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Original article

Sympatry of *Amblyomma eburneum* and *Amblyomma variegatum* on African buffaloes and prevalence of pathogens in ticks

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ABSTRACT

The *Amblyomma* genus is represented on the African continent by 24 species, out of which 17 are known to occur in different ecological niches of southern Africa. *Amblyomma*, known for their aggressive hunting behaviour and aptitude as pathogen vectors, are of main concern to travellers, mainly in rural and conservation areas of Africa. In this study, we highlight the overlapping distribution of *Amblyomma eburneum* and *Amblyomma variegatum* found on African buffaloes (*Syncerus caffer*) at Coutada 11, Central Mozambique. In total, 1,039 *Amblyomma* ticks were collected and morphologically identified using taxonomic keys, and genomic DNA was extracted. They were subjected to reverse line blotting for pathogen identification followed by molecular analysis (*COI* sequencing) of both tick species. Pathogens such as *Ehrlichia ruminantium, Anaplasma centrale, Theileria* sp., *Babesia* sp. and *Rickettsia africae* were detected, of which *R. africae* is zoonotic. *Ehrlichia ruminantium, R. africae, Theileria mutans* and *Theileria velifera* are well-established pathogens transmitted by *Amblyomma* ticks; however, *Anaplasma* spp. and *Babesia* spp. are not, suggesting residual parasite DNA in the bloodmeal. Little is mentioned in the literature about *A. eburneum*, including its role as a vector and reservoir for pathogens. In Mozambique *A. eburneum* is currently restricted to wildlife but the spread of the tick may be observed given the climate change that is occurring. The infection rates for the pathogens in both *Amblyomma* tick species were lower than expected, but this may be due to the low host density in the forest niche and the innate immunity of these hosts. With the propensity of ticks of the *Amblyomma* genus to form parapatric distributions, the mechanisms that allows for the overlapping distribution of these two *Amblyomma* species while maintaining tick species identity is of great interest.

1. Introduction

In south-east Africa, 21 *Amblyomma* species have been documented, including: *Amblyomma astrion, Amblyomma cohaerens, Amblyomma compressum, Amblyomma eburneum, Amblyomma exornatum, Amblyomma falsomarmoreum, Amblyomma gemma, Amblyomma hebraeum, Amblyomma latum, Amblyomma lepidum, Amblyomma loculosum, Amblyomma marmoreum, Amblyomma nuttalli, Amblyomma personatum, Amblyomma pomposum, Amblyomma rhinocerotis, Amblyomma sparsum, Amblyomma sylvaticum, Amblyomma tholloni, Amblyomma transversale*, and *Amblyomma variegatum* (Walker, 1991; Horak et al., 2018). Although the diversity of *Amblyomma* species is substantial, the majority of research has been conducted on *A. hebraeum* and *A. variegatum* due to their extensive geographical spread across southern Africa (as well as other African regions and the Caribbean in the case of the latter), and their competence as vectors of pathogens (Petney et al., 1987; Walker, 1991; Walker et al., 2003).

Amblyomma ticks are three-host ticks. During their life cycle, the ticks detach from the host after full engorgement during the larval, nymphal, and adult (females only) stages. While some *Amblyomma* spp. are specialists in all life stages, the majority of *Amblyomma* are generalists in the larval and nymphal stages. Larvae and nymphs are reliant on host availability and have been documented on birds, reptiles, and wild mammals, while the majority of adult *Amblyomma* spp. prefer domestic animals, including livestock, as hosts (Walker, 1991; Horak et al., 2018). While adult infestations on wildlife are less well-documented, several

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species have been found infesting African buffalo (*Syncerus caffer*) (Dias, 1991; Walker, 1991). The African buffalo has been documented to be the primary host for *A. astrion, A. cohaerens, A. eburneum, A. lepidum* and *A. sparsum* (Dias, 1991; Walker, 1991; Voltzit and Keirans, 2003). *Amblyomma cohaerens. A. gemma, A. hebraeum, A. pomposum, A. tholloni* and *A. variegatum* have been documented occurring on buffalo but have alternative preferred hosts (Dias, 1991; Walker, 1991; Voltzit and Keirans, 2003; Horak et al., 2018). Although there is a variety of *Amblyomma* that prefers buffaloes as their primary hosts, they are generally separated by their geographical distributions, rarely co-occurring in the same area on the same hosts. The geographical distribution of different *Amblyomma* species has limited overlap and forms parapatric boundaries in most cases, with sympatry being observed in geographical transitioning areas or when ticks use different hosts (Petney et al., 1987).

