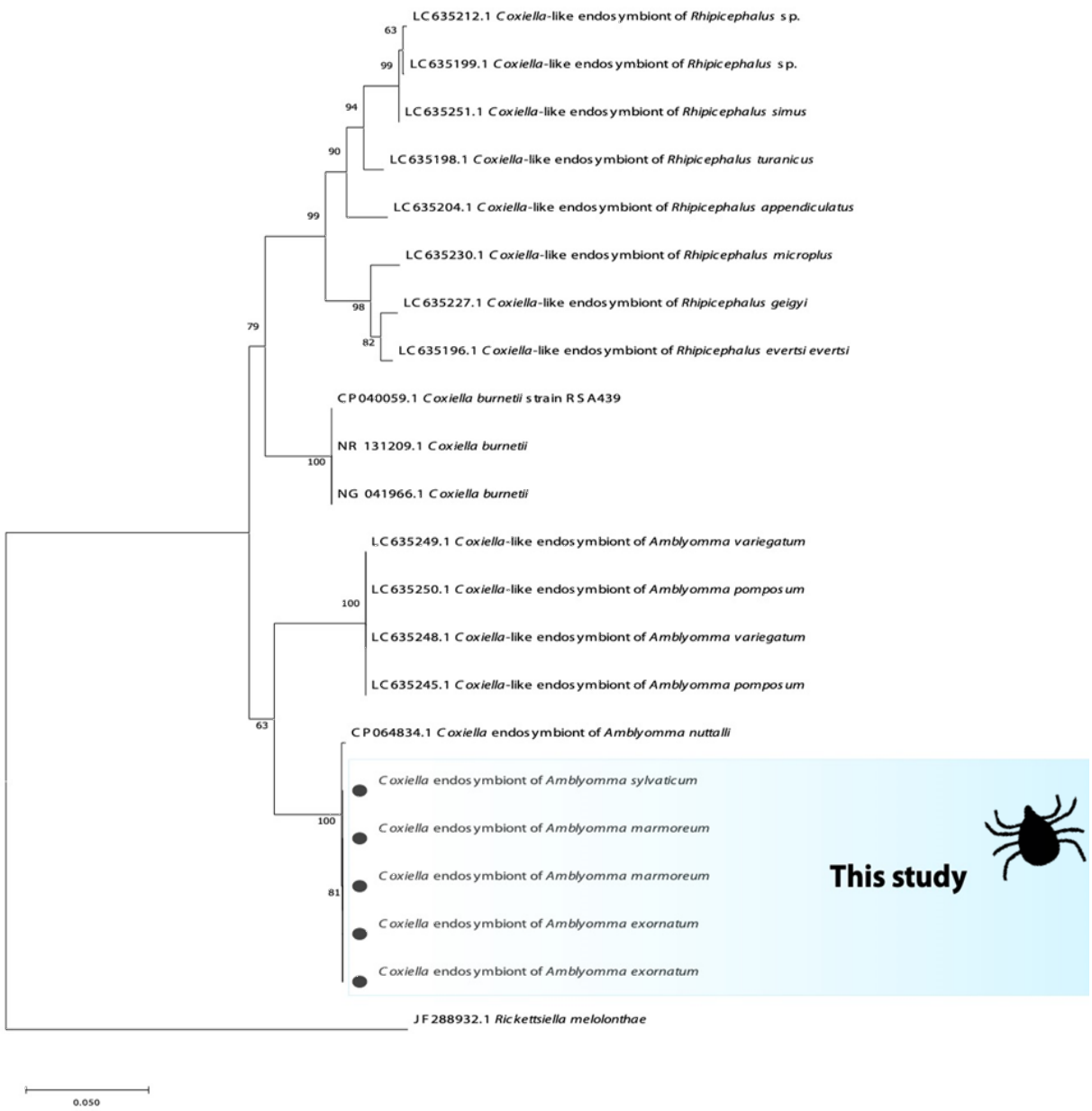


Figure 7-5: Phylogenetic tree based on the 807 bp sequences of the *Coxiella* 23S rRNA gene. The tree was constructed using MEGA 11 based on the maximum likelihood, using the Kimura 2-parameter Model. The sequences obtained in this study are indicated with bullets.

7.4. Discussion

Notwithstanding the fact that tick-borne bacterial microorganisms represent serious emerging and re-emerging health problems to humans and other vertebrates, the surveillance of these pathogens in wildlife has focused mainly on mammals, birds and their ectoparasites, whereas reptiles and their associated ectoparasites remain unexplored (Lane et al. 2006; Sánchez-Montes et al. 2019). Reptiles from Africa have been reported to carry *Borrelia* and *Rickettsia* infected ticks (Andoh et al 2015; Halajian et al. 2016). However, studies focusing on the detection of bacterial microorganisms in these ectothermic hosts, these hosts' ability to act as reservoirs of infection or their ability to be refractory to infection (Lane et al. 2006), and their associated ticks, are only recent and scarce, making their inventory incomplete. This demonstrates the critical need to investigate their potential role in introducing and spreading new tick-borne bacterial diseases in South Africa in order to understand their transmission and zoonotic potential.

Beati et al. (1992) isolated *R. massiliae* for the first time from *Rhipicephalus sanguineus s.l.* collected from canines in France, identifying it as a distinct species within the spotted fever group of rickettsiae. It has been mainly associated with ticks of the genus *Rhipicephalus*. It was then detected from various tick species associated with several vertebrate hosts in Europe, Africa and the Middle East (Parola et al. 2013; Halajian et al. 2016; Blanda et al. 2017; Demir et al. 2020). Even though a human case of *R. massiliae* has not been reported in South Africa, it has been reported in Europe and Argentina, and as such should be considered in patients with tick bites (Vitale et al. 2006; García-García et al. 2010; Cascio et al. 2013). Recently, this *Rickettsia* species was also detected in *Rhipicephalus* ticks infesting domestic small ruminants in Nigeria (Elelu et al 2022). The finding of *R. massiliae* in reptilian ticks from South Africa suggests that human cases of this pathogen can occur. Therefore, ticks associated with reptiles should be considered in the epidemiology of *R. massiliae*, although more studies investigating the potential role of these ticks as vectors and reservoirs of this pathogen are needed. In the current study, *R. massiliae* was detected from *A. sylvaticum* and *A. marmoreum* collected from tortoises in South Africa, and these results are in accordance with a previous report on the presence of *R. massiliae* in *Amblyomma* ticks in this country (Halajian et al. 2016). However, it is unfortunate that the *R. massiliae* detected by Halajian et al. (2016) could not be compared by phylogenetic analysis to *R. massiliae* in the present study, as different genes were amplified between these two studies.

Anaplasma species are tick-transmitted intracellular bacteria that infect a multitude of wild and domestic animals, as well as humans (Han et al. 2019). Although *Anaplasma* species have been documented from ticks and reptiles (Nieto et al. 2009; Kho et al. 2015), they have not been

recorded from African reptiles and their associated ticks, and we did not detect *Anaplasma* species in the current samples.

Coxiella burnetii is the causative agent of Q fever, a zoonotic disease with a global distribution, and it has been reported throughout the African continent in ticks, animal production and humans (Dupont et al. 1995; Vanderburg et al. 2014; Mtshali et al. 2017; Halajian et al. 2018). Ticks may become infected with *C. burnetii* during feeding but are not recognized as the actual vectors of the pathogen (Maurin et al. 1999). However, the current study could not detect *C. burnetii*. Nevertheless, for the first time in South Africa, the present study reports on the occurrence of *Coxiella*-like endosymbiont in reptilian ticks. These endosymbionts have been documented as vitamin provisioning endosymbionts, and it was reported that the biosynthetic pathways for various vitamins are found in all CLE (Gottlieb et al. 2015; Guizzo et al. 2017). Furthermore, Nardi et al. (2021), in their genomic study, have suggested that these CLEs have independently evolved from *C. burnetii*. The prevalence of CLE in the current study was 41.1% among all the screened ticks, and it was detected in three of four *Amblyomma* species identified with different percentages. This was not surprising, as it has been documented that the prevalence of these bacteria varies widely in various species of ticks. This prevalence was in accordance with studies by Arthan et al. (2015) and Rahal et al. (2020), who reported prevalence's of 42.2% and 51.7% in ticks from vegetation and cattle, respectively. Taking this into consideration, the potential of these CLEs to adapt to vertebrate hosts and evolve into pathogenic *Coxiella* cannot be ruled out. Recently, CLE was confirmed to be a new agent of human infections such as neck lymphadenopathy syndrome and scalp eschar through tick bites (Guimard et al. 2017). Furthermore, Shivaprasad et al. (2008) and Vapniarsky et al. (2012) reported avian infections of *Coxiella*-like bacteria even though there was no link to ticks. Therefore, the pathogenicity and zoonotic potential of these *Coxiella*-like bacteria merits further investigation. The negative results for *Coxiella*-like bacteria in *A. latum* may be due to the presence of other symbionts such as *Francisella*-like bacteria, as it was detected in some of the *A. latum* using bacteria specific primers, thus the presence of other endosymbionts in these ticks must not be ruled out.

