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; Guglielmine et al., 2014).

Species of *Hepatozoon* Miller, 1908 are frequently observed as intraerythrocytic or intraleucocytic haemoparasites of reptiles (Smith, 1996; Telford, 2009). These adelie 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-Kociková 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; 

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2.1. Sample collection and identification

2. Materials and methods

Some comments on the potential of these acarine parasites as potential tiles and to molecularly screen these ectoparasites for species of the stages, findings of potential life cycle stages in sensu stricto moegregarine was originally described as belonging to Maia et al. (2016) and Borges-Nojosa et al. (2017), and the recent genus was considered premature (Maia et al., 2016; Borges-Nojosa et al., 2014). As such, the taxonomic changes associated with this new rRNA gene. As such, the taxonomic changes associated with this new study. Therefore, as suggested and recommended by Maia et al. (2016) and Borges-Nojosa et al. (2017), and the recent phylogenetic findings of Hrazdílova et al. (2021) opposing Bartazoon, we will conservatively continue to refer to species parasitizing reptilian hosts as species of Hepatozoon and not Bartazoon. 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 fitsimonsi (Dias, 1953) (Cook et al., 2014, 2018). This species of haemogregarine was originally described as belonging to Haemogregarina (sensu stricto) by Siddall (1995) 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).

Despite the importance of Amblyomma ticks from a one-health perspective, knowledge on their taxonomic status, potential of reptiles as their hosts, and the pathogens they may transmit, is limited (Sánchez-Montes et al., 2019; Mendoza-Roldan et al., 2021). In this sense, the current study aimed to identify tick species parasitizing reptiles and to molecularly screen these ectoparasites for species of the blood apicomplexan genus Hepatozoon. Furthermore, this study provides molecular findings of H. fitsimonsi in species of Amblyomma, with some comments on the potential of these acarine parasites as potential hosts for this haemogregarine.

2. Materials and methods

2.1. Sample collection and identification

Adult ticks (n = 100) were collected from reptiles (tortoises, snakes and monitor lizards) in the Cape Columbine region, Western Cape (WC) (17° 51’ 56” E, 32° 49’ 2” S) (2011) and the Ndumo Game Reserve, KwaZulu-Natal (KZN) (32°18’49”E, 26°54’33”S) (2014–2017), thereafter they were allowed the opportunity to digest their blood meals for approximately 10–20 days (Desser et al., 1995; Smith, 1996), and subsequently stored in 70% ethanol until further analysis. Sampling was carried out under the relevant permits (WC: 0035-AAA004-00383; KZN: OP 839/2014, OP 4374/2015, OP 4092/2016, OP 4264/2017), and received the relevant ethical approval (reference numbers: 920203595 and NWU-00372-16-A5 respectively). Morphological identification to species level was done with the aid of a Nikon AZ100M microscope and NWU-00372-16-A5 respectively). Morphological identification to species level was done with the aid of a Nikon AZ100M microscope and NWU-00372-16-A5 respectively. Representative tick samples from the study area were also used for molecular studies.

2.2. DNA extraction from tick specimens

Whole individual tick specimens were used for DNA extraction following the standard protocol method for animal tissue as detailed in the DNeasy Tissue Kit (Qiagen, Germany). Following filtration, the filtrate was collected and stored at –20 °C.

2.3. PCR amplification and phylogenetic analysis

DNA samples extracted from the ticks were used as a template for PCR amplification. For the identification of ticks, a 710bp long fragment of the cytochrome c oxidase subunit I (CO1) gene was amplified in a conventional PCR with the primer pairs HCO2198 (5′-GGTCAACAATCATAAGATTTGG-3′) and LCO1490 (5′-TAAACTTCAGGGT-GACCCCCAAAATCA-3′) (Folmer et al., 1994). Another PCR was used to amplify an approximately 460bp long fragment of the 16S rDNA gene of Ixodidae (Black and Piesman, 1994), with the primers 16S + 1 (5′-CGTCTCAATGATTTTTTAATTGTGTTG-3′) and 16S-1 (5′-CCGCTT CGAACTCATGATCAAG T-3′). For the screening of Hepatozoon spp., PCR was conducted using P1 (5′-CACAGGGAGGTGACAGA-G-3′) and P2 (5′-AAAGATTTCACCTATGACAG-3′) primers that amplified 430bp of the hypervariable region of 18S rDNA gene of pirolaplasms (Schnittger et al., 2004). The primer set HAMF-5′-GCCAGTAGCTCAT-ATGTTGTC-3′ (Criadó-Formelo et al., 2006) and Hep900 5′-CAAATCTAA-GAATTCCACCTGAC-3′ (Uyvarí et al., 2004) were also used for screening of Hepatozoon spp. However, none of the sequences amplified by this latter set were of good quality.

Conditions for PCR of all genes were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles, entailing a 95 °C denaturation for 30 s, annealing at 58 °C for 30 s with an end extension at 72 °C for 2 min, and following the cycles a final extension of 72 °C for 10 min (Netherlands et al., 2018).

All PCR reactions were performed with volumes of 25 μl, using 12.5 μl Thermo Scientific DreamTaq PCR master mix (2×) (2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM MgCl2), 1.25 μl of each primer (10 μM), and 2 μl of DNA. Double distilled water (ddH2O) was used to make up final reaction volume. Reactions were undertaken in a Bio-Rad C1000 Touch™ Thermal Cycler PCR machine (Bio-Rad, Hemel Hempstead, UK). An agarose gel (1%) stained with gel red was used to visualise resulting amplicons under UV light.

