A Surveillance Study on Tick and Tick-Borne Pathogen diversity in Shimba Hills National Reserve, Kenya

By

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I56/80800/2012

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE (GENETICS)

UNIVERSITY OF NAIROBI

2015

DECLARATION

This thesis is my original work and to the best of my knowledge has not been submitted to any other university for examination.

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DEDICATION

To my grandmothers, ‘Wawe-Muruu’ and ‘Wawe-Amina’.

ACKNOWLEDGEMENT

I am heartily grateful for the various means of support from individuals, who indeed deserve a special recognition for their help in facilitating my successful studies. I am particularly indebted to my thesis supervisors, Drs David Odongo, Jandouwe Villinger, and Edward Kariuki for their guidance, encouragement and support in undertaking this study and thesis writing. Special thanks to Dr. Dan Masiga for mentorship and help especially in my conceptualization of this study. I'm also thankful to Mr. James Kabii who has been supportive throughout including during the fieldwork.

I additionally thank fellow students and colleagues in Molecular Biology and Bioinformatics Unit (MBBU) and the Martin Lüscher Emerging Infectious Disease Laboratory at *icipe* for their conviviality.

I also extend my acknowledgments to the School of Biological Sciences, University of Nairobi for awarding me a scholarship to cover my tuition and subsistence costs. I'm indebted to *icipe* through the capacity building department for awarding me a Dissertation Research Internship Programme (DRIP) scholarship to facilitate the completion of my M.Sc. studies.

Finally, to my family, I thank my mum and dad for their love and patience, my brothers Mathew, Stephen and Edwin for being a source of inspiration. Without a doubt this work would not have seen the light of the day had it not been by the grace of my God in His divine guidance and care. All glory is to Him alone.

ABSTRACT

Outbreaks of emerging and re-emerging tick-borne pathogens are becoming more frequent world-wide. Surveillance is critical to improve our understanding of pathogen diversity and their tick vectors. This will elucidate disease transmission dynamics that can inform the development of better disease prevention and control strategies. A total of 4,324 questing ticks (209 adult ticks, 586 nymphs and 3,502 larvae) were collected from six sites in Kenya's Shimba Hills National Reserve (SHNR). Morphologically, adults were identified to species level while nymphs and larvae were identified to the genus level. Three species from two genera of the Ixodidae family were identified; *Amblyomma eburneum*, *Amblyomma thollonii,* and *Rhipicephalus maculatus.* Molecular analysis of CO1, ITS2 and 16S rRNA genes was used to further confirm adult species identifications and to assign species identities to the nymphs and larva which were difficult to identify based on morphological characteristics. Ticks were grouped into pools of varying sizes, depending on species and life cycle stages, then analysed for tick-borne pathogens including arboviruses, bacteria as well as protozoa using PCR with high resolution melting (HRM) analysis and sequencing of unique melting profiles. Detection of *Anaplasma phagocytophilum,* two *Rickettsia*-like and two *Ehrlichia*-like bacterial species in *R. maculatus* ticks, and *Theileria velifera* along with *Rickettsia africae* in *A. eburneum* ticks was recorded for the first time. Molecular evidence in this study suggests that there is a broad diversity of novel *Rickettsia* and *Ehrlichia* species that occur in ticks within the SHNR. Moreover, unique tick diversity and previously unknown associations between *R. maculatus* and *A. eburneum* ticks with bacterial pathogens were identified. As such, the importance of routine systematic efforts to monitor both known and novel pathogens that are likely to emerge in the future is re-emphasised.

Key words: Surveillance, tick-borne pathogens, diversity

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LIST OF ABBREVIATIONS

EIDs – Emerging Infectious Diseases

TBDs – Tick-Borne Diseases

ICIPE – International Centre of Insect Physiology and Ecology

ML-EID laboratory – Martin Lüscher Emerging Infectious Disease laboratory

IUCN – International Union for Conservation of Nature

PCR – Polymerase Chain Reaction

GPS – Global positioning system

HRM Analysis – High Resolution Melting Analysis

SHNR – Shimba Hills National Reserve

RT-PCR – Reverse Transcription-PCR

cDNA – Complementary DNA

CHAPTER ONE

INTRODUCTION AND LITEREATURE REVIEW

1.0 General Introduction

Ticks are important arthropod vectors and reservoirs of a broad range of pathogens including viruses, bacteria, and protozoa which are capable of causing diseases in humans, livestock and wildlife (Crowder et al., 2010; Estrada-Peña & Jongejan, 1999). Their interaction with different vertebrate hosts ranging from mammals, birds and reptiles presents them with an opportunity of acquiring an array of different pathogens that may be harboured in these hosts (Basu et al., 2012). Unlike insects, they are exclusively parasitic obligatory blood feeders at all stages of their life cycle.

Ticks and tick-borne pathogens are on the rise marked with increasing incidences of human illnesses annually throughout the world (Dantas-Torres et al., 2012; Piesman & Eisen, 2008). Similarly in global livestock production, ticks and tick-borne pathogens are a major animal health concern with an estimated 80% of the world's cattle being infested with ticks (Bowman & Nuttall, 2004). A disturbing global trend in the emergence and re-emergence of infectious diseases is also on the increase, significantly burdening global economies with a significant proportion of these being dominated by zoonoses originating from wildlife (Jones et al., 2008; Tabish, 2009). Some of these pathogens such as *Ehrlichia* bacteria and some viruses have been identified in ticks (Lu et al., 2013).

Identification of emerging and re-emerging pathogens has recently drawn research in ticks as disease vectors and reservoirs. This has been underscored by the discovery of previously unknown mechanisms of pathogen transmission such as the non-viremic transmission of Tick-Borne Encephalitis (TBE) virus, the transovarial transmission of

Anaplasma species, and the colonisation by tick species in habitats where they were previously known to be absent.

Ticks exhibit a remarkable range of form, structure and size with three important families of ticks currently recognized (Parola & Raoult, 2001). The Ixodidae (Hard ticks) comprising 702 species, the Argasidae or "soft ticks" comprising 193 species and the Nuttalliellidae, which has only one known species (Guglielmone et al., 2010). Approximately 10% of the identified tick species are known to be pathogen carriers (Lu et al., 2013). Numerous taxonomic keys are available for identification of ticks to the species levels; however species differentiation of immature or damaged ticks may be challenging, necessitating the use of molecular methods.

Ticks have a world-wide distribution illustrating a degree of adaptation to habitat diversity. Major determinants for maintenance of their populations include availability of vertebrate hosts and host behaviour. Accordingly, human activities can shape the expansion of ticks in different ways, both by habitat modification and by altering host populations and their composition (IOM, 2011). Habitat modification by humans may result in reduced biodiversity by creating environments that favour particular hosts, vectors and/or pathogens (Foley et al., 2005).

Incidentally, the reduction of biodiversity is a risk factor linked to the emergence of infectious diseases (EIDs). Extinction of natural hosts for vectors and pathogens as well as the introduction of new hosts in areas they were previously absent may lead to altered host-vector-pathogen interactions (Munderloh & Kurtti, 2010). Additionally, ecological disturbances due to climate and habitat changes are likely to induce phenological shifts in

many species, which can encourage parasite transmission and host switching. The discovery of an active transmission of *Rickettsia rickettsii* in Arizona involving the exotic dog tick *Rhipicephalus sanguineus*, is evidence of host-switching possibilities (Munderloh & Kurtti, 2010).

Increasing incidences of both known and novel tick-borne pathogens has led to renewed of interest in ticks and tick-borne pathogens (Estrada-Peña et al., 2013). Consequently, a proper understanding of ticks and their associated pathogens is critical. With many demographic shifts accompanied with persistent human encroachment to wildlife habitats, the distribution of ticks and TBDs is likely to expand. Moreover, there has been an increase in the number of epidemiological important tick-borne pathogens with global epidemic potential thereby making it necessary to conduct routine surveillance in all countries (Estrada-Peña et al., 2012).

To better understand ticks and their associated pathogens in wild habitats, ticks were collected from Shimba Hills National Reserve (SHNR), one of Kenya's biodiversity hotspots. Human activities within and around the reserve include tourism, gum harvesting and livestock farming. These form an important wildlife/livestock/human interface, and expose humans to risks of contracting zoonotic infections. This human-animal interface is enhanced further by the conflicts between wildlife and humans due to the proximity of the reserve to human settlement.

The human-animal interface in SHNR potentially facilitates the transmission of zoonotic diseases. In addition, ticks, among other vectors may be facilitating the movement of infectious pathogens among these groups of potential hosts. Specific tick-pathogen

interactions may be facilitating the maintenance and transmission of zoonotic pathogens, hence, understanding tick biodiversity and identifying associated pathogens in SHNR should contribute to better disease control measures and have implications for other localities with intensified human - wildlife interactions.

1.1.1 General Objective

The overall objective of this study was to undertake a systematic description of ticks and tick-borne pathogens circulating within the SHNR ecosystem and to provide baseline information to inform future strategies in tick and TBD control and management.

1.1.2 Specific Objectives

The specific objectives in this study were:

1. To determine the species composition of ticks found within the SHNR.
2. To assess the prevalence of tick-borne pathogens in questing ticks collected from SHNR.
3. To estimate the molecular diversity among tick-borne pathogens indentified from the questing ticks in SHNR.

1.2 Literature review

1.2.1 Tick Taxonomy and diversity

Kenya is rich in wildlife and several communities keep domestic animals as a source of livelihood. Both wild and domestic animals support diverse populations of ticks that affect their health and production. However, a comprehensive documentation of all tick species across the various eco-regions in Kenya is not up to date, the last one having been conducted by Walker, 1974. The lack of such recent information is likely to undermine the control of diseases transmitted by ticks. Additionally, climate-change associated shifts alongside frequent contact of humans, livestock and wildlife could exacerbate TBDs occurrence.

Despite the increasing interest in ticks and their associated pathogens, adequate taxonomic expertise on tick identification is still not advanced in Kenya. Tick identification has remained largely morphological, although identification based on DNA and proteomic analysis is also gaining importance (Estrada-Peña et al., 2013). Ticks closely resemble mites along with spiders and can be classified taxonomically according to the following hierarchy;

Phylum: Arthropoda

Class: Arachnida

Order: Acarina

Sub-order: Ixodoidea

Family: Argasidae, Ixodidae, Nuttalliedae

Ixodidae and Argasidae are the main families of ticks with Nuttalliedae comprising of a single species confined to southern Africa identified to-date (Guglielmone et al., 2010). There are fourteen genera within the Ixodidae family with approximately 702 species, while the family Argasidae has approximately 193 species but their genera status is not fully resolved (Guglielmone et al., 2010).

Studies on the phylogeny of tick families has been progressing over the years especially with the application of molecular markers in tick identification and phylogenetic analyses (Lu et al., 2013). A consensus on the phylogenetic relationship of the three tick families despite several changes in the nomenclature of some species is outlined in the figure below.

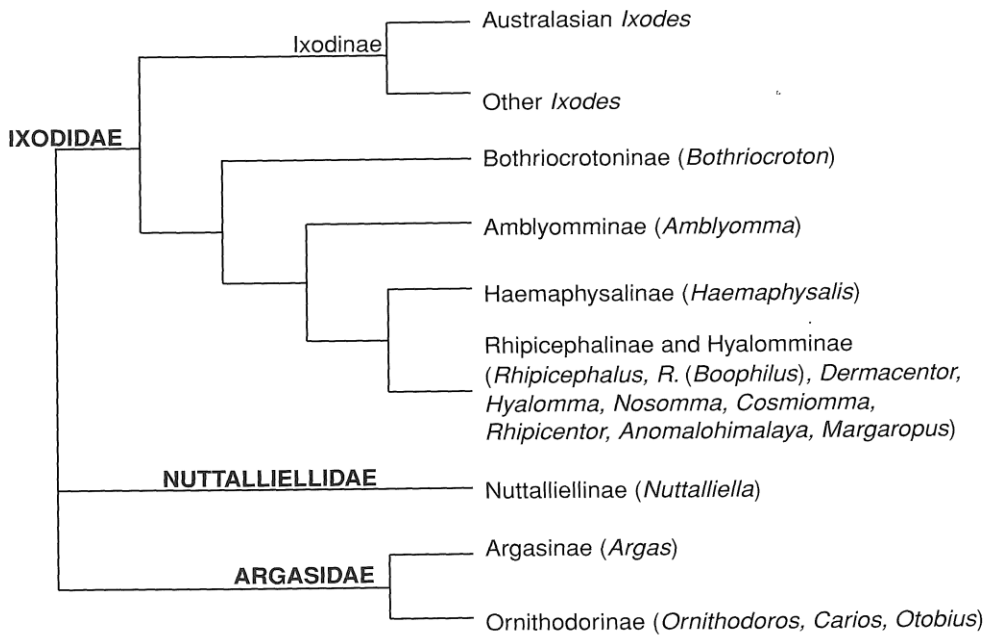

Figure 1: Current hypothesis of the phylogeny of tick families indicating the relationships between different tick taxa proposed according to analyses on nucleotide sequences as well as phenotypes (Barker & Murrell, 2008).