The *Amblyomma* genus is known for its aggressive hunting behaviour and is well-documented as vectors of pathogens in Africa, including but not limited to, *Ehrlichia ruminantium, Rickettsia africae, Theileria mutans* and *Theileria velifera* (Walker, 1991; de la Fuente et al., 2008; Horak et al., 2018). Of the aforementioned pathogens, *E. ruminantium*, the causative agent of heartwater, is the most lethal, responsible for 80% - 95% case fatality in cattle and small ruminants (Allsopp, 2010). Although wildlife are able to contract heartwater, fatalities and severe infections are lower in comparison to livestock (Andrew and Norval, 1989; Wesonga et al., 2001). Another pathogen transmitted by *Amblyomma* ticks is *R. africae*, which causes African tick bite fever (ATBF) in humans. *Rickettsia africae* is the most widespread spotted fever group rickettsia that commonly affects individuals travelling to southern Africa. Although it is rarely fatal, severe disease has been recorded (Kelly et al., 1996).

Mozambique is the 35th largest country in the world with an area of 801,537 km^2. Located on the south-eastern coast of Africa, Mozambique is bordered by Eswatini (formerly known as Swaziland), South Africa, Zimbabwe, Zambia, Malawi, Tanzania, and the Indian Ocean. In recent years (excluding 2020–2022) international tourism to Mozambique has increased for activities including game watching, hunting and safari expeditions (World Bank Group, 2022). Currently, Mozambique boasts an increasing population of African buffalo, mainly in the Marromeu Complex which consists of four hunting grounds, luring many hunters worldwide to the country. With the increase in tourism, there is an increased risk of human interactions with ticks and their associated pathogens. The Marromeu Complex consists of four main areas, Coutadas 10, 11, 12 and 14, located within the Zambezi delta (Loft, 2018; Couto and de Nazaré Mangueze, 2020). The majority of the buffaloes within the Marromeu Complex inhabit the swampy grasslands of the Zambezi delta, which is difficult to access by either boat or via land vehicle. However, there is a sub-population of buffalo that thrives in the Miombo forest niche on the edges of the swampy areas that are more accessible as targets for legal hunting activities (Beilfuss et al., 2010; Loft, 2018). This study aimed to investigate the *Amblyomma* ticks found on African buffaloes from the Marromeu Complex and investigate the haemoparasites they carry.

2. Materials and methods

2.1. Sampling and identification

Sampling was conducted in 2021 from November until December in a wildlife utilization area, Coutada 11, Mungari, Sofala province, Mozambique (S 18.39371; E 35. 59856). Coutada 11 is located within the Miombo forest niche. *Amblyomma* ticks were collected from hunted wildlife that was processed at a nearby abattoir. Wildlife included: African buffalo – *Syncerus caffer*, eland – *Tragelaphus oryx*, nyala – *Tragelaphus angasii*, reedbuck – *Redunca arundinum* and warthog – *Phacochoerus africanus*. All ticks were collected, when possible, from all incoming animals. Ticks were identified under a stereo microscope with the use of taxonomic keys obtained from Walker et al. (2003) and Voltzit and Keirans (2003). Morphological characteristics were documented with the use of a Nikon SMZ 25 camera and NIS-Elements software (Why, 2022).

2.2. DNA extraction and PCR amplification

DNA was extracted using the Chelex 100 resin method as described by Nishiguchi et al. (2002) with modifications. In brief, each individual tick was cut in half. One half was stored at −20 °C for other projects. The other half was added to a 1.5-ml microcentrifuge tube containing 200 µl of 5% Chelex® 100 resin preparation and two 2 mm glass beads. The tick half was homogenized, incubated at 56 °C for 1 hour, and then its DNA was denatured by incubation at 95 °C for 30 min. Centrifugation at 12, 000 x g for 3 min separated the mixture into aqueous and solid phases. The denatured DNA is suspended in the top aqueous phase while the Chelex® 100 resin preparation and other cellular components (proteins, lipids, and carbohydrates) remained in the lower solid phase. The samples were stored at −20 °C for downstream use. Molecular characterization was conducted by targeting the Cytochrome c oxidase I (*COI*) gene as described by Folmer et al. (1994) with slight modifications. A 708-bp length fragment was amplified in a 20 µl reaction consisting of 10 µl Phusion Flash High-Fidelity PCR Master Mix (1X final concentration), 0.5 µl each of primers LCOI490 and HCO2198 (final concentration of 0.5 µM) (Table 1), 7 µl double distilled water and 2 µl sample DNA. The PCR cycling conditions comprised an initial denaturation at 98 °C for 10 s, followed by 10 cycles of denaturation at 92 °C for 1 s, annealing at 50 °C for 5 s, extension at 72 °C for 15 s. Next another 30 cycles of amplification was conducted; denaturation was performed at 92 °C for 1 s, annealing at 56 °C for 5 s, and extension at 72 °C for 15 s. Final extension was performed at 72 °C for 2 min. The PCR products were separated on a 1.5% agarose gel and visualized using the Bio-Rad gel documentation system with assisted visualization programming. All samples that had visible bands were sent to the Central Analytical Facility (CAF), Stellenbosch, South Africa for Sanger sequencing.