Although *Anaplasma* spp., and *C. burnetii* were not detected, they cannot be completely disregarded, as their absence could be attributed to the limited number of ticks and localities analysed in the current study. More studies should be done in more localities to assess the impact of these bacterial microorganisms in reptiles and the risk of transmission to domestic animals and humans.

Takano et al. (2010) identified reptile-associated *Borrelia* spp. as a novel group of *Borrelia* spp. from imported reptiles and/or their ectoparasites, although this group have been found to be

associated with species of the order Monotremata (Loh et al. 2017, Panetta et al. 2017; Gofton et al. 2018). The transmission of these species is thought to be through hard-bodied ticks belonging to the genera *Hyalomma* or *Amblyomma*, while they appear to have evolutionarily diverged from an ancestor shared with RF *Borrelia* spp. (Takano et al. 2010). In Africa, these species were only documented from *Amblyomma* ticks. Reptile-associated *Borrelia* isolated from *Amblyomma* ticks clustered with the RF *Borrelia* based on a phylogenetic analysis study conducted by Takano et al. (2011). However, in 2014, based on the analysis of the 16S rRNA gene, Adeolu and Gupta (2014) grouped reptilian *Borrelia* species in a third distinct clade.

In this study, *Borrelia* spp. were detected in two species of *Amblyomma* ticks collected from *C. angulata* and *Kinixys* spp., and it was closely related to the REP borreliae group. This group has been previously detected in reptiles and their ticks imported from Africa into Japan (Takano et al. 2010). These findings showed the presence of REP *Borrelia* in South Africa for the first time. *Amblyomma marmoreum* have been reported to feed on mammals and exported reptiles (Horak et al. 2006), which causes a serious concern on the spreading of pathogens by these *Amblyomma* ticks. Thus far, the impact of REP borreliae in humans and animals in South Africa is unknown. However, more detailed phylogenetic studies of the four groups of the genus *Borrelia* are needed to elucidate the origin of REP borreliae and that in turn may aid in clarifying the taxonomic status of species in this genus (Margos et al. 2020; Morales-Diaz et al. 2020).

Moreover, co-infection of *Coxiella*-like endosymbiont and *Borrelia* sp. was found in *A. marmoreum* and *A. sylvaticum* collected from tortoises. This could be due to co-transmission of the pathogens during blood feeding on the host, or as a result of infection during different feedings (Randolph et al. 1996; Del Cerro et al. 2022).

Recently, many species of reptiles have been and continue to be traded and kept as pets in several countries across the world. As a result, tick-borne pathogens pose potential health threats to humans, associated pets and livestock; therefore, knowledge of the dynamics of the circulation of tick-borne bacteria in the environment is important to assess the risk of infection to both livestock and humans. Although the direct evidence remains to be determined, observations from this study provide clues for the possibility that reptile associated *Amblyomma* ticks can play a role in the maintenance and transmission of pathogenic bacteria to homothermic animals. The accumulation of this epidemiological information from multiple individuals and/or species of reptiles and different stages of ticks in future studies should be informative for this goal. More work will need to be done to determine the prevalence and pathogenic potential of *R. massilae*, REP *Borrelia* and *Coxiella*-like endosymbionts in South Africa. Recognition of the potential of *Rickettsia* species, *Borrelia* species and CLEs in reptiles and their *Amblyomma* ticks will help

further our understanding of the emerging bacterial diseases in the country and the potential threat they pose to a global market in reptiles and their associated products.

Besides the presence of *Rickettsia, Coxiella,* and *Borrelia* spp, an environmental bacterium genus (*Brevibacterium*) was observed from *A*. *marmoreum*. This bacterium has been observed from various other tick species, although ticks have been sterilised prior to DNA extraction (Zhang et al. 2019). The presence of this bacterium may be attributed to inadequate sterilization or that ticks may have ingested it when feeding and therefore present in their midgut (Zolnik et al. 2016; Greay et al. 2018). This study also observed *Francisella*-like endosymbionts from *A. latum* using bacteria-specific primers. Though the role of most bacteria has not yet been clearly elucidated, the essential role of *Francisella*-like endosymbionts have been reported in several tick species in which it may provide essential nutrition for the tick (Panetta et al. 2017; Zhang et al. 2017; Duron et al. 2018).

7.5. Conclusion

The present study demonstrates for the first time the presence of REP *Borrelia* and *Coxiella*-like endosymbionts in *Amblyomma* ticks infesting South African reptiles, and corroborates the presence of *R. massilae* in South Africa. These results should increase the awareness on the emergence of these pathogens in this region, and additional studies should elucidate the role of these ectotherms in the transmission of different tick-borne bacterial microorganisms to domestic animals and humans. Findings from this study should stimulate further wide-scale investigation to determine the role of *Francisella*-like endosymbionts and *Coxiella*-like endosymbionts in tick physiology and vector competence.

SUMMATIVE DISCUSSION

Reptiles are critical in many aspects, occupying both roles as predator and prey in the habitats/biomes that they occupy, as well as being part of human food systems, culture, and as research models, in conservation activities, and as pets (Huey 1982, Böhm et al. 2013). One of the main threats to reptiles is the global trade of these animals, especially the illegal trade (Auliya et al. 2016). As reptiles are host to a wide range of vectors, particularly ticks, which may be responsible for a number of vector-borne parasites such as TBPs and haemoparasites, this trade can have a number of implications, such as the movement of vectors and their associated vector-borne pathogens, some zoonotic, to naïve populations of reptiles, other animals and humans around the globe (Burridge et al 2001; Burridge and Simmons 2003; Karesh et al. 2005). South Africa has a rich biodiversity of reptiles (Bates et al. 2014), however, knowledge on the biodiversity, taxonomy and phylogenetic relationships of their associated ticks, and these parasites' TBPs and other vector-borne pathogens is lacking.

Ticks reported from reptiles in South Africa to date include both soft and hard ticks of different genera (Horak et al. 2006; Cook et al. 2014, 2016; Hornok et al. 2020). However it must be noted that the phylogeny of these ectoparasites as well as the diversity of bacterial microorganisms they may harbour, remains poorly studied (Hornok et al. 2020; Kelava et al. 2021).This is probably due to the fact that most studies are more focused on ticks infesting domestic animals, as well as their associated bacterial microorganisms and the fact that reptiles are more difficult to find and capture. The paucity of studies in these ectotherms and their associated vectors is one of the reasons that these vectors' relevant diversity and evolutionary data remains scarce. This data together with the phylogenetic positions of these vectors and possible life stages of the parasites will provide a better insight on the transmission modes, species identification, and effects of parasites on the hosts. This study aimed to address these problems.

As such, in this thesis the main aim was to identify reptile associated ticks, the prevalence, taxonomy and diversity of blood parasites (haemogregarines and haemosporidians) and tick-borne pathogens (*Ehrlichia, Anaplasma*, *Rickettsia, Coxiella,* and *Borrelia* spp.) in selected reptiles and their ectoparasites (particularly ticks) in South Africa.

Reptile-associated ticks and their potential to host haemoparasites and tick-borne pathogens

In order to better understand the potential of reptiles and their associated ticks to host such parasites and pathogens, one needs to identify the species of tick found infesting reptiles and the

occurrence of haemoparasites and TBPs in reptile and reptile-associated ticks. In this study, three main groups of reptile, namely monitor llizards, snakes and tortoises, formed the focus, as these groups are popular in the pet trade (Burghardt 2017). A total of 71 reptiles (16 monitor lizards, 30 snakes and 25 tortoises), collected from localities in KwaZulu-Natal province (henceforth KZN), were examined for ticks, with a total of 174 ticks collected from these reptiles. An additional 67 archived ticks were also included in the study, having been collected from tortoises in the Western Cape province during 2012.