1.2.2 Argasidae

These are generally referred to as ‘soft ticks’ because they lack a hard plate called scutum on their dorsal surface that is present in hard ticks. Species of this genus have a worldwide distribution. They are dorso-ventrally flattened with a general oval outline, although their shape varies between species. Medically important soft-tick species belong to the genus *Ornithodoros* (Walker et al., 2003).

1.2.3 Ixodidae (Hard ticks)

Ixodid ticks also known as ‘hard ticks’ have a worldwide distribution but are more frequent in the temperate world than soft ticks. All hard ticks have a sclerotized dorsal plate that is absent in soft ticks. Adults are dorso-ventrally flattened and oval in shape (Service, 2012). Medically important species in this genus include *Ixodes, Amblyomma, Dermacentor, Haemaphysalis, Rhipicephalus,* and *Hyalomma.*

1.2.4 Ticks as vectors and reservoir of disease pathogens

Ticks transmit a greater variety of infectious pathogens to man and animals than any other haematophagous arthropods (Estrada-Peña & Jongejan, 1999). These pathogens range from viruses and bacteria to protozoa (Crowder et al., 2010). Ticks have been rated to be second only to mosquitoes in their importance as disease vectors of human pathogens (Parola & Raoult, 2001).

An estimated 38 viral species belonging to at least 6 families and 9 genera are transmitted by ticks in a fairly highly specific tick-virus-host relationship (Labuda & Nuttall, 2004). Important tick-borne viral diseases include Crimean-Congo hemorrhagic fever caused by a *Nairovirus* from the Bunyaviridae family transmitted by ticks within the *Ixodid* and

Hyalomma genera. Tick-borne Borne Encephalitis (TBE) which is endemic to Europe and Asia and is caused by a *Flavivirus* transmitted by *Ixodes scapularis* (Lindquist & Vapalahti, 2008).

Tick-borne rickettsioses are among the oldest known vector-borne zoonoses (Parola et al., 2005) with Rocky Mountain spotted fever being the main life threatening rickettsial infection and primarily transmitted by *Dermacentor variabilis* in the USA. Other important tick-borne rickettsia genera include *Anaplasma spp.* and *Ehrlichia spp. Rickettsia africae* the causative agent of African tick fever, is transmitted by *Amblyomma hebraeum* (Estrada-Peña & Jongejan, 1999).

Tick-borne bacterial diseases include Lyme disease, a worldwide bacterial infection caused by *Borrelia burgdorferi* and transmitted by Ixodes ticks (Grubhoffer et al., 2005). Lyme disease has been reported in Northern Africa, and whereas its occurence in sub-saharan africa is poorly known, two cases were described in Kenya (Jowi & Gathua, 2005). Tick transmitted protozoan infections such as babesiosis caused by *Babesia* sp. infect both human and animals. *Babesia* is mainly transmitted by *Ixodes scapularis* ticks (Hunfeld, et al., 2008) however in Kenya, *Boophilus* ticks are the principal vectors (Wesonga et al., 2010). *Theileria* spp are apicomplexan protozoan parasites responsible for huge losses in livestock and are transmitted by various ixodid tick species of the genera *Rhipicephalus* and *Haemaphysalis* (Norval et al., 1992).

In addition to being involved in pathogen transmission, ticks also act as reservoirs of various pathogens including *Francisella tularensis,* spotted fever group rickettsiae and recurrent fever borreliae (Parola & Raoult, 2001). Sprong et al., (2009) observed a nearly

constant annual prevalence rate of *Rickettsia helvetica* in *Ixodes ricinus* ticks of all stages collected from the Netherlands, where they observed a seasonal fluctuation in nymphs and adults collected in the same locality for *Borrelia* infection. They explained their findings by proposing that *I. ricinus* is a major reservoir for *R. helvetica.* The ability of ticks in maintaining pathogenic agents has been attributed to transstadial and transovarial transmission mechanisms.

1.2.5 Tick – host – pathogen interactions

The occurrence of ticks in a particular geographic area is determined by the presence of suitable hosts which may also act as carrier (reservoir hosts) of pathogens and thus capable of infecting naïve ticks (Estrada-Peña et al., 2013). Ticks acquire pathogens when feeding on an infected reservoir host but the capacity of a tick to transmit the pathogen will depend on whether the pathogen is capable of surviving and multiplying within the tick.

Most pathogens possess various adaptations which enable them be maintained within the tick vector thus ensuring their successful transmission (Labuda & Nuttall, 2004). For example, most tick-borne bacteria express surface proteins which function as adhesion molecules onto tick cells. Moreover, others including *Anaplasma marginale* grow within membrane bound vacuoles of host cells (Kocan et al., 2004) . In addition, the ability of some pathogens to infect and multiply within tissues such as ovaries enables successful transovarial transmission (Howell et al., 2007).

1.2.6 The Human/Animals and Ticks interface

The increase in human population has led to land use changes leading to extensive modification of ecosystems. Human encroachment of wildlife habitats and virgin land has lead to significant loss of biodiversity as land is converted into crop and pasture land (Foley et al., 2005). Such land use changes have favoured the establishment and expansion of ticks as well as adaptable hosts (both wild and new domestic hosts) for ticks. Moreover, reduced biodiversity sets a stage for host-switching as a result of depletion of natural hosts (IOM, 2011). Consequently, human beings and animals interact with ticks frequently. This has provided opportunities for pathogens from the wild to spill over to domestic animals and human beings (Jones et al., 2013).

1.2.7 Emerging and re-emerging infectious diseases (EIDs)

At the turn of the new millennium, infectious diseases accounted for 25% of global annual deaths (Feldmann et al., 2002; Morens et al., 2004). More than a decade later, this trend seems to be barely improving despite significant strides in public health backed by an enormous scientific base and technological advancements. There has been a steady stream of newly recognized (emerging) infectious diseases, while previously recognized (re-emerging) infectious diseases that were thought to be under control have persisted (Satcher, 1995; Fauci, 2005).

The definition of an emerging infectious disease (EID) has remained to some extent subjective. Nonetheless, an EID can be defined as one which has newly appeared in the population or has existed before but is rapidly increasing in incidence or geographic range (Morse, 1995). More than 60% of EID have been shown to be zoonotic with a majority of these originating from wildlife (Taylor et al., 2001).

A review on the circumstances under which the most notable emerging infectious diseases have occurred indicates that EIDs have remained a continuous challenge to man in all millennia (Morens et al., 2008). This has repeatedly caused immense global consequences in terms of public health and global economies as EIDs emerge and re-emerge periodically. Despite their consequences, EIDs have remained neglected for a long time with significant attention only being given to them in the past two decades (Morens & Fauci, 2012).

Recent scientific advances have redefined the concept and focus of research in the area of EIDs, promising important breakthroughs in the fight and management of EIDs (Morens & Fauci, 2012). For example, recent molecular-genetic techniques have greatly facilitated the discovery of EIDs in a timely and cost effective manner. They have also enabled easy active surveillance to determine frequency and distribution of infectious pathogens in the environment (Brooks & Hoberg, 2006; Crowder et al., 2010). Moreover, a previously inconceivable genomic diversity among microbes has been revealed by whole-genome sequencing technologies (Fraser et al., 2000).

Ticks have received significant attention in the recent years as EIDs threat due to the increasing number of tick-associated pathogens. More than 15 new tick-borne pathogenic bacteria including *Rickettsia aeschlimannii* and *Ehrlichia chaffeensis* have been discovered in the last 20 years (Parola et al., 2005). The number of new viruses identified from ticks has also been increasing drastically. Recently described pathogenic viruses include a haemorrhagic fever causing tick-borne virus from *Haemaphysalis* ticks in China (Zhang et al., 2011). With the current trend of discoveries, it can be speculated that there are yet more tick transmitted pathogens to be identified.

1.2.8 Evolutionary basis of EIDs

With ticks being among the important vectors of emerging zoonotic infections in recent times, an understanding of how they influence the evolution of their associated pathogens that might lead to outbreaks is critical. EID outbreaks may involve the biological expansion due to population increase or geographic spread of parasites and pathogens from their original areas into new areas or the ecological changes resulting in phenological shifts among species (Gorškov et al., 2000). The latter has been a primary mechanism for the spread of the pathogens in the past, in addition to ecological changes due to climate change (Hoberg, 1997).

When hosts and their associated pathogens move outside their areas of origin and ecosystems, host-switching may occur in otherwise phylogenetically conserved host-parasite interactions, through a process known as ecological fitting (Brooks & Hoberg, 2007). Ecological fitting enables rapid host-switches especially when parasites and hosts colonize and persist in new environments where they are exposed to novel resource use. In this scenario the parasites and pathogens do not necessarily evolve novel host resource utilization capabilities, but rather employ a suite of traits that they carry at the time they encounter novel conditions. This has been argued to be the evolutionary basis of EIDs (Brooks & Ferrao, 2005).

Nevertheless, whether host-switching occurs in ticks has been a subject of debate for a long time because this question has an important bearing in understanding tick population dynamics, changing tick biodiversity, circulation and evolution of associated pathogens (McCoy et al., 2013). Recent studies have however continued to highlight the increased

possibilities of host-switching in ticks based on observations of zoonotic tick-borne pathogens in unlikely hosts apart from their normal reservoir hosts (Munderloh & Kurtti, 2010; Paștiu et al., 2012).

1.2.9 Emerging infectious diseases factors

Increasing anthropogenic activities have largely contributed to the current trends of EIDs. The escalating human population has led to worldwide changes in land use patterns that have incurred global consequences (Foley et al., 2005). Among them is biodiversity loss, which is intrinsically intertwined with climate change, both of which have been accelerated by human activities.

This has led to altered tick population structures as well as their boundaries in recent years. The habitat range for generalist tick species has been observed to continue increasing whereas certain tick species which are co-distributed with their endangered host species have been faced with a decreasing geographical and habitat range (Paștiu et al., 2012). It is therefore worth noting that the consequences of biodiversity loss is not only extinction of species, but is also a crisis of introduced species and emerging diseases (Brooks & Hoberg, 2006).

Frequently identified factors contributing to disease emergence include ecological changes involving those due to agricultural or economic development, human demographics and behaviour, international travel and commerce, technology and industry, microbial adaptation and change, and changing ecosystems (Morse 1995; Morens et al., 2008).

1.3.0 Tick Ecology and climate change

Ticks have to adapt to feeding on their host and surviving on the physical environment. Availability of hosts and host behaviour is important in maintaining tick populations because suitable hosts are required for reproduction by adults. Consequently, the distribution of hosts will influence the distribution of ticks on them (Estrada-Peña et al., 2013). The physical environment is an important factor for ticks during the moulting and questing stages which are affected with adverse environmental conditions (Walker et al., 2003).

Tick life cycle dynamics are modulated among other factors by the complex interaction between climate, hosts, and landscape characteristics (Estrada-pena, 2009). Climate has a major impact on the dynamics of tick populations as it directly and indirectly affects the availability of hosts, and the conditions of the physical environment. Thus, the recent and largely man-made ecological disturbances that have resulted in global climate changes are heavily shaping the distribution of ticks world-wide. These changes have promoted the establishment and expansion of the geographical range with conditions favourable to survival of ticks and other arthropod vectors (Munderloh & Kurtti, 2010).

CHAPTER 2

MATERIALS AND METHODS

2.0 Study site

This study was conducted in Shimba hills (-4.25S 39.39E) which is a protected area that was first gazetted as a forest reserve in 1903, and subsequently gazetted as a national reserve in 1968. The International Union for Conservation of Nature (IUCN) lists the Shimba hills area in the world database of protected areas as IUCN category II - which are areas managed mainly for ecosystem protection and recreation. It lies within the eastern African coastal forest eco-region, an area with high species endemism (Younge et al., 2002).

The geography of Shimba hills consists of a plateau that ascends steeply from the coastal plains and an escarpment. It has an area of approximately 22,425 ha and an altitude range between 95 – 466 meters. The climate is hot and humid but made cooler by sea breezes with an average temperature of 24ºC. It has an exceptionally rich flora, some of which is endemic to Shimba, and diverse fauna making it an important conservation site. Nevertheless significant human/animal conflicts are a common occurrence mainly from baboons and elephants straying from the reserve boundaries to the surrounding homesteads. These conflicts and other human activities within the reserve such as camping and adventure walks by tourists make interactions between human and wildlife frequent.