A random subset of 15 samples per species (4 females and 11 males) were subjected to reverse line blotting (RLB) for screening to determine initial pathogen presence. In brief, a touchdown PCR targeting two areas was done; the first was the V4 hypervariable area of the 18S rRNA gene using *Theileria/Babesia* genus-specific primers, and secondly the V1 hypervariable area of the 16S rRNA gene using *Ehrlichia/Anaplasma*

Table 1
Set of primers and probes used for the Reverse Line Blot, COI, ompA and pCS20 PCR's.

Target area	Primer/probe name	Sequence (5′−3′)	Reference
COI	LCOI490	GGT CAA ATC ATA AAG ATA TTG G	Folmer et al. (1994)
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	
18S rRNA	RLB F2	GAC ACA GGG AGG TAG TGA CAA	Nagore et al. (2004)
	RLB R2	Biotin-CTA AGA ATT TCA CCT CTG ACA GT	
16S rRNA	Ehr-F	GGA ATT CAG AGT TGG ATC MTG GYT CAG	Schouls et al. (1999)
	Ehr-R	Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT	Bekker et al. (2002)
ompA	Rr190.70F	ATG GCG AAT ATT TCT CCA AAA	Mazhetese et al. (2022)
	Rr190.701R	GTT CCG TTA ATG GCA TCT	
pCS20 gene fragment	Sol1 F	ACA AAT CTG GYC CAG ATC AC	Steyn et al. (2008)
	Sol1 R	CAG CTT TCT GTT CAG CTA GT	
	Sol1TM	6-FAM-ATC AAT TCA CAT GAA ACA TTA CAT GCA ACT GG- BHQ1	

genus-specific primers (Table 1). The 25 µl reaction consisted of 12.5 µl TaqMan Master Mix (1X final concentration), 0.625 µl of each primer (final concentration of 0.25 µM) (Table 1), 9.5 µl double distilled water and 2.5 µl sample DNA. A no-template negative control and two positive controls, verified *B. bigemina* and *E. ruminantium* DNA (Onderstepoort Biological Products, South Africa), were used as standard controls. The PCR conditions were as described in Table 2. The PCR products were then hybridized to a Biodyne® C blotting membrane, containing *Theileria/Babesia/Ehrlichia/Anaplasma* genus-and species-specific probes, as described by Nagore et al. (2004).

All of the collected *Amblyomma* ticks were screened with a quantitative real-time PCR for *E. ruminantium* with the use of pCS20 gene fragment as described by Steyn et al. (2008). The 25 µl reactions consisted of 12 µl TaqMan Master Mix (1X Final concentration), 0.625 µl each of primers Sol1F and Sol1R (final concentration of 0.25 µM), 1.25 µl of probe Sol1P (final concentration of 0.4 µM) (Table 1), 8.5 µl double distilled water and 2 µl sample DNA. The PCR cycling conditions comprised an initial UNG incubation step at 50 °C for 2 min followed by an AmpliTaq Gold pre-activation step at 95 °C for 10 min. This was followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 55 °C for 1 min. A positive control and no-template negative control were included for each set of reactions. The positive cut-off value was set at 38 CT.

We calculated the required tick sample size by assuming a confidence interval of 95% with a hypothesised infection rate of 10% (for *R. africae* and *E. ruminantium* separately) and an infinite population (Cannon and Roe, 1982). Thus, to provide statically significant results, 132 ticks were required. Thus, a random subsample of 160 ticks per species were screened for *R. africae* using the *ompA* gene as described by Mazhetese et al. (2022).

Table 2
Thermocycling conditions for Theileria/Babesia and Ehrlichia/Anaplasma touchdown PCR.