These ticks were morphologically identified to species level, identifying four species of *Amblyomma* (*A. exornatum*, *A. latum*, *A. marmoreum* and *A. sylvaticum*), these identifications were further supported through the use of molecular methods including PCR, amplifying both nuclear and mitochondrial genetic markers (see chapter 3). Phylogenetic analysis estimated the phylogenetic position of *A. sylvaticum* for the first time, whilst providing more sequences for *A. marmoreum, A. exornatum* and *A. latum*. Furthermore, during the morphological identification of these ticks, abnormalities were observed in *A. latum* (chapter 3). As these anomalies could lead to identification mistakes and taxonomic errors if not reported (Buczek 2000), reporting on these is considered a contribution towards these parasites' taxonomy. Even though the vector efficiency of these abnormal ticks was not clear in this study, we strongly encourage that more studies be undertaken to determine their vector competency.

In this study, the four species of *Amblyomma* were, in large, associated with certain reptile groups, *A. exornatum* from varanid lizards, *A. latum* from snakes, *A. marmoreum* from tortoises and lizards, and *A. sylvaticum* from tortoises (chapter 3). These findings were not surprising based on Horak et al. (2006), and as such do not present any unusual findings. As all three reptile groups are known to be parasitised by apicomplexan haemoparasites, with few to none of the vectors identified (Telford 2009), ticks were molecularly screened for the presence of two groups of these haemoparasites – haemogregarines and haemosporidians. The presence of the haemogregarine genus *Hepatozoon* was found in 14/253 (5.53%) ticks, 1/4 (25%) *A. marmoreum* parasitizing *Varanus albigularis* and 13/173 (7.51%) *A. marmoreum* and *A. sylvaticum* parasitizing tortoises. No *Hepatozoon* was detected from *A. latum* (chapter 3). As this latter tick species is known to primarily infest snakes (Horak et al. 2006), this may have been expected. Even though snakes are known to be infected with *Hepatozoon*, as was found during this study (chapter 5), where the vectors are known or suspected, these in most species of snake *Hepatozoon* are attributed to biting insects such as mosquitoes (Telford, 2009). Furthermore, *Hepatozoon* of snakes is proposed to follow a three host life cycle, where the snake host is infected when ingesting another intermediate host and not directly through the ingestion of the invertebrate vector. As such *A.*

latum may play no role in the development of species of *Hepatozoon* nor the transmission thereof. Finding the presence of *Hepatozoon* in *A. marmoreum*, a tick species commonly associated with tortoises however was more novel/interesting (chapter 3). This was even more so finding that these sequences compared to the tortoise haemogregarine *Hepatozoon fitzsimonsi*, supporting the theory that ticks act as vectors for this species.

The first contribution to studies on phylogeny of *Amblyomma* ticks in this study was determining the phylogenetic position of *A. sylvaticum* for the first time using both nuclear and mitochondrial genetic markers (see chapter 3). More sequences for *A. marmoreum, A. exornatum* and *A. latum* were also provided. Furthermore, based on morphology, abnormalities were observed in *A. latum* (chapter 3), contributing towards this specie's taxonomy. This chapter also provided the first insight on the phylogenetic position of *A. sylvaticum.* At the same time as identifying ticks, ticks were screened molecularly for the presence of haemoparasites including haemogregarines , particularly members of the genus *Hepatozoon*, and haemosporidians. Screening for haemogregarines identified the presence of a tortoise haemoparasite *H. fitzsimonsi* from *A. marmoreum.* As ticks screened in this study were allowed to complete digestion of their blood meals, and taking into consideration the findings of previous studies, in particular that of Cook et al. (2014) that identified sporogonic stages in tortoise associated ticks, further study into whether these ectoparasites have the potential to be vectors of this haemogregarine was required.

Linking developmental stages of H. fitzsimonsi *in tortoises and ticks*

As ticks screened in this study were allowed to complete digestion of their blood meals, and taking into consideration the findings of previous studies, in particular that of Cook et al. (2014) that identified sporogonic stages in tortoise-associated ticks, further study into whether these ectoparasites have the potential to be vectors of this haemogregarine was required (chapter 4). This included the morphological screening of tortoise tissues and ticks collected from tortoises for developmental stages. In this particular part of the study blood smears prepared from blood collected from 25 tortoises was screened for the presence of *H. fitzsimonsi*. However, only a subset of these, comprising only *Stigmochelys pardalis* was used for further research (see chapter 4). This was done as *S. pardalis*, with the exception of one individual in literature (see Cook et al. 2009), does not appear to naturally harbour infections with *Hemolivia parvula* as do species of *Kinixys* from KZN. From these three tortoises (2/3 showing a peripheral blood infection of what appeared to be *H. fitzsimonsi*), three species of ticks were collected *A. hebraeum*, *A. marmoreum* and *A. nuttalli*, from which impression slides were made, these showing what appeared to be sporogonic stages. In one case a *S. pardalis* showed a peripheral blood infection, potential

merogonic stages in the liver tissue, and sporogonic stages in an individual of *A. marmoreum* collected from this same tortoise. Sequence fragments of the 18S rRNA gene from both this tortoise and associated ticks compared to *H. fitzsimonsi* on Genbank. As the chromatograms did not show any evidence of double peaks, this provided further support that the sporogonic stages observed in ticks were that of *H. fitzsimonsi*. What followed was a detailed description of these stages, with sequences supporting the vector potential of three species of ticks for this parasite.

Reptiles and their associated ticks as reservoirs and hosts of tick-borne pathogens

The occurrence of different tick transmitted bacterial microorganisms were observed in *Amblyomma* ticks (chapter 7) through molecular screening, but none were observed using the same methods in reptiles, This was the reptile associated *Borrelia* sp. that was observed for the first time in the country. Only one species, *Borrelia turcica* has been named along with other unknown *Borrelia* species within the reptile-associated *Borellia* group, and reported from the blood and organs of tortoises and hard bodied ticks of genera *Amblyomma* and *Hyalomma* (Takano et al. 2010). The latter authors suggested that tortoises are reservoir hosts of this *Borrelia* group with hard ticks acting as vectors. Even though the *Borrelia* from this study clustered with other reptile-associated *Borrelia* species, culturing of this bacteria as well as the imaging within the vector would be beneficial in naming the species and genotypes of these bacteria circulating in reptilian ticks as well as determining whether or not they all invade the salivary gland of the vector as seen by Takano et al. (2010), as DNA was extracted from whole ticks in the present study. Prior to this study, *Rickettsia* species were also documented from *Amblyomma* ticks infesting tortoises and lizards in South Africa (Halajian et al. 2016; Hornok et al. 2022).

Reptiles as host to haemoparasites

Even though species of *Hepatozoon*, besides *H. fitzsimonsi*, were not molecularly detected in any of the ticks collected from reptiles (chapter 3), blood smears from monitor lizards and snakes showed the presence of these parasites (chapter 5). In both species of monitor lizard a species of *Hepatozoon,* morphologically comparing to *Hepatozoon camari* described from *Varanus niloticus* in Mozambique, was observed. Sequence comparison supported the identification of this haemogregarine as a species of *Hepatozoon*, with this species clustering with other 18S sequences of *Hepatozoon* from other varanids and a snake (*Varanus panoptes* and *Liasis fuscus)*. Though this haemogregarine closely compares morphologically with *H. camari*, was observed in the type host *V. albigularis* and was observed in this host from Ndumo Game

Reserve, a reserve bordering Mozambique, it would be premature to redescribe this study's *Hepatozoon* as that of *H. camari*. Unfortunately, the type locality was not mentioned in the original description by Dias (1954), so until such a time as samples from Mozambique can be acquired, we will refrain from naming this study's species.