2.1 Tick collection and identification

Ticks were collected in three field sampling trips during the dry and wet seasons in 2013 and 2014. The collection dates were October 4th – 6th, 2013; November 24th, 2013 – December 2nd, 2013 and March 26th – 29th, 2014 for the 1st, 2nd, and 3rd trips respectively. Questing ticks were collected from six sites that were purposefully chosen within the logistical constraints, and included host resting areas and burrows, host routes and areas surrounding watering holes. The sampling sites (Figure 2); Marere (MR), Buffalo-ridge (BR), Campsite (CS), Kivumoni (KV), Kidongo (KD), Airstrip (AR) were mapped by determining their GPS locations.

Collection methods included flagging, hand picks from vegetation and CO_2 trapping using improvised CO_2 traps (Figure. 3). Following collection, the ticks were transported live in falcon tubes plugged with cotton swabs to the Martin Lüscher Emerging Infectious Disease (ML-EID) laboratory at the International Centre of Insect Physiology & Ecology (*icipe).*

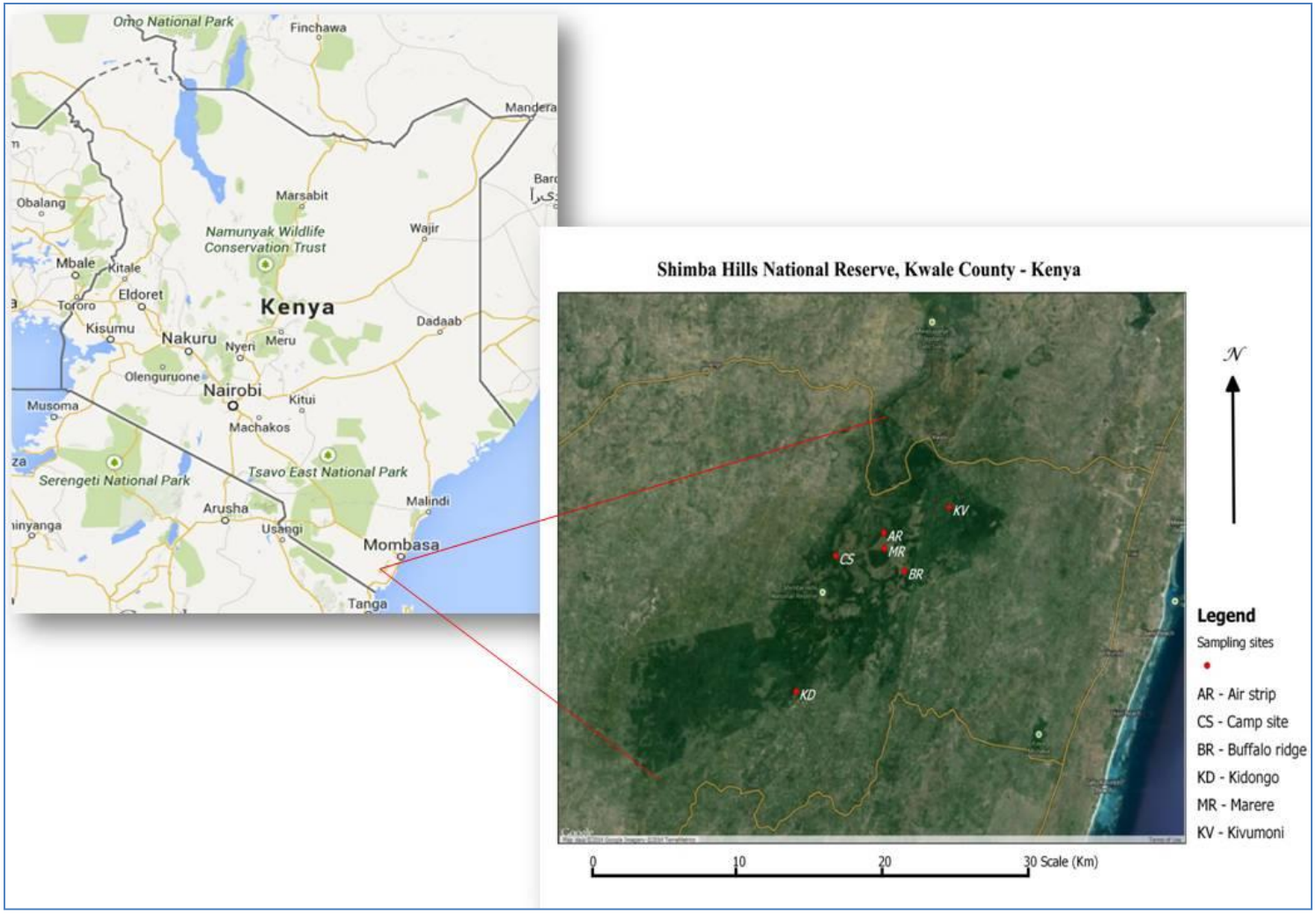

Figure 2: Map of Shimba Hills indicating the sampling sites from where ticks were collected (Map data © Google 2015)

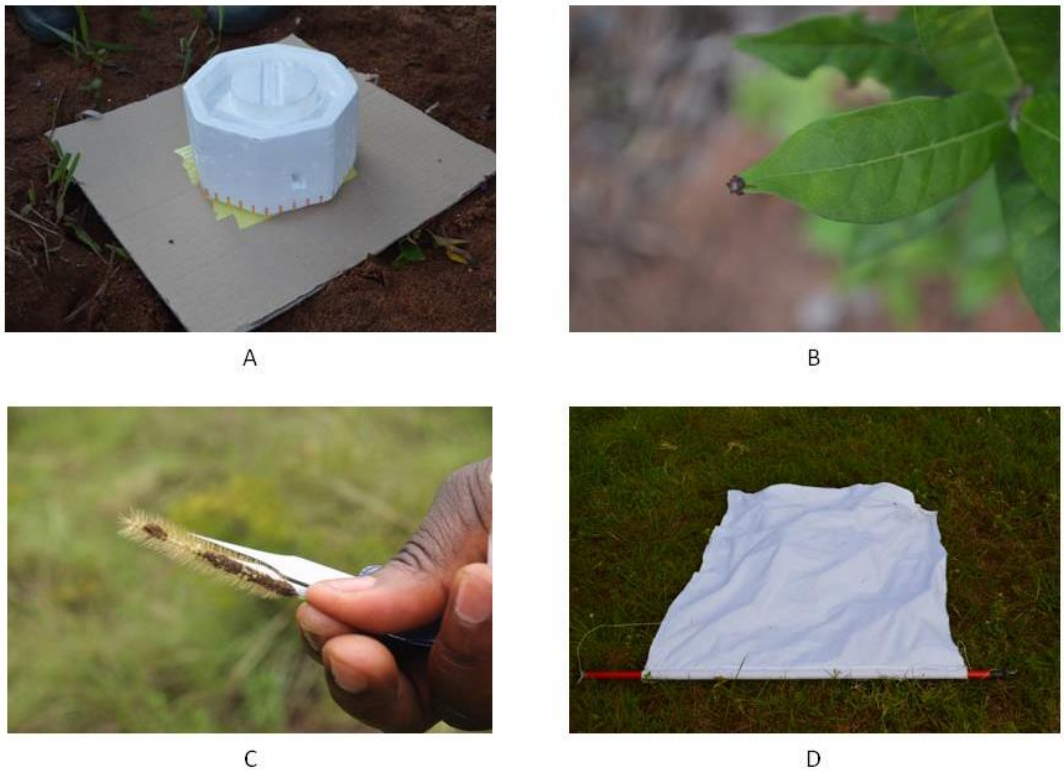

Figure 3: Different tick collection Methods that were used to collect ticks for this study.

Panel A: Improvised CO_2 trap made of perforated Styrofoam container with a cardboard base. Dry ice was placed inside the container and left for at least three hours. Ticks that drew near the traps were picked using a pair of forceps.

Panel B: Questing tick on a leaf; ticks were collected from leaf blades using a pair of forceps.

Panel C: Questing tick larvae on grass inflorescence. These were maintained in falcon tubes before sorting in the lab.

Panel D: Tick flagging cloth: A white piece of cloth (1 M^2) was dragged along 100 m transects on ground vegetation and then inverted to collect ticks from the underside using forceps.

2.2 Tick identification

Both morphological characteristics as well as molecular analyses were used to identify ticks as described in the sections below.

2.2.1 Tick morphological identification

Identification of ticks was done using morphological characteristics based on modified procedures described by Walker et al., 2003. Ticks were first sorted to distinguish different developmental stages and genera based on taxonomic keys. Adult ticks were staged and photographed before plucking a leg for use in tick molecular characterization.

2.2.2 Tick molecular identification

2.2.2.1 DNA extraction from ticks

Tick genomic DNA was extracted using the Hot Sodium Hydroxide and Tris (HotSHOT) protocol that is used to prepare PCR-quality genomic DNA (Truett et al., 2000). Briefly, one tick leg was plucked off and a small snip (approximately 0.2 cm) was placed in a PCR tube and 40 µl of alkaline lysis reagent (25 mM NaOH, 0.2 mM Na_2EDTA) was added. The samples were then heated to 95 ºC for 30 min and cooled to 4ºC for 15 min before adding 40 µl of neutralization buffer (40 mM Tris pH 5). The tick DNA samples were maintained at 4 ºC before PCR amplifications of the 16S rRNA, ITS2 and CO1 genes.

2.2.2.2 PCR amplification of 16S rRNA, ITS 2 and CO1 genes

Amplification of a 630 bp CO1 gene fragment, a 1500 bp ITS2 gene fragment and a 500 bp 16S rRNA gene fragment was done for each of the tick DNA extracts using specific primer pairs listed in Table 1. The PCR reactions were performed in 10 µl

volumes that included 1 µl of DNA template, 2 µl of 5x HOT FIREPol® Blend Master Mix (SOLIS BIODYNE) and 0.5 µl of 10 mM forward and reverse primers. PCR grade water was used to bring the total reaction volume to 10 µl. DNA Engine® Dyad Peltier Thermal Cycler (BIO-RAD) was used for the PCR amplifications with the following cycling conditions: Initial denaturation at 95 ºC for 15 min followed by 35 cycles of denaturation at 95 ºC for 20 s, annealing at 55 ºC (16S rRNA and CO1) and 65 ºC (ITS2) for 30 s and 1 min extension at 72 ºC. Final extension was at 72 ºC for 1 min. A non-template control was included in each run. Following amplification, 2 µl of the PCR products was resolved in a 1 % (W/V) agarose gel by electrophoresis using 1x TAE as the running buffer at 90 V for 40 min. The Solis Biodyne ready to load 100 bp ladder was loaded and run alongside the amplified samples as a molecular size DNA marker. The gels were stained with Ethidium Bromide and visualised under UV transillumination.

Table 1: Sequences of oligonucleotide primers used for PCR amplification of tick DNA

Gene	Primer ID	Sequence	Reference
16S rRNA	F-RMI16S	5′-AATTGCTGTAGTATTTTGAC-3′	(Brahma et al., 2014)
	R-RMI16S	5′-TCTGAACTCAGATCAAGTAG-3′	
CO1	LepF1	5'- ATTCAACCAATCATAAAGATATTGG-3'	(Hebert et al., 2004)
	LepR1	5'- TAAACTTCTGGATGTCCAAAAAATCA-3'	
ITS 2	F1-ITS2	5′-CGAGACTTGGTGTGAATTGCA -3′	(Chitimia et al., 2009)
	R1-ITS2	5′-TCCCATACACCACATTTCCCG -3′	

2.3 Total Nucleic Acid extraction from ticks

All the field collected ticks were pooled into groups of 3-8 adults, 8-15 nymphs and 30 larvae. The pools were homogenized using a lysing matrix consisting of 750 mg of 2.0 mm and 120 mg of 0.1 Yttria stabilized Zirconium Oxide (Zirconia/Yttria) beads (Glen Mills, Clifton NJ) in 0.5 ml screw caps as previously described (Crowder et al., 2010). Homogenization was done in 360 µl PBS buffer (pH =7.4) using a mini beat-beater-16 (Biospec products Inc) for 90 seconds. Two hundred microlitres of the homogenate was transferred into a 96-well MagNA pure cartridge (Roche) under a biosafety cabinet. *Hazara*, and *Dhori* virus cell culture isolates (200 µl of each) were used as positive controls while sterile water was used as a negative control. The total nucleic acid extraction was done on 93 pools per single run using DNA/RNA small volume kit (Roche) in a MagNA pure 96 robotic work station.