Cycle	Time	Temperature	Purpose
1 cycle	3 min	37 °C	
1 cycle	10 min	94 °C	Initial denaturation
2 cycles	20 s30 s	94 °C	Denaturation of double stranded DNA
	30 s	67 °C	template
		72 °C	Annealing of primers
			Extension of PCR products by *Taq* polymerase
2 cycles	20 s30 s	94 °C	Denaturation of double stranded DNA
	30 s	65 °C	template
		72 °C	Annealing of primers
			Extension of PCR products by *Taq* polymerase
2 cycles	20 s30 s	94 °C	Denaturation of double stranded DNA
	30 s	63 °C	template
		72 °C	Annealing of primers
			Extension of PCR products by *Taq* polymerase
2 cycles	20 s30 s	94 °C	Denaturation of double stranded DNA
	30 s	61 °C	template
		72 °C	Annealing of primers
			Extension of PCR products by *Taq* polymerase
2 cycles	20 s30 s	94 °C	Denaturation of double stranded DNA
	30 s	59 °C	template
		72 °C	Annealing of primers
			Extension of PCR products by *Taq* polymerase
40 cycles	20 s30 s	94 °C	Denaturation of double stranded DNA
	30 s	57 °C	template
		72 °C	Annealing of primers
			Extension of PCR products by *Taq* polymerase
1 cycle	7 min	72 °C	Final extension

2.3. Statistical analysis

A Chi-square analysis was conducted in Microsoft Excel to evaluate the independence between infection rate and sex of tick species, as well as to evaluate the independence between infection rate and tick species.

2.4. Phylogenetic analysis

Sequences were manually corrected on CLC main workbench version 8.1.2 (developed by CLC Bio, http://www.clcbio.com), and compared with those available in the GenBank database (Supplementary data A) using BLAST (https://www.ncbi.nlm.nih.gov/genbank/) (Appendix A). Assembled sequences were aligned alongside reference sequences using the online MAFFT version 7 (developed by http://mafft.cbrc.jp/alignment/server/index.html) with default parameters. The aligned matrices were manually viewed, edited, and truncated using MEGA 7 (Kumar et al., 2016). The best fit model was determined using the jModelTest2 (Darriba et al., 2012) on the CIPRES Science Gateway (https://www.phylo.org/) platform. The file format was changed depending on program requirements using FaBox version 1.61 (https://users-birc.au.dk/palle/php/fabox/index.php). Bayesian analyses were performed in MrBayes version 3 (Ronquist and Huelsenbeck, 2003). For Bayesian analysis, the Jukes-Cantor (JC) was used with five Monte Carlo Markov Chains (MCMC) for 5000,000 iterations, saving every 1,000th tree. Tracer version 1.6 (Rambaut et al., 2014) was used for inspection of estimated sample size (ESS) (>>200) and parameter sampling using graphical plots indicating parameter stabilisation. The resulting tree was visualised and edited in FigTree v1.4.2 (Rambaut, 2014).

2.5. Ethical considerations

This study was approved by the Research Ethics Committee of the University of Pretoria (REC 121–20) and approval was obtained from the Department of Agriculture, Land Reform and Rural Development (DALRRD), South Africa, under Section 20 of the Animal Diseases Act 1984 (Act no. 35 of 84) (12/11/1/1 (1937SS)).

Results

A total of 1039 *Amblyomma* ticks were collected from African buffalo, Eland, Nyala, Reedbuck and Warthog (Fig. 1). The adult ticks were morphologically and molecularly identified as belonging to two species: *A. eburneum* ($n = 208$; 49 females and 159 males) and *A. variegatum* ($n = 801$; 248 females and 553 males), alongside 30 nymphs of unidentified species (Fig. 2). The Bayesian analysis of the phylogeny based on *COI* indicated unambiguous separation (probability of 1) between the *A. eburneum* and *A. variegatum* branches (Fig. 3). Moreover, the *A. eburneum* samples were differentiated into two main clades, one of which contained only two of our Mozambican samples, whereas the other exhibited further sub-structuring between our samples and those from other countries (Fig. 3). Of the 1039 ticks collected, 924 were obtained from African buffalo. A general screening was conducted on 15 ticks of each species with RLB. *Babesia* sp. (7/15 and 3/15), *Theileria* sp. (4/15 and 3/15), *Ehrlichia* sp. (3/15 and 1/15) and *Anaplasma* sp. (1/15 and 0/15) were detected in *A. eburneum* and *A. variegatum,* respectively. One *A. variegatum* and two *A. eburneum* ticks were co-infected with *Babesia* and *Theileria* species.