Two species of *Hepatozoon* were observed infecting *Naja mossambica* from Ndumo Game Reserve. Phylogenetic analysis of the 18S rDNA sequences of these two species showed these clustering with other sequences of *Hepatozoon* from snakes, but in two distinctly separate clades. The one species (designated *Hepatozoon* sp. A) was found to be most closely related to *Hepatozoon ayorgbor* from a snake (*Python regius*) host from Ghana, whilst the second species (designated *Hepatozoon* sp. B) was most closely related to *Hepatozoon colubri* from a snake (again species) from Iran. As mentioned above *Hepatozoon* species of snakes are thought to follow a three host life cycle (Smith 1996), and as such the diet of snakes likely play an important role in the diversity of *Hepatozoon* species infecting these hosts. Studies have already reported clustering of *Hepatozoon* sequences from snakes with those of either anuran or saurian hosts (Haklová et al. 2014). Given the varied diet of *N. mossambica* that includes rodents, lizards, toads and even grasshoppers (Branch 1998), as well as other snakes and records of cannibalism like many other species within the genus *Naja*, the potential sources of infection are great. Varied diets are observed in many snake species, such as in species of *Psammophis* that are known to prey on various small vertebrates (Bates et al. 2014). As *Hepatozoon* sp. A, clustered with sequences of *Hepatozoon* from *Psammophis* species known to have a saurophagous diet as well as with a sequence from *Hemidactylus alkiyumii* (a gekkonid host), it may suggest that this snake was infected through ingestion of lizard hosts. Regarding *Hepatozoon* sp. B, no inferences can be made at this stage, but the clustering of these two species in distinctly separate clades may suggest a different group of hosts as the source of infection.

Screening of blood smears from tortoise and snake hosts revealed the presence of haemosporidians (chapter 6). Two *Kinixys natalensis* and one *Naja mossmbica* were found parasitised with what appeared morphologically to be species of *Haemoproteus* (*Haemocystidium*), based on the absence of division stages in the peripheral blood, which would have suggested the genus *Plasmodium*. Molecular screening, amplifying the cyt-b gene, further picked up the presence of *Haemoproteus* (*Haemocystidium*) in two species of *Dendroaspis*, which appeared negative during screening of the blood smears. Morphological comparison of the haemosporidian in the tortoises did not compare to *Haemoproteus natalensis* or *Haemoproteus testudinalis* previously described from tortoises in South Africa. Unfortunately, sequences of the haemosporidian in this study could not be compared to these known species as no molecular

work was carried out for these (Cook et al. 2010), but phylogenetic analysis showed clustering of this parasite's sequence with others from Chelonia. The morphological differences seen between this study's species and those of the known *Haemoproteus* (*Haemocystidium*), as well as the lack of further research into these parasites of tortoises in South Africa, suggest the potential for a much higher diversity of these parasites to be present in tortoises in South Africa.

Molecular analysis of the haemosporidian in *N. mossambica* and the two *Dendroaspis* species suggested these to be the same species, sequences clustering with a sequence of *Haemocystidium mesnili* provided in GenBank. However, this specie's sequence does not have any morphological data to compare with, so as to assist with species confirmation. Furthermore, this sequence was amplified from a host that is not the type host for this species and from a locality that is not the type locality (Pineda-Catalan et al. 2013). As such there is doubt that this sequence is that of *H. mesnili*. Recently, Smit (2022) in her dissertation provided morphological and molecular descriptions of this *Haemocystidium* from different African cobras. The finding of this study as well as those of Telford (2009) and Smit (2022) may indicate that this parasite presents different morphological traits in the blood of each host species. Morphological similarities were observed in these studies but the shape and size of this parasite somewhat differed depending on the host species. It is worth noting that to date, this particular *Haemocystidium* from snakes has only been documented in different species of African cobras, and it might be possible that either the parasite or the invertebrate host has a strong host-specificity towards these cobras. Furthermore, as proposed by Javanbakht et al. (2015) the presence of *Haemocystidium* in ectotherms may be influenced by climate and ecology, which may allow the co-distribution of invertebrate and vertebrate hosts. Moreover, the parasite's ability to successfully infect a host might be due to the relative abundance of vertebrate hosts and vectors in the environment and the host's susceptibility to the parasite (Javanbakht et al. 2015).

Future research

The current study provides a baseline for future studies on taxonomy, diversity and phylogenetic relationships of reptilian haemoparasites, ticks, as well as tick-borne bacterial microorganisms. Moreover, this study highlights the importance of continuing to describe new species based on both morphology and molecular characterization, to document their vertebrate hosts as well as their geographical distributions. Comparison of reptilian haemoparasites, tick species and tick-borne microorganisms found in the current study with other areas across South Africa and globally, may provide better insights to the phylogenetic position of some of the *Amblyomma* ticks, the biogeographical distribution of the parasites as well as the co-evolutionary relationships of

these parasites with their vertebrate host. This study sequenced reptilian *Amblyomma* ticks using both mitochondrial and nuclear genes. However, future research should consider sequencing mitochondrial and complete genomes of these ticks in order to gain a better insight into their phylogenetic positions as well as into their evolution. Even though the vector efficiency of these abnormal ticks was not clear in this study, more studies needs to be undertaken to determine their vector competency. Only ticks of genus *Amblyomma* were found on reptiles within the study area and these invertebrates have the potential to be vectors of some haemoparasites and several bacterial species and genera. If these invertebrates could be reared under laboratory conditions, then life cycle as well as the transmission mode could be studied in more detail. For example, even though the developmental stages of *Hepatozoon fitzsimonsi* were observed in both ticks and tortoises in the current study, the life history of this parasite still needs to be elucidated. From the data collected in this study, *Hepatozoon* species of squamate reptiles and the Haemosporidia infecting both tortoises and snakes needs to be described in order to determine their diversity and the possibility of identifying new species. As data of this study was based on a small sample size, future research should involve a larger data set consisting of various reptilian hosts from different habitat types. Furthermore, if possible, it would be beneficial if bacterial species observed from ticks in the current study could be isolated and cultured. This would help in gaining better understanding of the genotypes/ isolates circulating in these invertebrates and their reptilian hosts. Future studies should also focus on virulent genes as well as antimicrobial resistance of these bacteria in South Africa and globally. Therefore, molecular techniques such as high throughput sequencing should be applied in future when screening these ectotherms for tick-borne bacterial microorganisms. The determination of genetic diversity in these bacterial microorganisms may require the use of multiple genes as most of the genes such as 16S rRNA used in this study are highly conservative. Even though the *Borrelia* from this study clustered with other reptile-associated *Borrelia* species, culturing of this bacteria as well as the imaging within the vector would be beneficial in naming the species and genotypes of these bacteria circulating in reptilian ticks as well as determining whether or not they all invade the salivary gland of the vector as seen by Takano et al. (2010), as DNA was extracted from whole ticks in the present study.

Although there is much more research to conduct in this area, we believe the work in this study has made a significant contribution to the studies focusing on the taxonomy and phylogeny of *Amblyomma* ticks; their potential as vectors of haemoparasites as well as bacterial microorganisms of public importance (veterinary and human health). The research covered in this

thesis also contributed to the diversity of haemoparasites in South African reptiles. Some of the main highlights from this thesis are listed below:

Main highlights of the study

- Determined the phylogenetic position of *A. sylvaticum* for the first time using both nuclear and mitochondrial genetic markers.
- DNA of *H. fitzsimonsi* was detected from *Amblyomma* ticks infesting tortoise and these findings provide further support for ticks as vectors of *H. fitzsimonsi* whilst considering the validity of *Bartazoon.*
- Developmental life stages of the haemogregarine, *H. fitzsimonsi* were observed from ticks and tortoises.
- Two morphologically and molecularly different species of *Hepatozoon* were observed in *Naja mossambica* as well as a *Hepatozoon* sp. resembling *H. camarai* in two *Varanus* spp.
- A *Haemocystidium* (*Haemoproteus*) morphologically incomparable to *Haemocystidium* observed from African terrestrial tortoises, as well as *Haemocystidium* comparable to *Haemocystidium mesnili* in South African snakes was identified and described.
- First detection of reptile-associated *Borrelia* and *Francisella*-like endosymbiont in South Africa and the detection relapsing fever in reptile associated ticks.