2.4 Reverse Transcription (RT)

RNA was reverse-transcribed into cDNA in 96-well PCR plates using the High Capacity cDNA RT kit (Applied Biosystems) in 10 µl reaction. The reaction master mix comprised of 1 µl of 10x RT buffer, 0.4 µl of 100mM dNTP mix, 0.25µl RNase inhibitor (Roche), 1 µl of 0.6mM non-ribosomal hexamers (Endoh et al., 2005), 0.25 µl Reverse Transcriptase and 5.0µl of template. PCR grade water was used to bring the reaction volume to 10 µl. First strand cDNA was synthesized in an Eppendorf MasterCycler® thermal cycler with the following temperature regime; 25 ºC for 10 min, 37 ºC for 2 hrs, 85 ºC for 5 min and a final Hold of 4 ºC. The cDNA was stored at -20 ºC for later use.

2.5 Molecular detection of tick-borne pathogens

Tick-borne pathogens were screened using PCR-HRM assays on a Rotor Gene Q cycler (Qiagen).

2.5.1 Viral pathogen screening

A multiplex PCR (See appendix 1 for cycling conditions) consisting of virus primer sets for *Flavi-, Alpha-, Nairo-, Phlebo-, Orthobunya-, and Thogoto*-viruses (Table 2) was used to screen ticks for the respective viruses followed by High resolution Melting (HRM) analysis. The reaction master mix included 5 µl of 2x MyTaq™ Mix (Bioline), 0.7 µl of primer mix, 1 µl of SYTO® 9 Green Fluorescent Nucleic acid stain (life technologies™) and 1 µl of cDNA template. PCR grade water was used to top up the reaction volume to 10µl. HRM analysis was performed between 75 ºC to 95 ºC with increments of 0.1 ºC done for 2 sec each.

2.5.2 Protozoan and bacterial pathogen screening

A similar protocol was applied to screen ticks for both protozoan and bacterial pathogens. The PCR-HRM assays were performed in single runs of 10 µl volumes that included 5x HOT FIREPol® EvaGreen® HRM mix (ROX) and 1 µl of template DNA. The primers used in the screening are listed in the Table 2. All thermo-cycling was done using the Rotor-Gene Q cycler (Qiagen) and melting profiles assessed with Rotor-Gene Q series Software 2.1.0 (Build 9). PCR conditions included and initial enzyme activation at 95 ºC for 15 min, followed by denaturation at 94 ºC for 20 sec. Annealing took 25 seconds with decrements of 1℃ on each cycle from 65℃ to 55℃ followed by an extension step at 72℃ for 30 sec. At the end of PCR cycling, the products were maintained at 72℃ for 3 minutes before proceeding with high resolution melting (HRM) analysis by increasing

the temperature from 75℃ to 95℃ at 0.05 ℃ increments with fluorescence acquisition after 2 sec incremental holding periods. Melt rates were visualized with pre- and post-melt fluorescence signals normalized to values of 100 and 0 respectively.

2.5.3 Sequencing of PCR amplicons

PCR products were first cleaned prior sequencing using ExoSap-IT purification protocol. Briefly, 5 µl of the PCR product was mixed with 0.5 µl of Exonuclease I (Thermoscientific) and 1 µl of FastAP™ Alkaline Phosphatase (Thermoscientific) in a 0.2 ml PCR tube. The mixture was gently vortexed and centrifuged then incubated at 37 ºC for 15 min. The reaction was stopped by heating at 85 ºC for 15 min and the samples were submitted for sequencing using the Sanger platform which was an outsourced service from Macrogen Inc. (http://www.macrogen.com/eng/).

Table 2: Sequences of oligonucleotide primers used to detect tick-borne pathogens

Pathogen	Primer name	Primer Sequence	Reference
Ehrlichia sp	16S rRNA- F	CACCTCAGTGTCAGTATCGAACCA	(Tokarz et al., 2009)
	16S rRNA-R	CGTAAAGGGCACGTAGGTGGACTA	
Anaplasma sp.	16S rRNA- F	GGGCATGTAGGCGGTTCGGT	(Tokarz et al., 2009)
	16S rRNA- R	TCAGCGTCAGTACCGGACCA	
Rickettsia sp.	rpmE *Rickettsia* F	TTCCGGAAATGTAGTAAATCAATC	(Nijhof et al., 2007)
	rpmE *Rickettsia* R	TCAGGTTATGAGCCTGACGA	
Theileria sp. and Babesia sp.	RLB-F	GAGGTAGTGACAAGAAATAACAATA	(Georges et al., 2001)
	RLB-R	TCTTCGATCCCCTAACTTTC	
Coxiella burnetti	IS1111F	GCTCCTCCACACGCTTCCAT	(Tokarz et al., 2009)
	IS1111R	GGTTCAACTGTGTGGAATTGATGAGT	
Phlebovirus	Phlebo JV3a F	AGTTTGCTTATCAAGGGTTTGATGC	(Lambert & Lanciotti, 2009)
	Phlebo JV3b F	GAGTTTGCTTATCAAGGGTTTGACC	
	Phlebo JV3 R	CCGGCAAAGCTGGGGTGCAT	
Nairovirus	Nairo L 1a F	TCTCAAAGATATCAATCCCCCCITTACCC	(Villinger et al., manuscript in preparation)
	Nairo L 1b F	TCTCAAAGACATCAATCCCCCTTWTCCC	
	Nairo L 1a R	CTATRCTGTGRTAGAAGCAGTTCCCATC	
	Nairo L 1b R	GCAATACTATGATAAAAACAATTMCCATCAC	
	Nairo L 1c R	CAATGCTGTGRTARAARCAGTTGCCATC	

	Nairo L 1d R	GCAATGCTATGGTAGAAACAGTTTCCATC	
	Nairo L 1e R	CRAKGCTGTGGTAAAAGCAGTTRCCATC	
Orthobunyavirus	Bunya group F	CTGCTAACACCAGCAGTACTTTTGAC	(Lambert & Lanciotti, 2009)
	Bunya group R	TGGAGGGTAAGACCATCGTCAGGAACTG	
Alphavirus	Vir 2052 F	TGGCGCTATGATGAAATCTGGAATGTT	(Eshoo et al., 2007)
	Vir 2052 R	TACGATGTTGTCGTCGCCGATGAA	
Flavivirus	Flavi JV2a F	AGYMGHGCCATHTGGTWCATGTGG	(Villinger et al., manuscript in preparation)
	Flavi JV2b F	AGCCGYGCCATHTGGTATATGTGG	
	Flavi JV2c F	AGYCGMGCAATHTGGTACATGTGG	
	Flavi JV2d F	AGTAGAGCTATATGGTACATGTGG	
	Flavi JV2a R	GTRTCCCADCCDGCDGTRTCATC	
	Flavi JV2b R	GTRTCCCAKCCWGCTGTGTCGTC	
Thogotovirus	Thogoto S6 F	GATGACAGYCCTTCTGCAGTGGTGT	(Villinger et al., manuscript in preparation)
	Thogoto S6 R	RACTTTRTTGCTGACGTTCTTGAGGAC	
	Dhori S5 F	CGAGGAAGAGCAAAGGAAAG	(Villinger et al., manuscript in preparation)
	Dhori S5 R	GTGCGCCCCTCTGGGGTTT	

2.6 Data analyses

Tick sequences were viewed and edited using the Geneious software (Version 7.1.4). Clustal*W* was used to align the sequences (Larkin et al., 2007). The Geneious software Version 7.1.4 was also used to analyse the pathogen sequences. The sequences were then blasted against the National Centre for Biotechnology Information (NCBI) database available at http://www.ncbi.nlm.nih.gov/ for similarities to sequences in the GenBank and identity confirmation.

For the phylogenetic analysis, nucleotide similarities were computed using Geneious software and phylogenetic relationships estimated with the maximum likelihood (ML) method with 1000 replications in the bootstrap test. jModelTest2 program (Darriba et al., 2012) was used to choose the nucleotide substitution models applied to construct phylogenetic trees.

An estimate of the true pathogen prevalence was computed using the frequentists' method applied in calculating the maximum likelihood (ML) estimates of pooled prevalence and confidence limits (Cowling et al., 1999). A generalized linear model (GLM) was employed due to the variable pool sizes in the data (Williams & Moffitt, 2001; Speybroeck et al., 2012). Pooled prevalence was calculated using the Epitools epidemiology calculators, an online tool available at http://epitools.ausvet.com.au. Uncertainties regarding the test characteristics are not incorporated in the computation and the method assumes 100% sensitivity and specificity for diagnostic tests.

CHAPTER 3

RESULTS

3.0 Tick distribution and abundance

A total of 4,324 ticks representing 209 adult ticks, 586 nymphs and 3502 larvae were collected from the 6 sites in Shimba hills national reserve (Figure 1). Of the adult ticks, three species from 2 genera of the Ixodidae family were identified by morphological examination (Figure 3 & 4) and these included; (number of ticks in parenthesis) *Amblyomma eburneum* (118), *Amblyomma thollonii* (3) and *Rhipicephalus maculatus* (88).

There was a variation in the numbers of ticks collected between the different sampling points and during the periods of collection. Highest number of all ticks growth stages was collected from the buffalo ridge which is an extensive grassland area.. The total numbers of ticks from the different sites are summarised in Table 3. Ticks collected from Kivumoni area were mainly *Rhipicephalus maculatus* and were mainly collected during the second trip coinciding with the short rainy season. The numbers of immature *Amblyomma sp.* were high on all occasions during collection, particularly at the buffalo ridge. A majority of the adult *Amblyomma eburneum* ticks were collected during the dry season just before the long rains season. The adult species of *Amblyomma thollonii* also known as the elephant tick were obtained from the camping site, an area frequented by elephants.

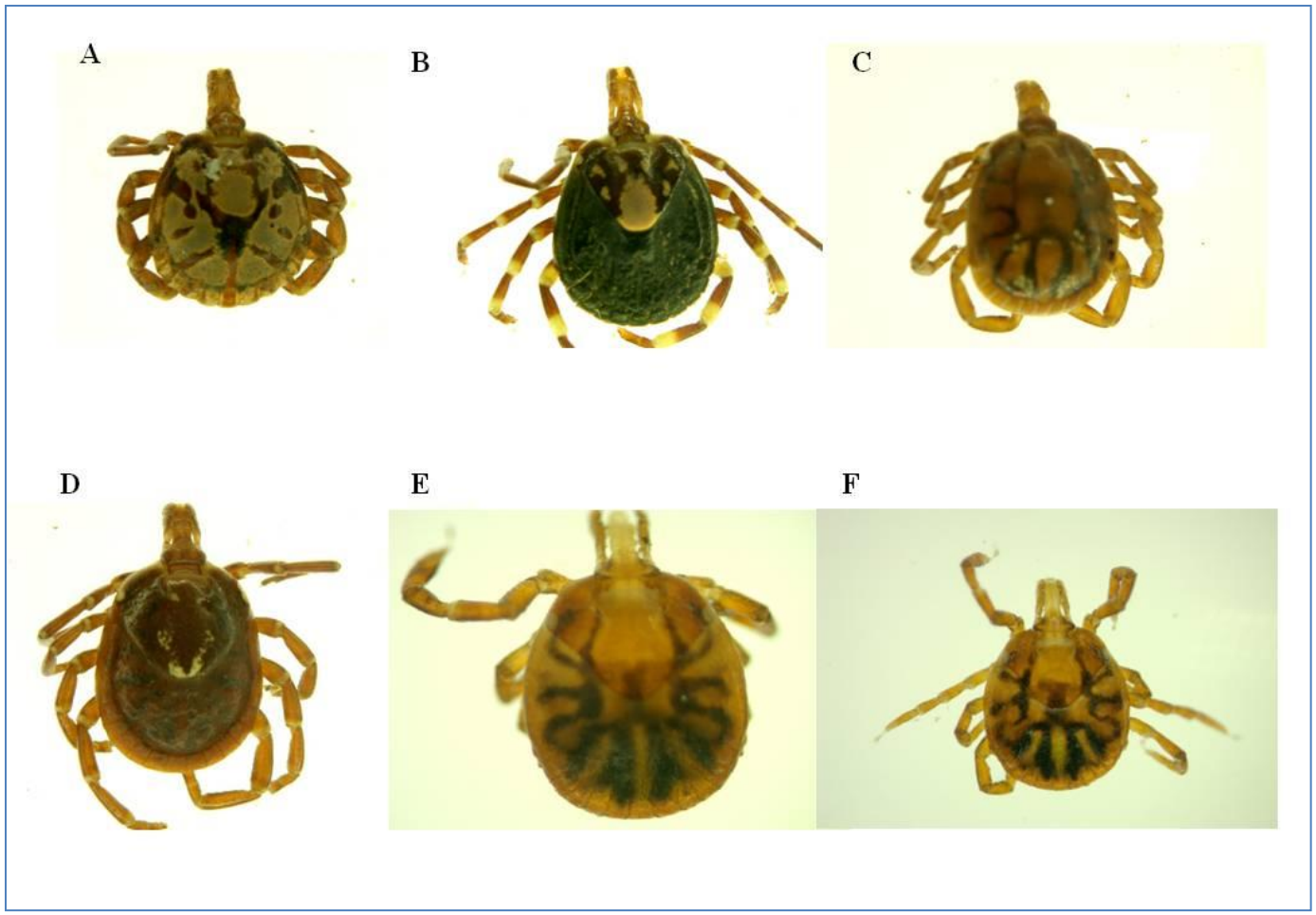

Figure 4: Photographed images of selected Amblyomma ticks collected from Shimba Hills in this study.