The qPCR indicated that in total, *A. eburneum* had a prevalence of 7.7% (16/208) for *E. ruminantium* with 15 males (9.4%) and one (2.0%) female testing positive with no significant difference between the sexes ($X^2(1, N = 208) = 2.883$ $p = 0.090$).The *A. variegatum* prevalence for *E. ruminantium* was 2.6% ($n = 21/801$) with 16 males (2.9%) and five females (2.0%) testing positive with no significant difference between the sexes ($X^2(1, N = 801) = 0.516$, $p = 0.473$). There is a significant difference between *A. eburneum* and *A. variegatum* prevalence for *E. ruminantium* ($X^2(1, N = 1009) = 18.187$, $p = 0.000$). For the *R. africae* conventional PCR, 22 of the 160 (13.8%) *A. eburneum* ticks tested positive of which 14 were male (12.2%) and eight were female (19.5%)

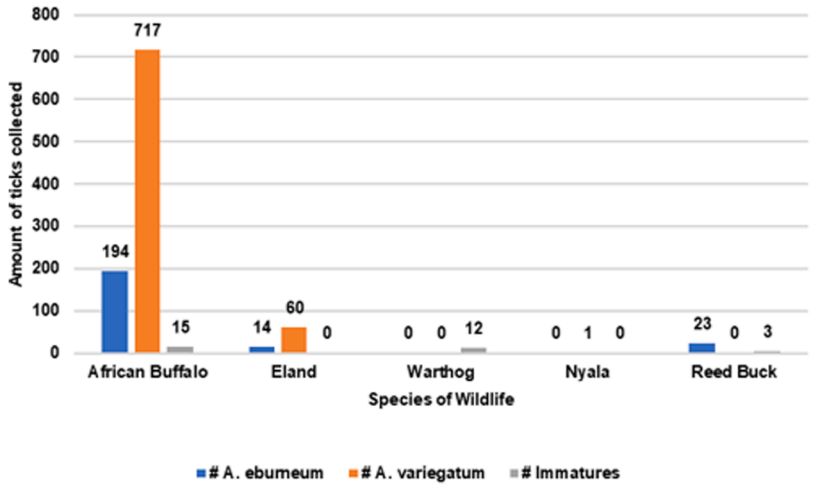

Fig. 1. The *Amblyomma* species and quantity collected from wildlife in Coutada 11, Mungari, Mozambique.

Fig. 2. Morphological comparison of male (indicated by ♂) and female (indicated by ♀) *Amblyomma variegatum* (A) and *Amblyomma eburneum* (B) collected from African buffalo.

with no significant difference between the sexes ($X^2(1, N = 160) = 1.344, p = 0.246$). Sample MSMun993 exhibited a co-infection with both *E. ruminantium* and *R. africae*. Among *A. variegatum* samples, 35 of the 160 ticks tested positive (21.9%), which included 27 males (25.2%) and eight females (15.1%) with no significant differences between the sexes ($X^2(1, N = 160) = 0.213, p = 0.144$) and no co-infections were detected. There was no significant difference between *A. eburneum* and *A. variegatum* prevalence for *R. africae* ($X^2(1, N = 320) = 6.929, p = 0.074$).

3. Discussion

This study aimed to investigate the *Amblyomma* ticks found on African buffaloes inhabiting the Miombo forest niche in the Zambezi delta and the pathogens that these ticks carry. During the study, two *Amblyomma* species were identified using both morphological identification and the *COI* gene as *A. eburneum* and *A. variegatum* (Fig. 3), occurring in a sympatric manner on wildlife. There is limited information on GenBank on *A. eburneum*, and sequences that are available are limited to the *COI* gene, therefore this gene was selected for the study. Unfortunately, during the study, we struggled to amplify *A. variegatum* DNA using the *COI* gene. Lv et al. (2014) experienced the same complications using this *COI* primer pair, with amplification success being lower than 27.4% in an attempt to barcode 84 specimens. It has been suggested that the universal *COI* primers (LCOI490 and HCO2198) are not as effective on all tick species, and other primer pairs for the *COI* gene should be considered (Lv et al., 2014; Low et al., 2015).

Amblyomma eburneum (Gerstaecker, 1873), known as the Ivory bont tick, is an Afrotropical tick species with a paucity of information in literature (Tandon, 1990; Robbins and Carpenter, 2011; Mwamuye et al., 2016a; 2016b, 2017; Oundo et al., 2020). Due to this knowledge gap, little is known about *A. eburneum*'s role as a vector, its full distribution and host range. Several studies have been reported in Kenya; however, some *A. eburneum* ticks have been collected from animals in Zambia, Somalia, Eritrea, Northern Ethiopia, Tanzania and Zimbabwe (Voltzit and Keirans, 2003; Mwamuye et al., 2017; Kutima et al., 2019; Oundo et al., 2020).