Conclusion

In conclusion, the overall aim of this study was to assess the diversity of haemoparasites, tick-borne bacterial microorganisms and tick infestation on reptiles (snakes, monitor lizards, tortoises) of South Africa. Lack of knowledge and information on the diversity of these ectoparasites, their associated bacteria, and haemoparasites is one of the limiting factors in understanding the biology of these parasites and this knowledges is very important when one is trying to gain information on their evolution and ecology. Data regarding the presence, genetic diversity and phylogenetic position of *Amblyomma* ticks, haemoparasites and tick-borne pathogens infecting reptiles is underestimated in South Africa and across the world. To date, the South African diversity of reptilian *Hepatozoon* and Haemosporidia parasite species comprises nine named and two unnamed *Hepatozoon* species (Laveran 1905; Sambon et al. 1907; Fantham 1925; van As et al. 2013, 2015, Cook et al. 2009, 2014,2016,2018; Harris et al. 2018) and nine species of Haemosporidia (Fantham and Porter 1950; Cook et al. 2010, van As et al. 2016). Together with the results of the current study that found four species of *Hepatozoon* and two species of Haemosporidia, as well as a peripheral blood stage of *Karyolysus*, these results indicate that

South Africa is not only rich in reptiles but also in haemoparasites infecting these ectotherms. The results of this study provide a foundation for future research on taxonomy and ecology of these haemoparasites in South Africa and Africa as a whole. Regarding the tick-associated microorganism in ticks infesting these reptiles, two reports of *Rickettsia* sp. from *Amblyomma* ticks infesting South African reptiles were documented by Halajian et al. (2016) and Hornok et al. (2022). The results of this study found the presence of reptile-associated *Borrelia*, *Rickettsia* sp., *Coxiella*-like endosymbionts and *Francisella*-like endosymbiont together with the findings of Takano et al. (2010) who found *Borrelia* in imported ticks from Africa into Japan, as well as the findings of Halajian et al. (2016) and Hornok et al. (2022) who found *Rickettsia* spp. in *Amblyomma* ticks of reptiles from South Africa indicates that these ecoparasites infesting reptiles harbour a rich diversity of bacteria that may pose a threat to human health as well as threatened species of reptiles.

Molecular techniques have proven to be advantageous in determining haemoparasite species identity and detecting the parasites with low parasitemia, however this was based only on 18S rRNA and a single set of primers for haemogragrines, which made it difficult to detect co-infection of different genera or mixed-infections, if any, of a single genus.

One of the hypotheses of this study was that South African reptiles are host to a diversity of blood parasites and tick-borne pathogens, acting as reservoir hosts for the latter infection. The results of this study indicated that reptiles are not hosts for tick-borne bacteria, which did not support the second part of the hypothesis. Although none of the reptiles were infected with tick-borne bacteria, data obtained from the current study indicated that ticks associated with these ectotherms harbour various tick-borne bacteria of zoonotic importance as well as some haemoparasites. Both reptiles and their associated ticks were found to be infected with different haemoparasites, which suggests that both these ectotherms and their vectors are host to a diversity of haemoparasites and this supported the first part of the hypothesis. The second hypothesis of this study was that there is a great genetic diversity among tick-borne pathogens harboured by reptiles in South Africa, and many of these tick-borne pathogens are important agents of zoonotic disease. This hypothesis was achieved when this study documented the occurrence of various tick-borne bacterial microorganisms such as Relapsing fever *Rickettsia* and *Coxiella*-like endosymbionts in ticks associated reptiles.

This study is yet another example of the urgent need of discovering and identifying parasitic infections in reptiles as well as well as their associated vectors, given their zoonotic potential and their important role in conservation, especially in both naïve and endangered species.

Furthermore, it highlights the importance of updating the taxonomy of *Amblyomma* ticks, haemosporidians, haemogregarines, and tick-borne bacterial microorganisms as well as to continue research on vector-borne and zoonotic pathogens in reptiles of South Africa. This study reports the presence of tick-borne pathogens that are causative agents of rickettsiosis, borreliosis and coxielliosis of importance to livestock and human health in the study area, and it further demonstrates that reptiles are often infected with different haemoparasites that may be of importance in the biodiversity of these ectotherms. The current study thus sets baseline data helpful for the identification of parasite host relationships that are important in transmitting diseases of domestic animals, wildlife, and humans.

The diagnosis and efforts to obtain a clear taxonomic picture of blood parasites (haemogregarines and haemosporidians) were made more difficult by co-infections as well as possible mixed-infections in the current study. Furthermore, except for the roadkill individual in Chapter 4, tissue samples were not available in the present study, and the limited/small sample size was one of the problems in the study. This emphasises the fact that future research will only be further strengthened once it is a collaborative effort with a variety of different stakeholders, in particular local ecologists and herpetologists, as well as community groups and local landowners.

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SUPLEMENTARY FILES

Table S1. list of *Borrelia, Coxiella*, and *Rickettsia* species associated with African reptiles and their ticks

Disease	*Rickettsia sp*.	Tick species	Host	Country	Reference
Rickettsiosis	*R. aeschlimannii*	*Hyalomma. aegyptium*	*Tesdudo graeca*	Algeria	Bitam et al. 2009
African tickbite fever	*R. africae*	*Amblyomma chabaudi*	*Astrochelys radiata*	Madagascar	Ehlers et al. 2016
Unknown	*R. hoogstraalii*	*Amblyomma transverale*	*Python regius*	Ghana	Andoh et al. 2015
Unknown tick-borne lymphadenopathy	*R. raoultii*	*Amblyomma sparsum*	*Stigmochelys pardalis*	Zambia	Andoh et al. 2015
Unknown	*Rickettsia* sp. related with *R. tumarae/R. monacensis*	*Amblyomma latum*	*Phelsuma dubia*	Madagascar	Andoh et al. 2015
	Rickettsia massilae	*Amblyomma sylvaticum*	*Chersina angulata*	South Africa	Halajian et al. 2016; Mofokeng et al. 2022
	Rickettsia massilae	*Amblyomma marmoreum*	*Chersina angulata*	South Africa	Mofokeng et al. 2022
	Borrelia sp.	*Amblyomma latum*	*P. regius*	Ghana	Takano et al. 2010
	Borrelia sp.	*Amblyomma transversale*	*S. pardalis*	Zambia	Takano et al. 2010
	Borrelia sp	*Amblyomma sparsum*	*P. regius*	Zambia	Takano et al. 2010
	Borrelia sp	*Amblyomma marmoreum*	*Kinixys zombesis & Chersina angulata*	South Africa	Mofokeng et al. 2022
Unknown	*Coxiella-like endosymbiont*	*Amblyomma marmoreum, Amblyomma sylvaticum, Amblyomma exornatum*	*Varanus albigularis; Kinixys zombesis, Chersina angulata,*	South Africa	Mofokeng et al 2022

Table S2. Characteristics of *Ehrlichia* species

Characteristic	***E. canis***	***E. ruminantum***	***E. ewingii***	***E. muris***	***E. chaffeensis***
Geographical distribution	Worldwide	Africa, Caribbean	North America	Japan	North America
Tissue tropism	Monocytes/ Macrophages	Endothelial cells/ Neutrophils	Neutrophils	Monocytes/ macrophages	Monocytes/ macrophages
In-vitro cultivation	Tick cells	Tick cells/endothelial cells/neutrophils	[c]	Macrophages	Tick cells
rologic reactions with[b]					
E. ruminantium	++		++	?	++
E. canis					
E. ewingii	+++				+++
E. muris	+++		+++		+++
E. chaffeensis	+++				

[a]Symbols: ± rarely present or difficult to identify, + present infrequently, + + present moderately often. [b]Symbols: – none, + weak, + + moderate, + + + strong? unknown or not reported. [c]Not cultivated in vitro. Adapted from Garrity et al. 2000, with modifications

Table S3. Characteristics of *Anaplasma* species

Characteristic	*A. bovis*	*A. marginale*	*A. phagocytophilum*	*A. platys*
Geographical disrtibution	Africa, Asia, North America?	Worldwide	Europe, Asia, North America, South America	North America, Europe, Taiwan
Tissue tropism	Monocytes	Erythrocytes	Neutrophils	Platelets
In vitro cultivation	-	Tick cells, erythrocytes, endothelial cells	Granulocyte cell lines, tick cells, endothelial cells	-
In peripheral blood[a]	±	++	++	+
Serologic reactions with[b]				
A. bovis	+++	-	-	?
A. marginale	?	+++	-	?
A. phagocytophilum	?	-	+++	-
A. platys	?	?	-	+++

[a]Symbols: ± rarely present or difficult to identify, + present infrequently, + + present moderately often. [b]Symbols: – none, + weak, + + moderate, + + + strong,? unknown or not reported. [c]Not cultivated in vitro. Adapted from Garrity et al. 2000, with modifications

Table S4. Classification of haemosporidians with their known vertebrate and invertebrate hosts. The number of described species as in Martinsen et al. (2008) are indicated in brackets. Adapted from Perkins (2014).