The tick species are illustrated as follows: **Panel A:** *A. eburneum*, male; **Panel B:** *A. eburneum*, female; **Panel C:** *A. thollonii*, male; **Panel D:** *A. thollonii*, female; **Panel E:** *Amblyomma sp.* Nymph; **Panel F:** *Amblyomma sp.* Larvae;

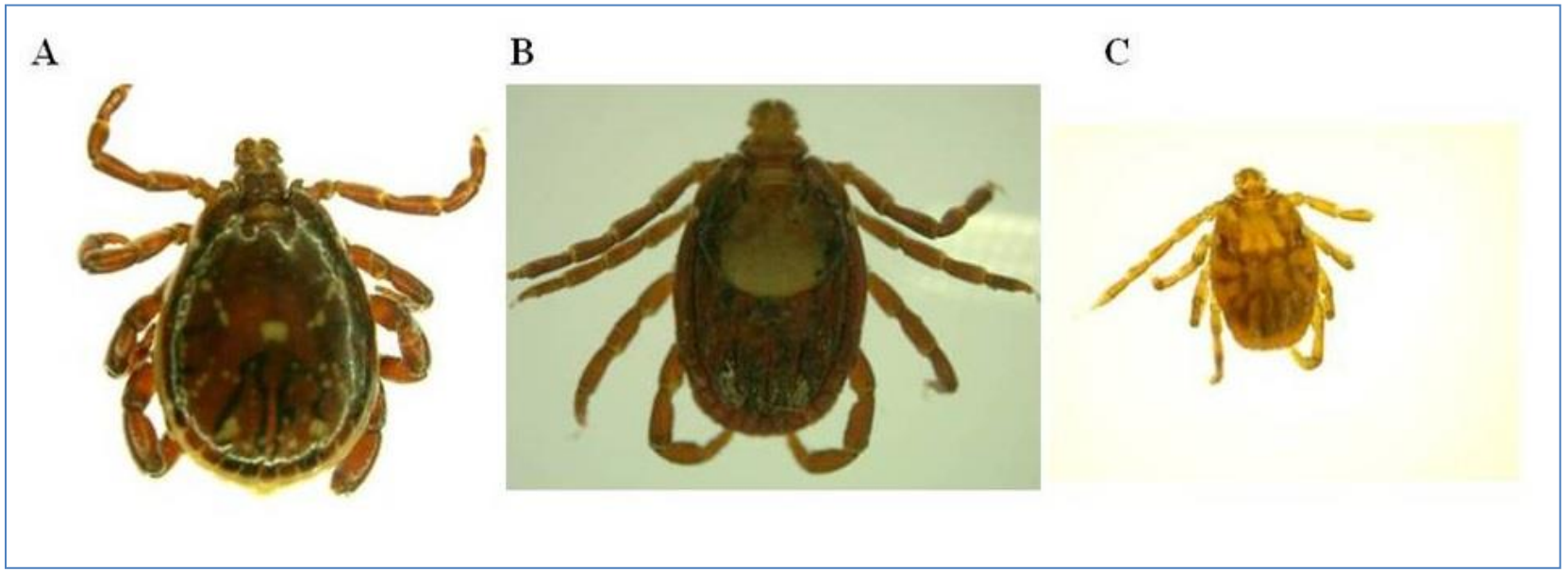

Figure 5: Photographed images of selected *Rhipicephalus* ticks collected from Shimba Hills in this study.

The ticks are illustrated as follows: **Panel A:** *R. maculatus*, male**; Panel B:** *R. maculatus*, female**; Panel C:** *Rhipicephalus sp.* nymph

Table 3: Total number of ticks collected from the different sampling sites in Shimba Hills National Reserve.

Location	No. of ticks			
	Adults	**Nymphs**	**Larvae**	**Total**
Marere	7	14	338	359
Buffalo ridge	119	312	3060	3491
Campsite	12	170	90	272
Kidongo	1	4	0	4
Kivumoni	67	23	0	90
Airstrip	20	47	14	81

3.1 Molecular identification and tick phylogeny

Representative individuals across the tick species were subjected to molecular characterization. Nymphs and larvae that had been identified only to genus level were also included in this analysis to assign their species level identity based on the molecular data. PCR amplification of 16S rRNA, ITS 2 and CO1 gene was successful (Figure 6).

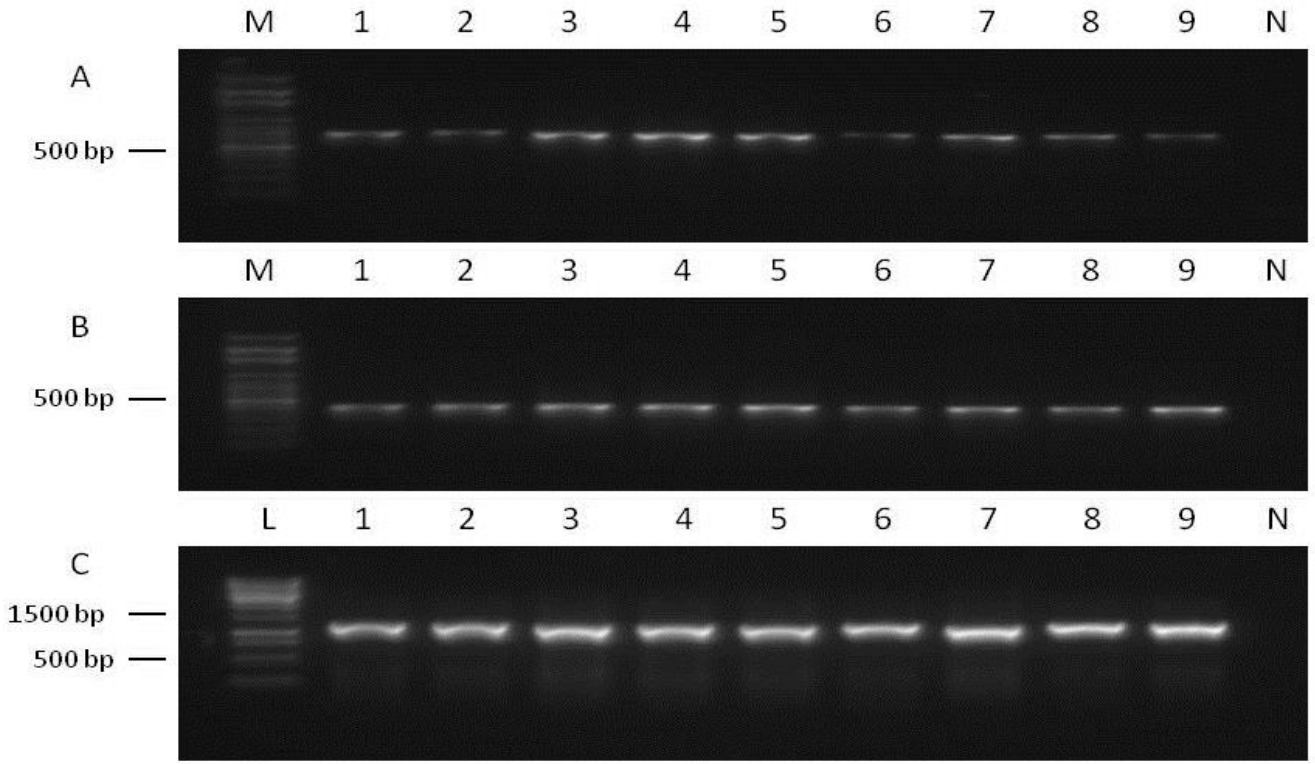

Figure 6: Gel images of representative PCR amplifications of tick DNA. Amplifications of the CO1 gene, 16s rRNA and ITS 2 genes are shown in Panels **A, B and C respectively**. Field tick samples are loaded on lanes 1 – 9 while Nis the negative control. M-is 100 bp molecular marker (Solis Biodyne) and L-is 1 kb molecular marker (Fermentas O'GeneRuler^{TM}).

3.1.1 Tick phylogenetic analysis of 16S rRNA sequences

The 16S rRNA sequences obtained from this study varied in length and ranged from 294bp in *A. eburneum* to 363bp in *R. maculatus.* BLAST results from the NCBI database for *A. eburneum* returned a 95% top match identity to *A. hebraeum* (GenBank Accession no. L34316.1). The *A. thollonii* collected and identified during this study was 89% identical to *A. variegatum* (GenBank Accession no. JF949794.1). An identity of 92% between R. *sanguineas* (GenBank Accession no. KF219732.1) and *R. turanicus* (GenBank Accession no. JX997393.1) was recorded for *R. maculatus* as the closest hits.

All *Amblyomma* larvae and nymphs that had previously been morphologically identified up to the genus level showed a nucleotide similarity of between 99.6% - 100% with *A. eburneum*. Juvenile rhipicephaline ticks returned identity top matches of between 90% - 92% with available *Rhipicephalus* sp. sequences in the GenBank database. However, the nucleotide intraspecific similarity within the juveniles ranged between 99.3% - 99.5%.

Molecular phylogenetic analysis of the tick sequences obtained in this study and the 16S rRNA sequences retrieved from GenBank was inferred using the soft tick, *Argus persicus* (GenBank Accession no. GU451248.1) as an out-group (Figure 7). The maximum likelihood model best fitting the 16S rRNA data by use of the Akaike Information Criterion (AIC) as computed using the jModeltest2 program was TvM+G. Two phylogroups comprising the genera *Amblyomma* and *Rhipicephalus* were formed consistent with the morphological identification. Within the *Amblyomma* phylogroup, all the immature forms obtained in this study clustered together with adult *A. eburneum* and

were monophyletic with *A. hebraeum*. The *A. thollonii* formed a separate clade that was paraphyletic to the rest of *Amblyomma* ticks on the tree.

Immature *Rhipicephalus* ticks were closely related and clustered into one clade which formed a separate lineage from *R. turanicus* although connected by a relatively weak branch with bootstrap support of 48%. Interestingly, *R. maculatus* species which were the only adult rhipicephaline ticks identified morphologically in this study were polyphyletic to the *Rhipicephalus* immature forms.