Based on the initial RLB screening, *A. eburneum* and *A. variegatum* tested positive for *Babesia* sp., *Theileria* sp., *Ehrlichia* sp. and *Anaplasma* sp. pathogens. While *Amblyomma* species are well-documented vectors of *E. ruminantium, R. africae, T. mutans* and *T. velifera*, they are not known as vectors of *Anaplasma* sp. and *Babesia* sp. (Allsopp et al., 1999; Allsopp, 2010; Teshale et al., 2015; Mazhetese et al., 2021). The detection of both *Anaplasma* sp. and *Babesia* sp. in the tick species could be a result of host blood found in the samples due to the crude extraction method that was used and the engorgement of some of the extracted

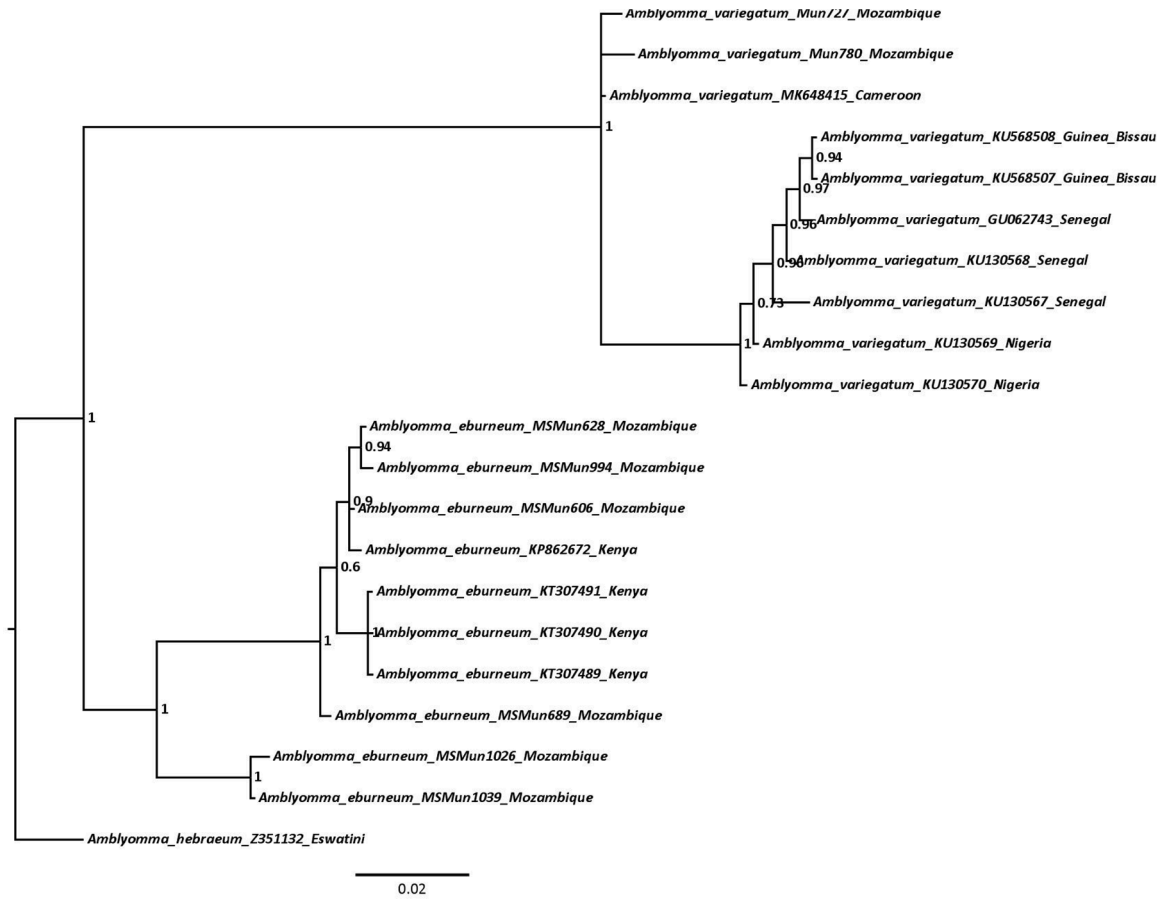

Fig. 3. Bayesian analysis of the cytochrome oxidase 1 (*COI*) gene (613 bp). Analysis was done using the JC model, with five Monte Carlo Markov Chains (MCMC) for 5000,000 iterations, saving every 1,000th tree. The resulting tree was visualised and edited in FigTree v1.4.2. Posterior probability is indicated at each branch node. Sequences obtained in this study have identification codes starting with "MSMun".