Classification	Vertebrate host	Vectors
Family Haemoproteidae		
Genus *Haemocystidium*		
Subgenus *Haemocystidium*	Squamates	?
Subgenus *Simondia*	Chelonians	Tabanids
Genus *Haemoproteus* (202)		
Subgenus *Haemoproteus*	Birds	Hippoboscids
Subgenus *Parahaemoproteus*	Birds	Culicoides
Family Leucocytozoidae		
Genus *Leucocytozoon* (91)		
Subgenus *Leucocytozoon*	Birds	Simulids
Subgenus *Akiba*	Birds	Culicoides
Family Garniidae		
Genus *Fallisia*		
Subgenus *Fallisia*	Lizards	?
Subgenus *Plasmodoides*	Birds	?
Genus *Garnia*	Lizards	?
Genus *Progarnia*	Crocodilians	?
Family Plasmodiidae		
Genus *Plasmodium* (199)		
Subgenus *Plasmodium*	Primates	Anophelines
Subgenus *Laverania*	Apes	Anophelines
Subgenus *Vinckeia*	Rodents	Anophelines
Subgenus *Bennettinia*	Birds	*Culex*
Subgenus *Giovannolaia*	Birds	Culicidae
Subgenus *Haemamoeba*	Birds	Culicidae
Subgenus *Huffia*	Birds	*Culex*
Subgenus *Novyella*	Birds	*Culex*, Culiseta
Subgenus *Asiamoeba*	Lizards	?
Subgenus *Carinamoeba*	Lizards	?
Subgenus *Lacertamoeba*	Lizards	Culicidae
Subgenus *Ophidiella*	Snakes	?
Subgenus *Paraplasmodium*	Lizards	?
Subgenus *Sauramoeba*	Lizards	?
Genus *Hepatocystis* (25)	Bats, Primates, Rodents, Ungulates	Culicoides
Genus *Polychromophilus*	Bats	?
Genus *Bioccala*	Bats	?
Genus *Bigueteilla*	Bats	?
Genus *Dionisia*	Bats	?
Genus *Saurocytozoon*	Lizards	?
Genus *Mesnilium*	Fish	?
Genus *Nycteria*	Bats	?

Table S5. *Hepatozoon* Miller 1908 described from Elapid snakes and Varanid lizards from Africa.

Parasite species	Snake host (Elapidae); Monitor lizards (Varanidae)	Localty	Description (Gamonts; nucleus)	Reference
***Hepatozoon* species A**	*Naja mossambica*	South Africa	11.5-15.4 × 3.7-5.9; 3.4-5.2 × 1.9-4.0	Present study
***Hepatozoon* species B**	*Naja mossambica*	South Africa	15.15-20.11 ×2.39-5.26; -	Present study
**Haemogregarina answanensis* (Mohammad et al. 1996)	*Naja haje*	Type: Egypt	12.5−17.5 × 2.5−6; 3.7 × 2.6	Mohammad et al. 1996
Hepatozoon enswerae (Hoare, 1932)	*Naja melanoleuca*	Type: Uganda	19 × 3, slender vermicular 15 × 3.8, bean-shaped; -	Hoare 1932, Smith 1996, Sloboda et al. 2007
Hepatozoon najae (Laveran, 1902)	*Naja naja*[1] , *Naja nigricollis*[2,4], *Naja haje*[3]	Type: India[1] Other: Egypt[2], Kenya[3], Tanzania[4]	14 × 3 (folded), 21−22 × 3 (when not folded1); -	Laveran 1902[1], Ball 1967, Bashtar and Abdel-Ghaffar 1987[2] , Smith 1996, Telford 2009[4]
Hepatozoon zumpti (Dias, 1952)	*Dendroaspis polylepis*	Type: Mozambique	14.3−16.3 × 4.3−5.3; 4.8−5.8 × 3.8−4.5	Dias 1952; Smith 1996
Hepatozoon species	*Varanus albigularis; V. niloticus*	South Africa		Present study
Hepatozoon borreli (Nicolle & Comte,1906) Smith, 1996b	*Varanus griseus*	Type:Tunisia	7–8 × 2/1–3 × 1–2	Nicolle & Comte 1906; Smith 1996

Hepatozoon camarai (Dias,1954), Smith, 1996b	*Varanus albigularis albigularis*	Type: Mozambique	2 forms observed: banana-shaped: 11.75 × 5.00; long curved: 14.25–18.25 × 1.25–4.25/nucleus irregular	Dias 1954; Smith 1996
**Haemogregarina roshdyi* Ramadan et al. 1996	*Varanus griseus*	Type: Egypt	13–20 × 1.5–2.5/6.0–8.5 × 1.5–2.5	Ramadan et al. 1996
Hepatozoon varani *(Laveran, 1905) Smith, 1996b*	Type: *Varanus niloticus* *Other: V. niloticus, V. griseus V. arenarius,*	Type: South Africa Other: Senegal, Portuguese Guinea, Gambia, French West Africa, French Sudan, Kenya	14 × 3/not given; 11–15 × 3/not given; 12.0 × 13/3.5–4.5 × 3.03; 10.3 × 2.5/not given; two forms: 12–14 × 2/not given, 10–12 × 4–5/ 5–6 × 4–55; 12.7 × 4.6/not given	Laveran 1905; Laveran and Pettit 1909; França 1911; Tood and Wolbach 1912; Wolbach 1914; Leger and Leger 1948; Rousselot 1943; Rousselot 1953; Ball 1967; Smith, 1996

*Likely a *Hepatozoon* species

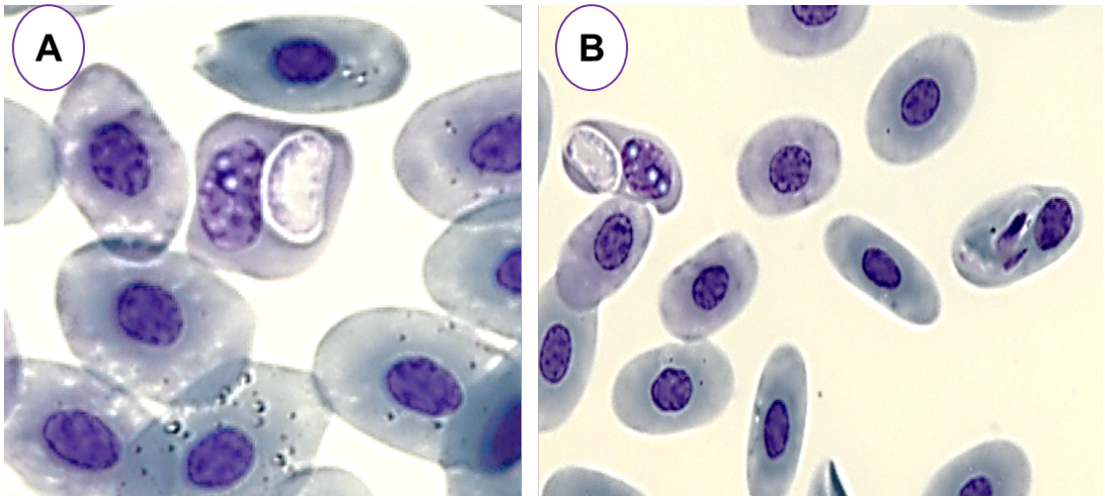

Figure S1: Co-infection of *Karyolysus* and *Hepatozoon* species in varanid lizard *Varanus niloticus*. Note the shrinkage of the host cell and vacuolated nucleus in *Karyolysus* infected cell (A-B).