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. Sequence and species identity were determined using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/). Sequences obtained in the current study were deposited in the NCBI GenBank database under the following accession numbers: [GenBank: MW290507-MW290509 (16S rRNA); MW513957-MW513958 (CO1)] for ticks and [GenBank: MW494678-MW494681] for Hepatozoon. For phylogenetic analysis, comparative sequences of Hepatozoon species 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 7.1. To infer phylogenetic relationships Maximum likelihood (ML) method was 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 Gasco, 2003; Darriba et al., 2012).

3. Results

3.1. Morphological analysis of Amblyomma ticks

Ninety-eight (98) adult ticks were identified as Amblyomma marmoratum and two (2) as Amblyomma latum based on their morphology. The A. marmoratum was found infesting tortoises (n = 5 from the Cape, n = 9 from Ndumo), as well as two monitor lizards (both Varanus albigularis) from Ndumo, while A. latum was found on one of the two snakes (both Naja mossambica) (Table 1). The characteristics of the adult ticks of the genus Amblyomma collected from reptiles in South Africa during this study.

| Tick species | Number | Host species |
|--------------|--------|--------------|
| A. latum     | 2      | Naja mossambica (2) |
| A. marmoratum (male) | 54 | Varanus albiculatris (2), Stigmochelys pardalis (25), Chersina angulata (8), Kinixys spp. (7), K. zombensis (12) |
| A. marmoratum (female) | 44 | V. albiculatris (2), S. pardalis (16), K. zombensis (13), C. angulata (7), Kinixys spp. (6). |

Number of ticks collected from hosts is indicated next to each host.
A. latum and adult A. marmoreum are as described by Theiler (1945) and Theiler and Salisbury (1959), respectively (Fig. 1).

3.2. Molecular analysis of Amblyomma ticks and their Hepatozoon spp.

The PCR analysis of the DNA isolates indicates amplification of 460bp for the 16S rRNA gene and ~700bp for the CO1 gene. BLASTn results for both mtDNA genes matched with the expected species, although notable divergence levels were observed in both cases (Fig. 1). The CO1 gene was not able to amplify the A. latum. Based on the 16S rRNA phylogenetic analysis, the A. marmoreum from the current study constitutes a monophyletic clade (99% bootstrap value) closely affiliated to the same lineage group of A. marmoreum, while A. latum from this study formed a monophyletic clade with A. latum with 99% bootstrap value. Both A. marmoreum and A. latum formed a monophyletic group with other Amblyomma species of reptiles, clearly distinct from other Amblyomma species of mammals with a >70% bootstrap value (Fig. 2).

The overall prevalence of Hepatozoon was 10/100 (10%). It was detected from 1/4 (25%) A. marmoreum parasitizing Varanus albogularis and 9/94 (10%) A. marmoreum parasitizing tortoises. No Hepatozoon was detected from A. latum. The BLASTn analysis for the 430bp 18S rRNA of the Hepatozoon sequences had a 99% similarity with H. fitzsimonsi (KR069084.1) from South African tortoise Kinixys zombensis, and clustered in a single clade with H. fitzsimonsi from another South African tortoise, a tortoise collected from Nigeria and a tick collected from a tortoise in Kenya (Fig. 2).

4. Discussion

Members of the genus Amblyomma are known as hosts and vectors of various pathogenic diseases that can cause considerable economic losses in tropical and sub-tropical regions of the world (Ogo et al., 2012, 2017; Vesco et al., 2011; Hornok et al., 2020). According to data encompassing several surveys, A. marmoreum is most frequently encountered on tortoises, as observed in this study (Theiler, 1959; Horak et al., 2006). On the other hand, A. latum is known to infest snakes with occasional records from lizards and even in some mammals, which represent incidental infestations (Walker, 1991; Theiler, 1945). The present study observed this tick species from a snake (Naja mossambica).

Even though the CO1 gene did not amplify A. latum DNA, both phenotypic characteristics and the 16S rRNA confirmed this species as A. latum. Based on the analysis of both genes in the current study, the phylogenetic trees showed a clustering of A. marmoreum tick sequences into two groups with high bootstrap values, indicating that the possibility of having a diverse population of A. marmoreum ticks in South Africa cannot be ruled out. However, a more detailed phylogenetic study of A. marmoreum needs to be undertaken to confirm any intraspecific variation. Ogo et al. (2017) found similar results in Nigeria based on the characterization of 16S rRNA of A. variegatum.

However, the current study was limited by the lack of A. marmoreum and A. latum sequences in GenBank, as it did not afford us the opportunity to draw inferences. Nevertheless, it 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.

Hepatozoon fitzsimonsi was reassigned from the genus Haemogregarina (sensu lato) 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 Amblyomma 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 × 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 × 5 μm (banana form) or 14.3 × 18.3 μm (curved form), with an irregular nucleus; whilst H. varani measures 14 × 3 μm, the gamont folded (suggesting a length of 24 μm), the nucleus occurring on the fold. Hepatozoon...
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.

In Kenya, during a study aimed at the molecular detection of tick-borne pathogens (TBPs), *Amblyomma sparsum* and *Amblyomma falsomarmoratum*, 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 prevalence of *H. fitzsimonsi* in these ticks. Furthermore, *A. falsomarmoratum* 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.

## 5. Conclusion

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, 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 at this stage that ticks may play a role as vector in the life cycle of *H. fitzsimonsi*. 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.

## Declaration of competing interest

All authors contributed in the draft of this manuscript and declare no conflict of interest.

## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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