ticks. Although the *Babesia* positive samples where only detected on the *Babesia* "catch-all" of the RLB, we can speculate that it is either *Babesia occultans* or an undescribed *Babesia* species. *Babesia occultans* is transmitted by *Hyalomma rufipes* and not *Amblyomma* species (Gray and De Vos, 1981; Ozubek et al., 2020). While not documented in the results, *Rhipicephalus maculatus* and *Rhipicephalus simus* were present on buffalo in the study area, but no *Hyalomma* were present. *Hyalomma rufipes* has been considered to be present in the arid areas of Sofala (Dias, 1991). In the absence of *Hyalomma* spp., we speculate that the *Babesia* detected might be an undescribed species that requires further investigation. For the *Anaplasma* positive samples detected by the *Anaplasma* catch-all of the RLB, we can speculate that it is either *A. marginale, A. centrale, A. sp. Omatjenne* or an undescribed *Anaplasma* species (Henrichs et al., 2016).

In this study, we found that *E. ruminantium* infection rates were within the expected range, although it was on the lower edge of the margin, 7.7% and 2.6%, for *A. eburneum* and *A. variegatum,* respectively. In Maputo, studies have previously been conducted to determine *E. ruminantium* infection rates in cattle blood, which ranged from 5% to 23.9% (Matos et al., 2019). Countries surrounding Mozambique have reported *E. ruminantium* infection rates ranging from 8.5% to 28.4% in ticks (Peter et al., 1999; Allsopp et al., 2007; Jongejan et al., 2020). In buffalo from Botswana, infection rates were 6%; while a 5% prevalence was detected in buffalo from Kruger National Park, South Africa; 3% was detected in buffalo from Namibia; and 0% was found in buffalo in Mozambique (Pascucci et al., 2011; Debeila, 2012; Eygelaar et al., 2015; Machado et al., 2016). We hypothesise that the low infection rates are linked to a number of factors. Based on previous unpublished observations, *Amblyomma* are relatively rare on buffaloes of the wet grasslands while in contrast, buffalo inhabiting the forest ecological niches are markedly infested by both *A. eburneum* and *A. variegatum*. The lower density of buffaloes occurring in the forest niches reduces the opportunity for *Amblyomma* ticks to transmit the pathogen between hosts (Peter et al., 2002). Furthermore, buffaloes are not efficient *E. ruminantium* hosts and their carrier status post-infection is suspected to be no more than six months (Peter et al., 2002). Gradwell et al. (1976) postulated that buffalo may have natural innate immunity to *E. ruminantium* after they attempted to artificially infect naïve buffaloes with *E. ruminantium* infected sheep blood and found that the buffaloes exhibited no clinical signs of infection. In ticks, transstadial maintenance of *E. ruminantium* occurs between larval and nymphal, and nymphal and adult, stages (Allsopp, 2010). The transstadial maintenance reduces the amount of *Amblyomma* progeny infected with *E. ruminantium*, reducing the infection load for the next season. Therefore, the low infection rate of *E. ruminantium* in buffaloes can be ascribed to the low host density in the forest niche and the innate immunity of these hosts.

Regarding *R. africae, A. eburneum* and *A. variegatum* had infection rates of 13.8% and 21.9%, respectively. A comprehensive systematic review of *R. africae* in sub-Saharan Africa indicated infection rates reaching 48% in *Amblyomma* species; however, some studies have found infection rates as high as 93% in endemic areas (Maina et al., 2014; Mazhetese et al., 2021; Pillay et al., 2022). In Mozambique, infection rates varied between 5.6% and 67% (Matsimbe et al., 2017; Magaia et al., 2020). The low host density, as described in previous studies, plays a significant role in the low infection rates that are observed. The infection rates are higher for *R. africae* as compared to *E. ruminantium* due to transovarial and transstadial maintenance that occurs in *Amblyomma* ticks infected with *R. africae* (Silva-Ramos and Faccini--Martínez, 2021). No information is available on the immunity of

buffaloes to *R. africae,* but it is speculated that buffaloes may have similar immune responses to *R. africae* as they do to *E. ruminantium,* or they may be refractory to *R. africae* (Obara et al., 2022).

For *E. ruminantium*, males of both tick species had higher numbers of infections as compared to females, although this was not significant. *Amblyomma eburneum* males had an infection rate significantly higher for *E. ruminantium* compared to *A. variegatum*. With regards to *R. africae, A. variegatum* males had higher infection rates as compared to their females and *A. eburneum*, although not significantly. Although, *A. eburneum* had fewer infections with *R. africae,* females had more infections as compared to their males. The higher infection rate in the males for both *E. ruminantium* and *R. africae* (in *A. variegatum*) can be attributed to their longer lifespan as compared to females and their migration from host to host looking for new mating possibilities, potentially increasing the chances of getting infected (Stachurski, 2006).