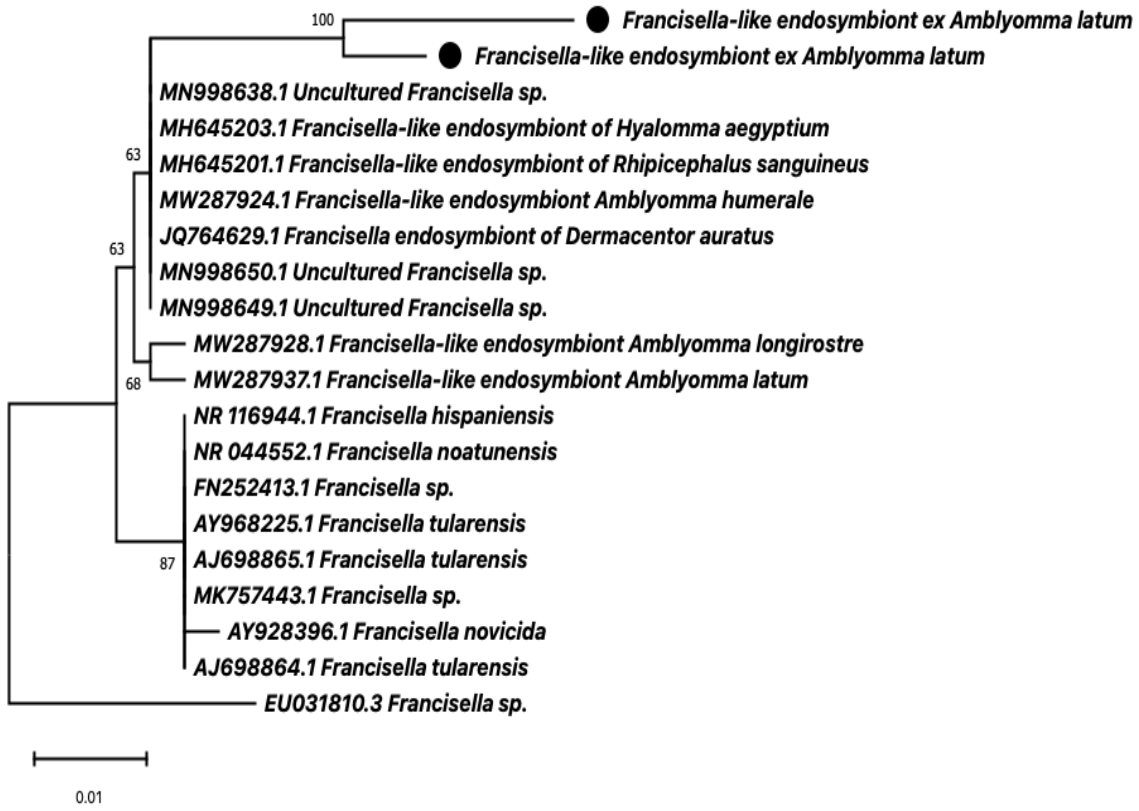

Figure S2: Phylogenetic tree based on the sequences of the *Francisella* 16S rRNA gene. The tree was constructed using MEGA 11 based on the maximum likelihood method, using the Jukes-Cantor Model. The sequences obtained in this study are indicated with bullets.

***Brevibacterium* sequences isolated from *Amblyomma marmoreum* in the present study**

>Seq1 [*Brevibacterium* spp. ex *Amblyomma marmoreum*]

GAAGTGGATCGGTGTCTTCCGTGAGTCGAACGCTGAAGCACTGTGCTTGCTCGGTGTGGA
TGAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTGACTTCGGGATAAGCCTG
GGAAACTGGGTCTAATACCGGATATGACTACTGGCCGCATGGTCTGGTGGTGGAAAGGGT
TTTACTGGTTGGGGATGGACTCGCGGCCTATCAGTTTGTTGGGGGGGTAGTGGCCTACCA
AGACGACGACGGGTAGCCGGCCTGAAAGGGCGACCGGCCACACTGGGACTGAAACACAG
CCCATACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGGAACCCTGATGC
AGCCACTCCGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTCAGTAGGGAAGAA
GCGAAAGTGACGGTACCTACGAAAGAAGTCCCGGCAAAGTCA

>Seq2 [*Brevibacterium* spp. ex *Amblyomma marmoreum*]

GAAAAACCTGCGGGACACACAACAGTCGAACGCTGAAGCCCTAGCTTGCTGGGGTGGATG
AGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTGACTTCGGGATAAGCCTGGG
AAACTGGGTCTAATACCGGATACGACCTGTCCTCGCATGAGGGTGGGTGGAAAGTTTTTTC
GGTTGGGGATGGGTTCGCGGCCTATCAGTTTGTTGGGGAGGTAATGGCTCACCAAAACAA
CAACGGGTAGCCGGCCTGAAAGGGCGACCGGCCACACTGGGACTGAAACACGGCCCAAA
CTCCTACGGGAGGCAGCAGGGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGA
CCCAGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTCAGCAGGGAAAAAGCCTT
CGGGTGACGGTACCTACAAAAAAAGTCCCGGCAAACTCA.

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Molecular screening of ticks of the genus *Amblyomma* (Acari: Ixodidae) infesting South African reptiles with comments on their potential to act as vectors for *Hepatozoon fitzsimonsi* (Dias, 1953) (Adeleorina: Hepatozoidae)

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ABSTRACT

In South Africa, the role of reptilian ticks in the transmission of haemoparasites is lacking, in part, due to limited information on tick diversity and their associated haemoparasites. The aim of this research was to identify tick species parasitizing reptiles and to molecularly screen these ectoparasites for species of the blood apicomplexan genus *Hepatozoon*. Samples were collected from Ndumo Game Reserve, KwaZulu-Natal, and the Cape Columbine region, Western Cape. Reptiles collected included 2 snakes, 2 monitor lizards of a single species respectively, as well as 17 tortoises of four species. Ticks collected from these were morphologically identified as *Amblyomma latum* (n = 2) and *Amblyomma marmoreum* (n = 98), this identification was molecularly confirmed using 16S rRNA and CO1 genes. Screening for *Hepatozoon* was done by amplifying the 18S rRNA gene. A species of *Hepatozoon*, *Hepatozoon fitzsimonsi*, was identified from *A. marmoreum* ticks, with an overall prevalence of 10%. This *Hepatozoon* species, has been described parasitizing tortoises from southern Africa, and has been reported from ticks infesting tortoises from Kenya, East Africa. Even though ticks have been suggested to be the likely vector of this *Hepatozoon* species, with this supported by the findings of *Hepatozoon*-like developmental stages in ticks collected off of infected tortoises, a recent systematic revision placed this species in a newly erected genus *Bartazoon*, a genus vectorised by biting insects. The present study thus provides further support for ticks acting as the potential vectors of *H. fitzsimonsi*.

1. Introduction

Members of family Ixodidae Dugès, 1834 are divided into two groups (Prostriata and Metastriata) that are distinctive in their morphology, physiology, phylogenetic and other characteristics (Hornok et al., 2020). They are the most significant vectors of emerging zoonotic pathogens, which can be severe and life-threatening to humans; second to mosquitoes in the number of human diseases they transmit (Kho et al., 2015; Luz et al., 2018). A number of tick species have formed a close relationship with their vertebrate hosts, with a marked preference for reptiles and amphibians. According to studies of valid names, *Amblyomma* Koch, 1844 was documented as the second largest group of Ixodidae ticks with 130 valid species distributed worldwide, and nine of these species, including three species that belonged to the former genus *Aponomma* Neumann, 1899, have been reported in South Africa (Walker, 1991; Barker and Murrell, 2004; Horak et al., 2006; Guglielmone et al., 2014).