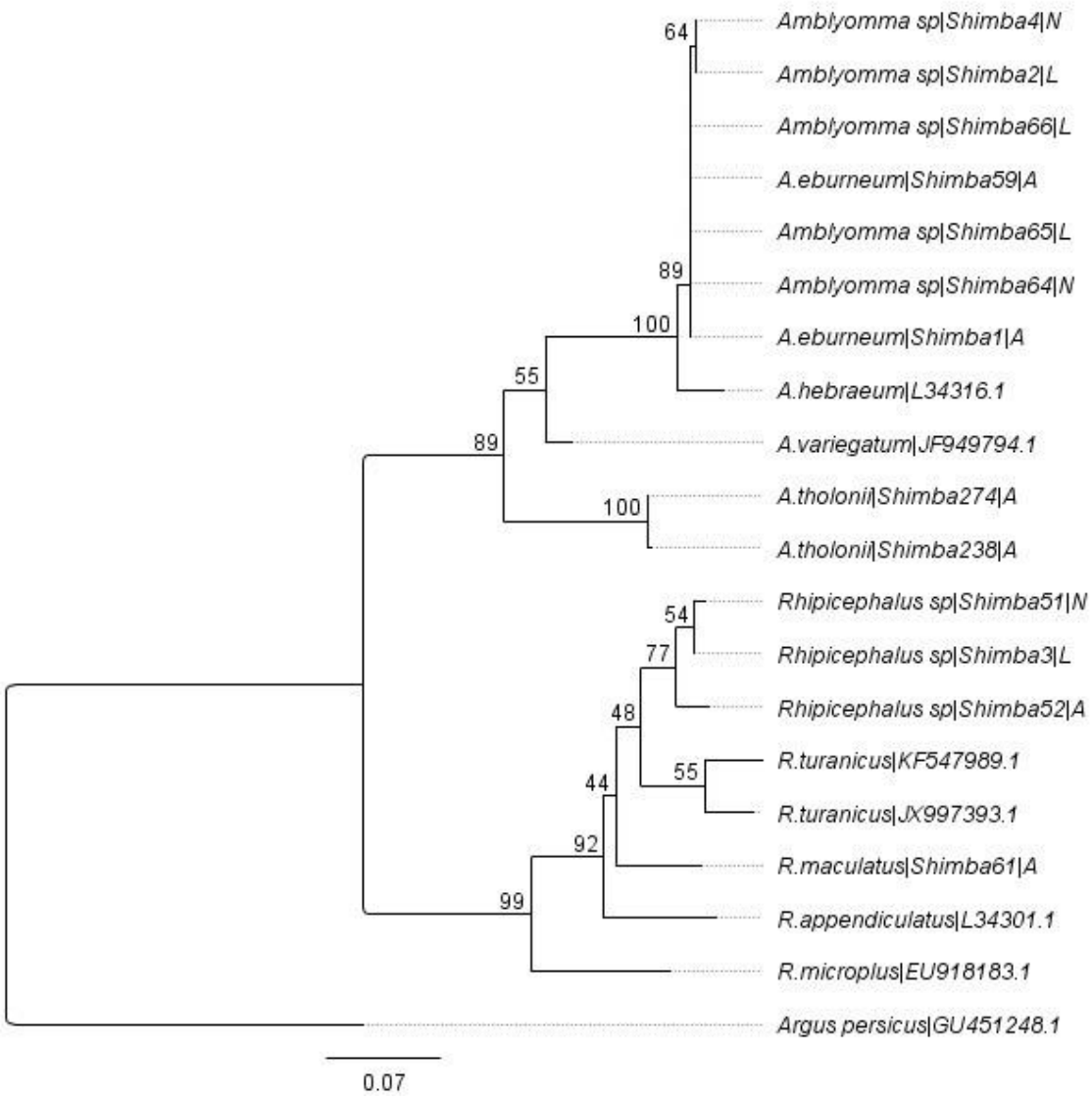

Figure 7: Maximum Likelihood tree inferring the phylogeny of SHNR ticks based on the 16S rRNA gene sequences. Numbers above the branches indicate bootstrap values. Sequences retrieved from GenBank have their accession number alongside the species name. The Shimba hills ticks are designated as follows, L=larva, N=nymph and A=adult indicated at the end of the names.

3.1.2 Tick phylogenetic analysis of ITS 2 sequences

The closest hit for *A. eburneum* ITS 2 gene fragment in the GenBank was *A. variegeatum* (GenBank Accession no. HQ856759.1) at 98% obtained from Mali, while the highest identity match for *A. thollonii* at 90% was an *Amblyomma* species (GenBank Accession no. KC538941.1) that has previously been known to infest nostrils of wild chimpanzees in Uganda (Hamer et al., 2013). *R. maculatus* from the study was 99% identical with *R. maculatus* (GenBank Accession no. AF271281.1) collected from Natal, South Africa.

Ixodes holocyclus was used to construct phylogeny due to the unavailability of ITS 2 sequences for soft ticks in the GenBank at the time of analysis. The maximum likelihood model best fitting the ITS 2 data by use of AIC was TpM2uf+G. Two phylogroups were formed consistent with the 16S rRNA phylogeny and the morphological identification (Figure 8). Within the *Amblyomma* ticks phylogroup, *A. thollonii* formed a paraphyletic clade to the rest of the *Amblyomma* species consistent with the 16S rRNA based phylogeny. Two sub-phylogroups were formed within the *Rhipicephalus sp.* with the *Rhipicephalus* immature tick stages obtained in the study being distant from *R. maculatus.*

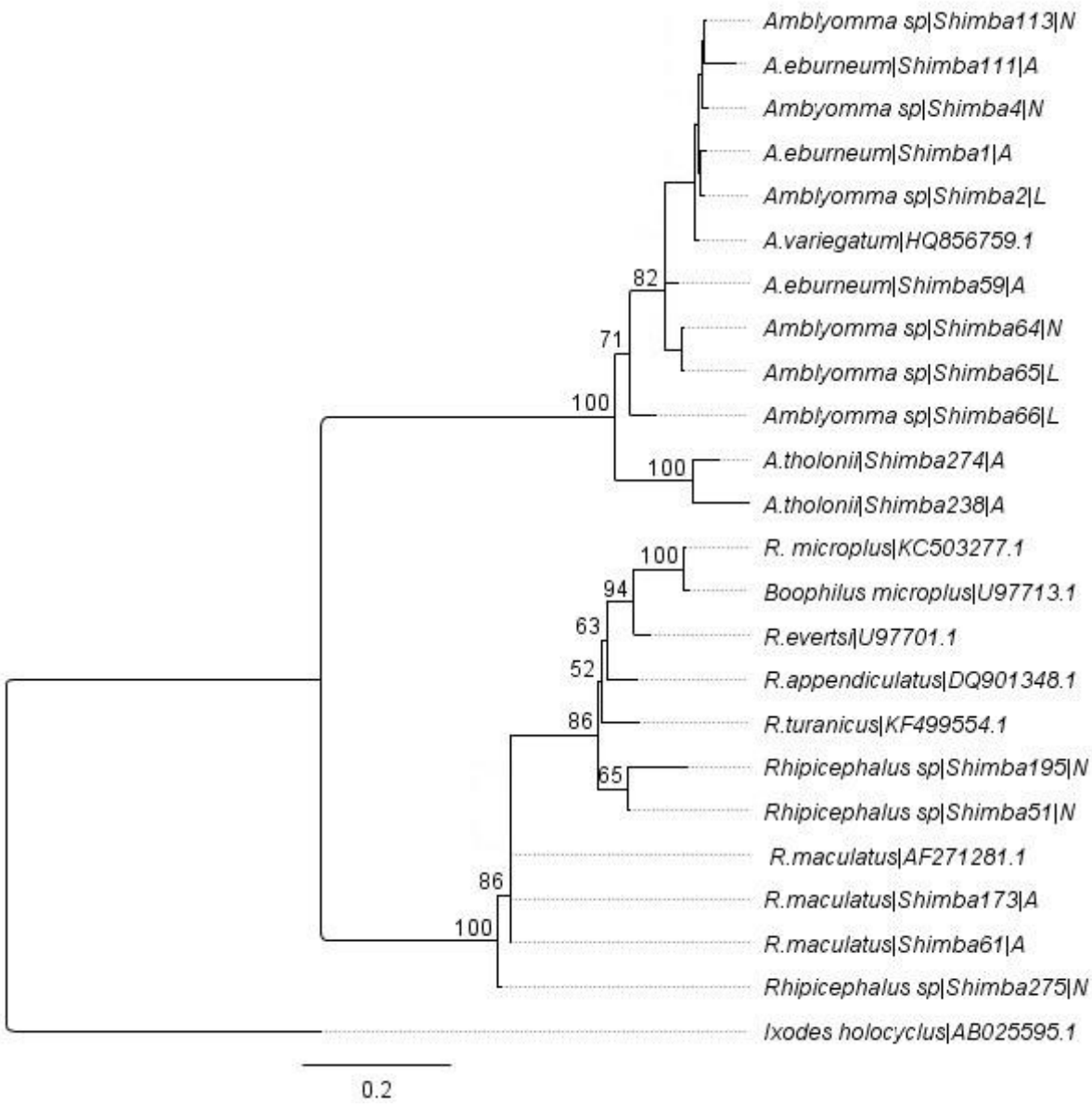

Figure 8: Phylogenetic analysis of ticks collected from Shimba hills using ITS 2 sequences. *Ixodes holocyclus* was used as an out-group. The accession numbers of the GenBank retrieved sequences are indicated alongside the species name.

3.1.3 Tick phylogenetic analysis of CO1 gene sequences

The highest BLAST match for CO1 gene sequences in *A. eburneum* was *A. hebrauem* (Accession no JX049266.1) with a 93% identity match. *R. maculatus* returned a 97% identity match with *R. maculatus* (GenBank record accession no. AY008681.1). The intraspecific pair-wise similarities of *A. eburneum* ticks from this study compared with three *A. eburneum* sequences retrieved from the BOLD database BIN (Cluster ID: BOLD: ACG8743) ranged between 98.9% to 99.2%. Intraspecific similarities of *R. maculatus* with 2 BOLD retrieved sequences were at 96.8% and 97%.

Phylogeny based on the CO1 sequences was inferred using a soft tick *Argas persicus* as the out-group (Figure 9). The maximum likelihood model best fitting the COI data by use of the AIC was TIM 2+I+G. The clustering pattern of the tick was similar to ITS 2 and 16S rRNA described above. *A. eburneum* and *Amblyomma sp.* juveniles formed a monophyletic clade with *A. eburneum* ticks sequences retrieved from the BOLD database. The juvenile *Rhipicephalus sp.* obtained in this study formed a polyphyletic clade to the rest of the rhipicephaline phylogroup.

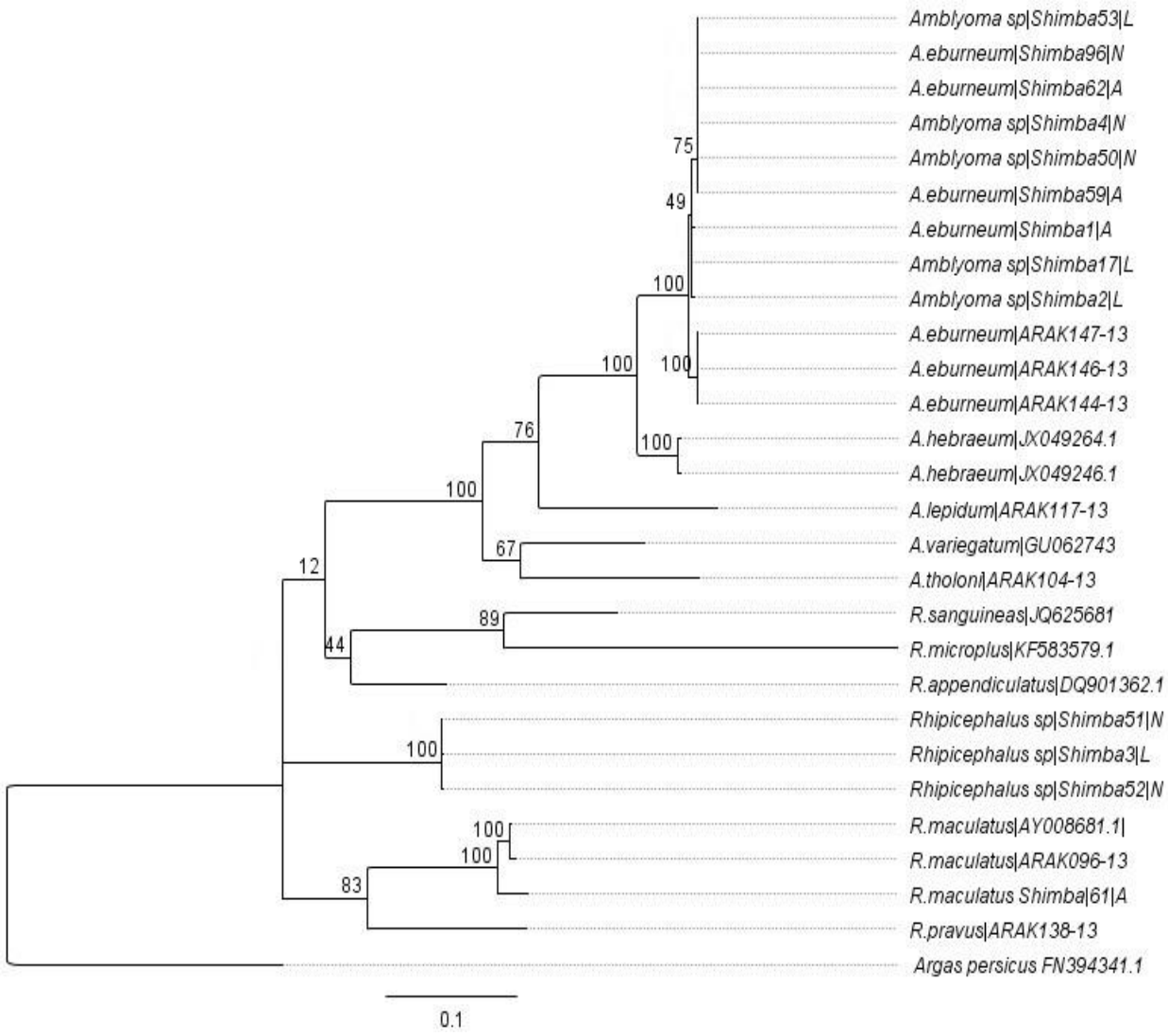

Figure 9: Phylogenetic relationships of Shimba hills ticks based on Maximum Likelihood analysis of CO1 gene sequences. Bootstrap values are shown on the branches. BOLD database published record numbers and GenBank accession numbers are shown in the figure. *Argas persicus* was used as an out-group.