In nature, *Amblyomma* ticks that parasitize large ruminants display clear geographical borders forming parapatric distributions, as can be seen for *A. hebraeum* and *A. variegatum* in southern Africa (north-eastern corner of Botswana, onward into north-western Zimbabwe and above the Save river in Mozambique), as well as for *A. pomposum* and *A. variegatum* in Angola. Sympatric distributions of *Amblyomma* ticks are rare in nature and generally occur in transitioning areas of geographical distribution zones (Walker and Olwage, 1987; Bournez et al., 2015; Cangi et al., 2015; Mandara, 2018). In both aforementioned examples, both species share the same host preferences, attachment site preference, similar mating behaviours and both species survive in the same climatic conditions (Bournez et al., 2015). The attraction-aggregation-attachment (AAA) pheromone is a multi-compound excretion that has been documented in a small subset of *Amblyomma* species, including *A. variegatum* and *A. hebraeum*. The pheromone is exclusively produced by fed males, after a few days of host attachment, and is essential in forming species-specific feeding clusters (Sonenshine, 2006; Bournez et al., 2015). The AAA pheromones in *A. hebraeum* and *A. variegatum* are similar and are believed to be the cause for cross-mating between the two species. Bournez et al. (2015) found that high rates of cross-mating occur between *A. hebraeum* and *A. variegatum*, suggesting that there is a failure to discern between species by means of the pheromones emitted. “Mating errors” such as these may lead to an increased probability of local extinction of the less frequently occurring species (Bournez et al., 2015). The cross-mating between the two species produces inviable eggs and leads to exclusive competition and parapatry between the two species (Rechav et al., 1982; Bournez et al., 2015). Here, the *A. eburneum* and *A. variegatum* found on wildlife, specifically the buffalo occurring in the forest niches, shared host and attachment site preferences; however, the *A. variegatum* outnumbered the *A. eburneum* roughly 4:1 on animals where collections were conducted. One of the main hypotheses for this sympatric occurrence is the possibility of a large divergence in the AAA pheromone excreted by these two tick species. Price et al. (1994) and Sonenshine (2006) hypothesised that the difference in the relative abundance of methyl salicylate and o-nitrophenol, as well as the presence or absence of benzaldehyde during the production and excretion of the AAA pheromone may contribute to sympatry found between *Amblyomma* species. With the limited research that has been conducted on *A. eburneum*, we are unsure whether the AAA pheromone is present or absent in *A. eburneum* and whether its composition is similar to that of *A. variegatum*’s AAA pheromone. If the pheromone is absent or sufficiently divergent, similar species may occur on the same hosts since no sexual or feeding competition would take place.

To conclude, *A. eburneum* and *A. variegatum* were found occurring sympatrically on African buffaloes in Mungari, Sofala, Mozambique. Limited information is available for *A. eburneum*. Whilst *A. eburneum* is currently restricted to wildlife species, the study has indicated its possibility as a vector of *E. ruminantium* and *R. africae*. The infection rates for both pathogens in both species were on the lower end of the expected range, but this may be due to the low host density in the forest niche and the innate immunity of these hosts to the pathogens. Some limitations of our study were the relatively small numbers of tick specimens subjected to RLB for initial pathogen screening and the exclusive focus on blood-fed adults. Future studies could investigate the ecological separation of *A. variegatum* and *A. eburneum* at the larval and nymph stages, and the pathogen prevalence in questing immature stages (as well as unfed adults) to focus on vectored pathogens without the impact of ingested blood. Additional examination should be conducted on sympatric tick species to investigate whether the dynamics of pathogen transmission are increased or reduced compared to regions where only one vector species is present. Lastly, investigation into the mechanisms that allow for the overlapping distribution of these two species while maintaining tick species identity, despite these species sharing the same geographical region, host and host predilection sites, would be of great interest. This is pertinent especially because propensity is observed in other species of the same genus to form parapatric distributions.

CRediT authorship contribution statement

Andeliza Smit: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Visualization. **Fernando C. Mulandane:** Methodology, Investigation, Writing – review & editing. **Stephané H. Wojcik:** Investigation, Writing – review & editing. **Ivan G. Horak:** Writing – review & editing. **Benjamin L. Makepeace:** Writing – review & editing, Supervision. **Darshana Morar-Leather:** Writing – review & editing, Supervision, Funding acquisition. **Luis Neves:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

None

Data availability

Data will be made available on request.

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Supplementary materials

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