Species of *Hepatozoon* Miller, 1908 are frequently observed as intraerythrocytic or intraleucocytic haemoparasites of reptiles (Smith, 1996; Telford, 2009). These adeleid haemogregarines display a heteroxenous life cycle requiring a vertebrate intermediate host and an invertebrate vector or definitive host (Smith, 1996; Telford, 2009; Karadjian et al., 2015). The systematics and diversity of species of apicomplexans such as *Hepatozoon* are still poorly understood, the genus *Hepatozoon* itself being paraphyletic based on estimated relationships using sequences of the 18S rRNA gene (see Barta et al., 2012, Haklová-Kočíková et al., 2014; Kvičerová et al., 2014, Cook et al., 2016). In an attempt to aid in the resolution of this, a new genus *Bartazoon* Karadjian et al., 2015 was erected, this genus included all species of reptiles, previously assigned to *Hepatozoon*. One of the characteristics of the genus *Bartazoon* is that the vectors of all members are biting insects, whilst species of *Hepatozoon* have ticks and mites as vectors (Karadjian et al., 2015). To date, the genus *Bartazoon* has not been widely accepted as the monophyly of this genus is not well supported (Maia et al., 2016;

microorganisms

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Molecular Detection of Tick-Borne Bacteria from *Amblyomma* (Acari: Ixodidae) Ticks Collected from Reptiles in South Africa

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Abstract: Reptiles are hosts for various tick species and tick-associated organisms, many of which are zoonotic. However, little is known about the presence and diversity of tick-borne bacteria infecting reptiles and their ticks in South Africa. *Amblyomma* ticks (n = 253) collected from reptiles were screened for the presence of *Coxiella*, *Anaplasma*, *Rickettsia*, and *Borrelia* species by amplification, sequencing and phylogenetic analysis of the 16S rRNA, 23S rRNA, *gltA*, *OmpA*, and *Flagellin* genes, respectively. This study recorded the presence of reptile associated *Borrelia* species and *Coxiella*-like endosymbiont in South Africa for the first time. Furthermore, a spotted fever group *Rickettsia* species was observed in 7 *Amblyomma marmoreum* and 14 *Amblyomma sylvaticum* from tortoises of genera *Kinixys* and *Chersina*. *Francisella*-like endosymbiont was observed from 2 *Amblyomma latum* collected from the Mozambique spitting cobra, *Naja mossambica*. *Coxiella burnetii* and *Anaplasma* spp., were not detected from the current samples. Although the direct evidence that reptiles can act as reservoir hosts remains to be determined, observations from this study provide indications that reptilian ticks may play a role in the transmission of pathogenic bacteria to homothermic animals. Furthermore, the absence of *Anaplasma* spp., and *C. burnetii* does not mean that these pathogens should be completely neglected.

Keywords: *Amblyomma*; endosymbionts; reptiles; South Africa; tick-borne pathogens

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1. Introduction

Tick-borne pathogens (TBPs) are responsible for some of the most severe emerging infectious diseases across the world [1,2]. The wildlife-domestic-human interaction has the potential to accelerate the spread of zoonotic tick-borne pathogens that cause significant diseases and eventually death [2,3]. To date, roughly 61% of human diseases have been documented to have a zoonotic origin, and wildlife is associated with approximately 75% of emerging zoonotic infections globally [4]. In South Africa, four tick-borne spotted fever groups (SFG) *Rickettsia* spp. (*Rickettsia africae*, *Rickettsia conorii conorii*, *Rickettsia aeschlimannii* and *Rickettsia sibirica mongolitimonae*) have been associated with human diseases [5–7]. Additionally, members of the family Ixodidae are involved in the epidemiology of other pathogens such as *Borrelia*, *Anaplasma* and *Coxiella* spp. An increasing number of studies have implicated reptiles and their associated ticks as potential reservoirs involved in the transmission cycles of TBPs [2,8,9]. This fact is of great concern in tick-borne disease control strategies, as these ectotherms are usually asymptomatic carriers that may serve as

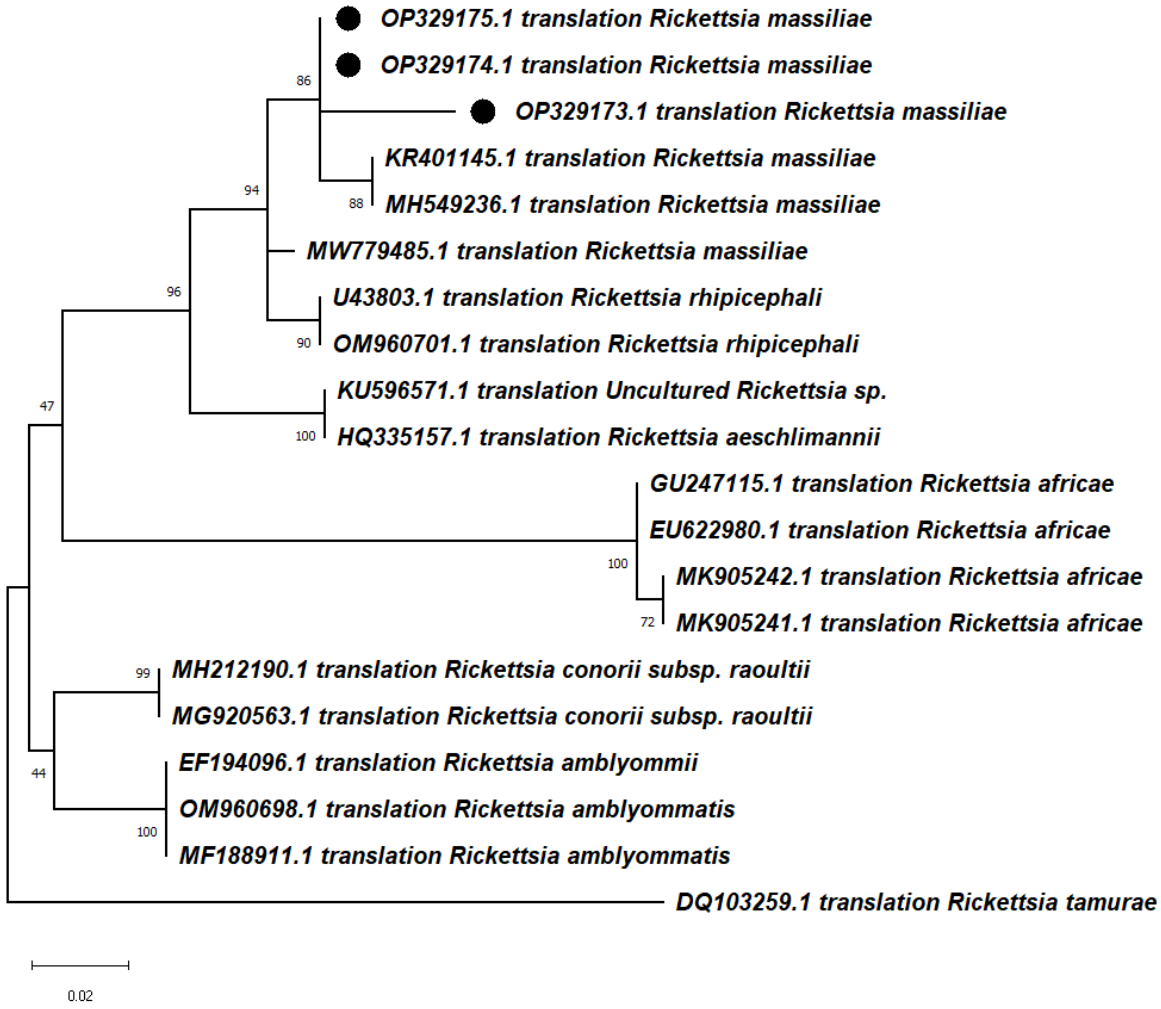

Figure S3: Phylogenetic tree based on the sequences of the *Rickettsia* ompA gene. The tree was constructed using mega 11 based on the maximum likelihood method, using the Jones-Taylor-Thoron (JTT) model. The were a total of 174 positions in the final data set. The sequences obtained in this study are indicated with bullets.

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North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
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28 November 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 28/11/2019, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Reptiles of South Africa as hosts for tick-borne pathogens and haemoparasites
Principal Investigator/Study Supervisor/Researcher: Dr C Cook
Student: LS Mofokeng - 29933870

Ethics number:

N	W	U	-	0	0	5	7	1	-	1	9	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 28/11/2019
Expiry date: 31/11/2020

Risk: **Category 2**

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of November annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal*

without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.

- Annually a number of studies may be randomly selected for active monitoring.
- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:
  - request access to any information or data at any time during the course or after completion of the study;
  - to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;
  - withdraw or postpone approval if:
    - any unethical principles or practices of the study are revealed or suspected;
    - it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;
    - submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or
    - new institutional rules, national legislation or international conventions deem it necessary.
- NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1206

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by
Christiaan B Brink
Date: 2019.11.29
13:08:26 +02'00'

Chairperson: NWU-AnimCareREC

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20 August 2019

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