3.2 Identification of tick-borne pathogens

The tick pools (adults = 69, nymphs = 84, larvae = 117) were screened for *Theileria, Ehrlichia, Babesia, Rickettsia, Anaplasma, Coxiella* and viruses (*Bunyamwera, Thogoto, Dhori, Sindbis* and *Flavi-viruses*). Positive pools were identified for *Rickettsia, Ehrlichia, Anaplasma,* and *Theileria* pathogens. The number of positive pathogen pools are summarised in Table 4 and 5 for the adult ticks and the juvenile ticks, respectively.

None of the pools was positive for *Babesia, Coxiella,* or arboviruses. One *R. maculatus* pool was positive for *Anaplasma sp* detected by PCR-HRM assay corresponding to an estimated prevalence of 1.0 % (95% CI: 6.00E-04 – 4.4). Sequencing of the PCR product and a subsequent search at GenBank returned a similarity of 100% with *Anaplasma phagocytophilum* (GenBank Accession no. JN558811.1) and *Anaplasma odocoilei* (GenBank Accession no. NR_118489.1). A distinct HRM profile was obtained that was different from an *Anaplasma sp.* amplified from ticks collected from a goat from a farmer near the reserve (Figure 10 D).

A total of 13 pools tested positive for *Ehrlichia sp.* Five pools of *R. maculatus* adult ticks were positive corresponding to an estimated prevalence of 5.5% (95% CI: 2.0 - 11.7) while 7 pools (prevalence estimate:1.0%; 95% CI: 0.4 - 2.0) of *Amblyomma sp* nymphal stage and 1 *Rhipicephalus sp* nymphal pool (1.1%; 95% CI: 0.6 – 4.3) were positive. Three unique HRM profiles were identified and sequencing of selected representatives confirmed one to be *Ehrlichia ruminatium* while the two other profiles did not match any strain in the GenBank. Furthermore, *E. ruminatium* infection was only detected in the nymphs of *Amblyomma sp.* nymphs while the unidentified *Ehrlichia sp.* was detected in both *Rhipicephaline* nymphs and adult ticks.

Table 4: The number of pathogen positive adult tick pools from Shimba Hills National Reserve.

Tick species	**Total pools**	**Positive pools**						
		Rickettsia	***Ehrlichia***	***Theileria***	***Viruses***	***Babesia***	***Coxiella***	***Anaplasma***
A. eburneum	45	2	0	0	0	0	0	0
A. thollonii	2	0	0	0	0	0	0	0
R. maculatus	22	2	5	0	0	0	0	1

Table 5: The number of pathogen positive juvenile tick pools from Shimba Hills National Reserve.

Tick species	**Total pools**	**Positive pools**						
		Rickettsia	***Ehrlichia***	***Theileria***	***Viruses***	***Babesia***	***Coxiella***	***Anaplasma***
Amblyomma sp. Nymphs	76	4	7	5	0	0	0	0
Amblyomma sp. Larvae	117	0	0	0	0	0	0	0
Rhipicephalus sp. Nymphs	8	0	1	0	0	0	0	0

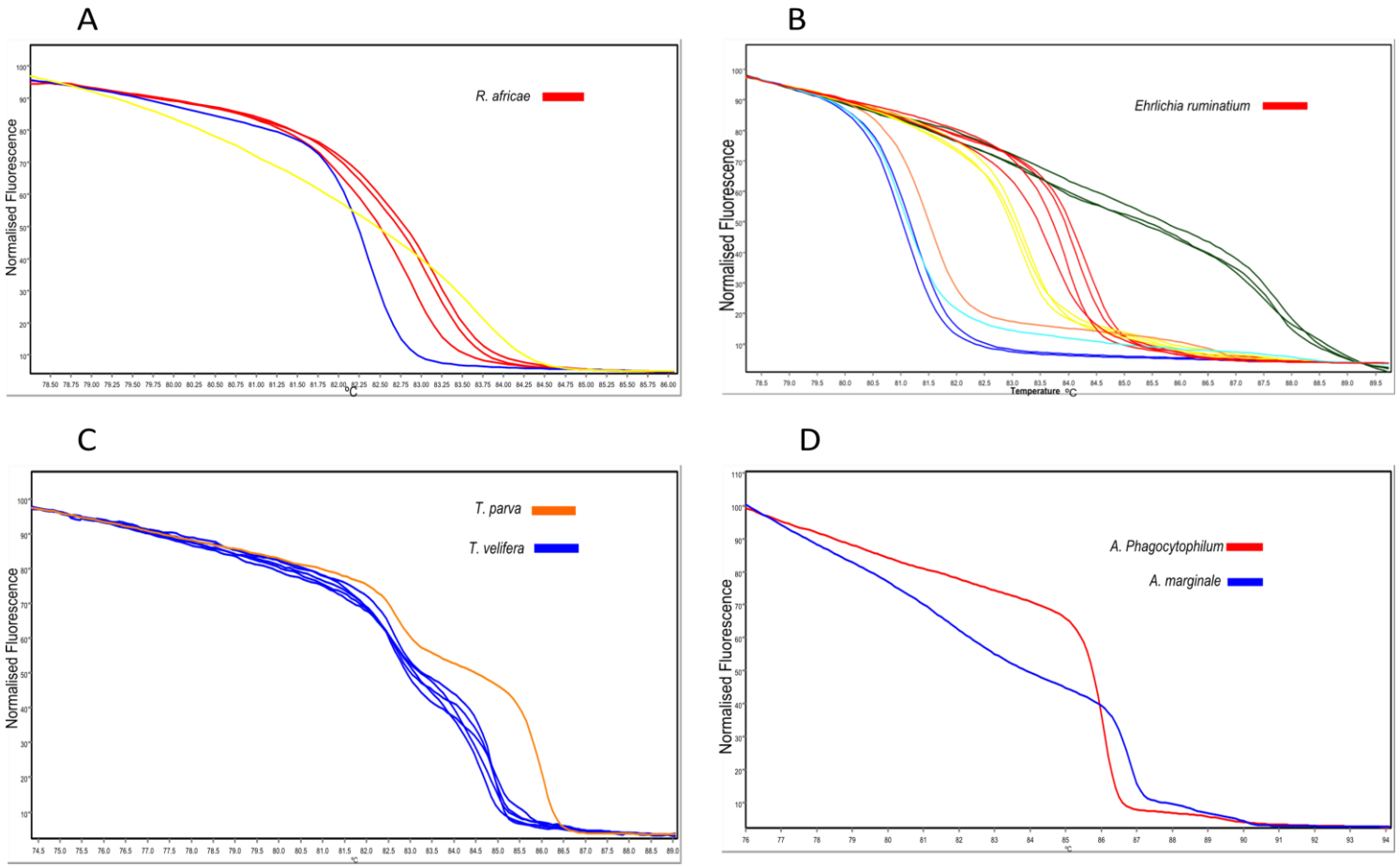

Figure 10: Normalized HRM graphs showing pathogen diversity detected in SHNR questing ticks.

Panel A: *Rickettsia sp.* graph; **Panel B:** *Ehrlichia sp.* graph; **Panel C:** *Anaplasma sp.* graph; **Panel D:** *Theileria sp.* graph

Table 6: GenBank BLAST hits of pathogen sequences

Sample ID	Tick species	GenBank Hit	Accession no.	Similarity (%)
ST53E	*Amblyoma sp.* Nymphs	*Ehrlichia ruminatum*	KF786048.1	100
ST98E	*R.maculatus* Adult	*Ehrlichia sp.*	JX629805.1	96
ST198E	*R.macualtus* Adult	*Candidatus ehrlichia shimanensis*	AB074459.1	95
ST242	*R.maculatus* Adult	*Candidatus ehrlichia shimanensis*	AB074459.1	95
ST250E	*Amblyoma sp.* Nymphs	*Ehrlichia ruminatum*	NR_074155.1	100
ST275	*Rhipicephalus sp* Nymphs	*Candidatus ehrlichia shimanensis*	AB074459.1	94
ST103A	*R.maculatus* Adult	*Anaplasma phagocytophilum*	JN558811.1	100
ST119R	*R. maculatus* Adult	*Rickettsia montanensis*	DQ008254.1	95
ST122R	*Amblyoma sp.* Nymphs	*Rickettsia africae*	CP001612.1	100
ST138R	*Amblyoma sp.* Nymphs	*Rickettsia africae*	CP001612.1	100
ST139R	*Amblyoma sp.* Nymphs	*Rickettsia africae*	CP001612.1	99
ST198R	*R. maculatus* Adult	*Rickettsia bellii*	CP000849.1	97
ST127TH	*Amblyoma sp.* Nymphs	*Theileria velifera*	JN572701.1	97
ST139TH	*Amblyoma sp.* Nymphs	*Theileria velifera*	GU733375.1	99
ST194TH	*Amblyoma sp.* Nymphs	*Theileria velifera*	GU733375.1	94
ST260TH	*Amblyoma sp.* Nymphs	*Theileria velifera*	JN572701.1	100
ST271TH	*Amblyoma sp.* Nymphs	*Theileria velifera*	JN572701.1	100

Seven pools were positive for *Rickettsia sp.*, four of which were *Amblyomma sp.* nymph pools (Prevalence estimate: 0.7%; 95% CI: 0.2 – 1.7). One positive pool belonged to adult *A. eburneum* while the other two pools belonged to *R. maculatus* adults (Prevalence estimate: 2.0%; 95% CI: 0.3 - 6.2). BLAST search on *Rickettsia sp.* infecting *Amblyomma* tick returned 97-100% similarity with *R. Africae. A* Similarity 95% and 97% with *Rickettsia montanensis* and *Rickettsia bellii* respectively were achieved for *Rickettsia* in *R. maculatus* (Table 6). These differences were also evident in the HRM profiles (Figure 9A). Pair-wise intra-specific similarities between the *Rickettsia sp.* detected from *Amblyomma sp.* ranged between 96.2 – 100%. Intra-specific similarities between the *Rickettsia sp.* identified from *R. maculatus* was at 50.8%.

R. belli was used as an out-group to infer phylogeny based on the *rpmE*-tRNA^{fMet} intergenic spacer (Figure 11). TpM1uf+G model was selected based on AIC to construct a Maximum Likelihood tree that included other sequences retrieved from the GenBank. A close relationship between *R. africae* Egyptian strain (GenBank Accession no. HQ335144.1) and the *Rickettsia sp.* amplified from *Amblyomma* ticks was obtained with 100% bootstrap support.

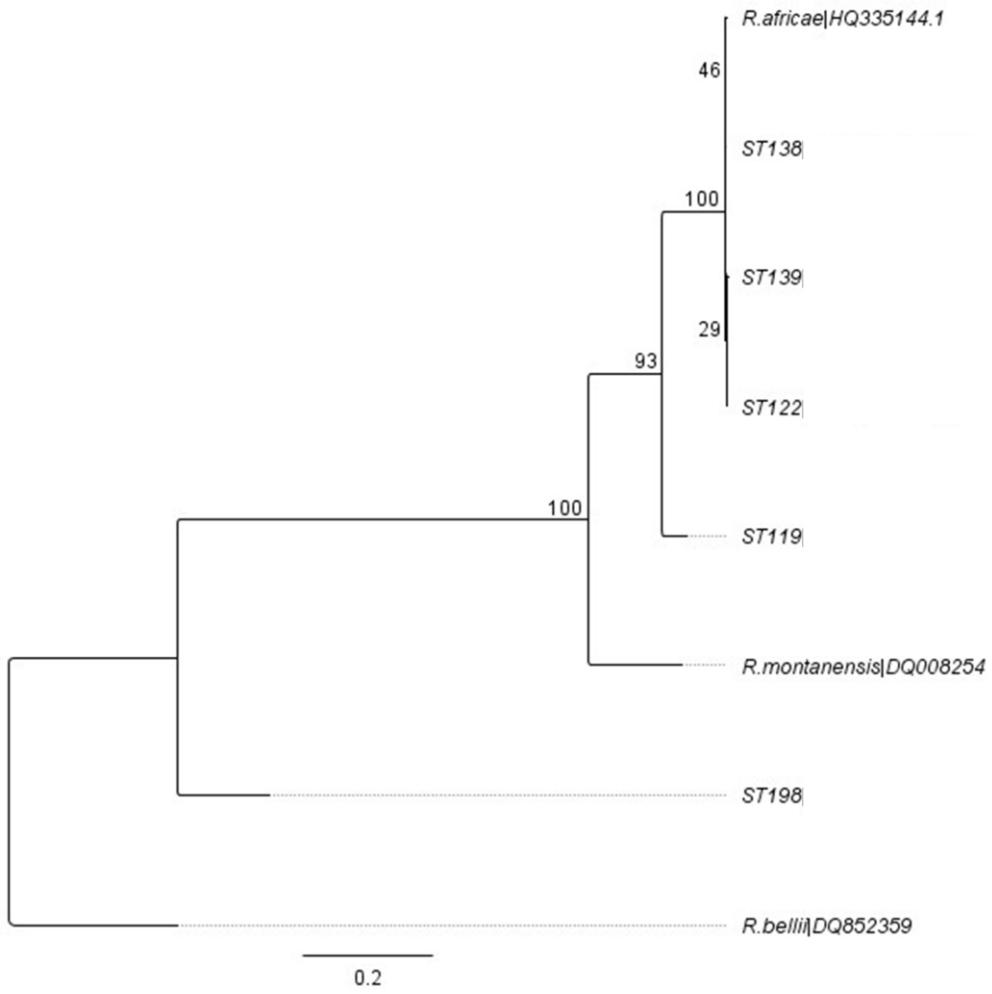

Figure 11: Maximum Likelihood phylogenetic relationship of *Rickettsia sp* based on the rpmE-tRNA^{fMet} intergenic spacer. The numbers ST138, ST138, ST122, represents *Amblyomma* ticks nymphal pools that were *Rickettsia sp.* positive while ST119 and 198 are *R. maculatus* adult ticks that were also positive for *Rickettsia sp.*

An estimated prevalence of 0.9% (95% CI: 0.3 – 1.9) was recorded for *Theileria sp.* in 5 *Amblyomma* nymphal pools. BLAST search returned 94 – 100% similarity with *Theileria velifera.* HRM analysis revealed distinct melt profiles between *T. parva* used as a positive control and *T. velifera.* Intraspecific similarities varied from 94.4% to 100% among the analyzed sequences.

CHAPTER FOUR

DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 Discussion

A unique human-livestock-wildlife interface which can potentially facilitate the transmission of infectious pathogens across different species exits in Shimba hills. With invertebrate vectors being significant in disease transmission especially in the tropics than in temperate regions (Wolfe et al., 2007), it is imperative to have an understanding of important vectors such as ticks and the pathogens they transmit particularly in such interfaces.

Molecular analysis of tick diversity and the tick-borne pathogens in this study led to the identification of a unique tick diversity and detection of three pathogens of human and veterinary importance. Among the pathogens detected, probable novel species are reported for the genus *Ehrlichia* and *Rickettsia.* Interestingly, these were identified in *R. maculatus*. Walker et al. (2000) reported that the disease relationship of *R. maculatus* was unknown. Accordingly, this finding implies previously unidentified disease relationships of this tick species that could be of public and animal health importance.

Rhipicephalus maculatus ticks have been observed to prefer large animal hosts such as elephants and buffalos and records of its collection are available especially along the coastal areas of the east African countries all the way to the north-eastern areas of the Republic of South Africa (Horak et al., 2007). The findings of the probable novel pathogens in this tick are of public and animal health interest especially when their pathogenicity is yet to be established.

Approximately seventy species of the genus *Rhipicephalus* (Acari:Ixodidae) are known to occur in Kenya, with *Rhipicephalus walkerae* (n) being among the recently described species infesting giraffes in North East of Kenya (Horak et al., 2013). Members of this genus are important vectors of human and animal pathogens although identification of species especially for immature ticks can be challenging (Walker et al., 2000).

Nevertheless, molecular techniques have been useful in tick identification to complement morphological examination (Lu et al., 2013). Notably, the *Rhipicephalus sp.* juveniles collected in this study had a high intraspecific similarity but still a low similarity with adult sequences of *Rhipicephalus* in the study and GenBank sequences. It was thus impossible to assign them into any known species group with confidence. This could imply that they belong to a tick species that is yet to be described using molecular markers applied in this study, evidently revealing unidentified tick diversity.

Given that this study focused on questing ticks, it cannot be ruled out that the adults of this tick species are mostly on their animal host or were out of season and therefore absent during time of collection. Nonetheless, this finding could imply a rare occurrence of tick species whose details are limited. Consequently, this could hamper control measures of such ticks and their associated pathogens. As such, molecular data on the ticks obtained in this study provide important references for future studies.

Additionally, important emerging technologies are now finding a useful application in tick identification requiring a limited entomological expertise. A protein profiling technique by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDITOF MS) has been developed for tick identification. This approach which uses

protein profiles to differentiate between species, promises to overcome challenges of molecular characterization such as difficulties in amplification of gene fragments in some tick species as well as, the limited the availability of gene sequences in GenBank as was encountered in this study (Yssouf et al., 2013).

Unavailability of sequences in the GenBank for *A. thollonii* and *A. eburneum* was a challenge in this study. Nevertheless, CO1 sequences for these tick species were available in the Barcode of Life Data systems (BOLD) database (http://www.boldsystems.org/) which enabled the verification of the adult ticks. The BOLDSYSTEM is an integrated database designed to support the acquisition, storage, analysis as well as publication of DNA barcodes based on the 648 bp CO1gene fragment for members of the animal kingdom (Ratnasingham & Hebert, 2007). Phylogenetic analyses of the CO1, 16S rRNA and ITS 2 were consistent with the morphological examination of the ticks forming two phylogroups comprising of *Amblyomma* and *Rhipicephalus* genera. All the unidentified *Amblyomma sp.* juveniles were assigned to *A. eburneum* due to high intraspecific similarity.

The occurrence of *A. eburneum* has already been documented in Kenya with its adults known to parasitize sheep, cattle and the African buffalo - *Syncerus caffer* (Voltzit & Keirans, 2003). Its occurrence in SHNR is therefore consistent with the presence of herds of *Syncerus caffer* in the park. *A. thollonii* commonly known as the elephant tick is primarily found on the African elephant, *Loxodonta Africana* (Voltzit & Keirans, 2003), and has been recorded in areas inhabited by elephants, which are also present in the SHNR (Spinage, 2012).

Although the distribution and relative abundance of the different tick species in the reserve was beyond the scope of objectives, important observations concerning these two aspects became apparent during the collection times. For example, the majority of *R. maculatus* ticks were obtained during the short-rains period, mainly at Kivumoni, whereas the *Amblyomma* sp. larvae were abundant on all collection times especially at vegetation near water. These are critical findings because the occurrence and localization of ticks and their associated pathogens is determined by several factors with some relating to the environment (Magnusson, 2012).

Most ticks show some adaptations to a specific hosts (Walker et al., 2003) that may be related to their ability to digest their host's blood (Spinage, 2012). The presence of a particular host is therefore an important factor affecting the distribution and occurrence particular tick species (Estrada-Peña et al., 2013). Only three adult *A. thollonii* were collected in an area frequented by elephants. Although SHNR has a significant elephant population, *A. thollonii*'s preference of elephants as the main host will adversely affect its population dynamics. Such vector-host relationships have important implications in supporting and maintaining various pathogen transmissions (Gortazar et al., 2014;Foley & Piovia-scott, 2014). Consequently, circulation and evolution of associated pathogens is also affected (McCoy et al., 2013).

Tick-borne ehrlichioses are among the important emerging infectious diseases with novel *Ehrlichia sp.* reported from several localities worldwide (Inokuma et al., 2004). Based on 16S rRNA gene PCR-HRM analysis and with subsequent sequencing, two unique *Ehrlichia* species that had limited homology with other GenBank sequences were detected, apart from *Ehrlichia ruminatium.* However, these two unique *Ehrlichia*

sequences could possibly be genetic variants of already existing ones, thus necessitating further molecular characterization.

Ehrlichia ruminatium was detected only in *Amblyomma sp.* nymphal pools confirmed to be *A. eburneum* based on sequence analysis. This is contrary to findings by Peter et al. (1999), in which the prevalence of *Cowdria* (*Ehrlichia*) *ruminatium* in heart-water endemic areas of Zimbabwe was higher in adult *Amblyomma hebraeum* than in their nymphal stages. This finding suggests complex dynamics in the ecology of *Ehrlichia* infections.

Nevertheless, depending on the species, some ticks display stage specific host relationships. This will clearly have an impact on the transmission of some pathogens to their vectors and consequently to different hosts (Randolph, 2004). As a result, variations of prevalence rates may be observed between adults and nymphal stages of a tick species in different sites within endemic foci of a known disease. Such variations have been reported in the prevalence of *Borrelia burgdorferi* Sensu Lato in *Ixodes ricinus* ticks (Ferquel et al., 2006). Unfortunately, there is limited literature on the biology and ecology of *A. eburneum* that may help bring into perspective the dynamics of *E. ruminatium* prevalence.

Rickettsia africae was detected only in *A. eburneum.* It is an intracellular spotted fever group (SFG) rickettsiae that causes African Tick-Bite Fever (ATBF), a flu-like acute illness accompanied with severe headaches (Jensenius et al., 2003). It's occurrence has already been documented along the east coast of Africa based on serological evidence mainly from travellers visiting rural sub-Saharan Africa and its presence in ticks has been

reported in *Amblyomma* ticks from Masai Mara, Kenya (Macaluso et al., 2003; Ndip et al., 2004; Rutherford et al., 2004). Although generally associated with ungulate ticks of the *Amblyomma* genus, *A. variegatum* species has been incriminated as its principal vector in East Africa (Jensenius et al., 2003).

The detection of *R. africae* in *A. eburneum* ticks in this study is novel, making this tick a previously unrecognized potential vector of this pathogen. African Tick Bite Fever is of major concern to public health because the disease symptoms can easily be confused with those of other febrile illnesses, leading to misdiagnosis. Moreover, serologic evidence of ATBF has mainly been from travellers visiting Africa. It is thus of interest to understand how local populations cope with this infection.

The detection of the novel rickettsiae in this study continues to add to the growing list of novel rickettsiae identified in recent years. Further molecular analysis revealed one of the *Rickettsia* sp. to be distantly related to *R. bellii*, which is considered together with *R. canadensis* to be ancestral in the overall *Rickettsia* sp. phylogeny (Weinert et al., 2009). This finding could have an important bearing in understanding the origin of *Rickettsia* pathogens. Investigations into the origin of infectious disease pathogens has been proposed as an important area of research focus for better understanding how diseases have emerged and continue to re-emerge (Wolfe et al., 2007).

Anaplasma sp. that had a 100% match with GenBank records of *A. odocoilei* and *A. Phagocytophilum* were detected in *R. maculatus. A. phagocytophilum* causes of human granulocytic anaplasmosis (HGA), an emerging zoonotic disease characterized by fever, myaglia, anorexia, headache and chills (Dunning Hotopp et al., 2006). This finding

additionally raises important question on the role and the vector capacity of this tick species in pathogen transmission. Seroprevalence of *Anaplasma sp.* antibodies in a Kenyan wildlife/livestock interface has been reported previously (Ngeranwa et al., 2008). These prevalence rates are however incomparable with this study due to methodological differences and design of the study. Furthermore, the study was not specific on the *Anaplasma* species detected.

Theilerial infections are a major concern in animal health. *T. velifera* was detected in *A. eburneum* nymphs in this study. In a recent study on detection of *Theileria* infection in Ethiopia, Gebrekidan et al. (2013) detected *T. velifera* among other piroplams in cattle. *A. variegatum, A. lepidum and A. hebrauem* are the proven vectors of *T. velifera* (Norval et al., 1992). Hence the detection of *T. velifera* in *A. eburneum* nymphs implicates this tick species as its principal vector in Shimba. The distribution of *T. velifera* has been associated with *Amblyomma hebrauem* ticks and the African buffalo (*Syncerus caffer*) which has been cited as the natural reservoir host for both pathogenic and non-pathogenic *Theileria* species (Yusufmia et al., 2010). Whereas *T. velifera* has been said to be apathogenic in cattle, its potential to cause disease in immunosuppressed cattle cannot be ruled out.

No arboviral pathogens, *Coxiella burnetti* and *Babesia sp.* were detected from ticks in this study. This by no means does it suggest the absence of these pathogens circulating within the park. Stefanoff et al (2012), based on a systematic search for published studies reported that TBE virus screening in questing ticks may not assure detection even in areas known endemic foci. Moreover, the ticks collected in this study are yet to be associated with any virus transmission to date.

Additionally, in a systematic literature search, no records have been found on incidences of *C. burnetti* and *Babesia* parasite infection associated with the tick species reported in this study. The main species of *Babesia* parasite infecting cattle in Kenya is *Babesia bigemina*, principally transmitted by *Rhipicephalus (Boophilus) decoloratus* (Wesonga et al., 2010). *C. burnetti* infection incidences have been recorded in *Haemaphysalis leachi* ticks collected from domestic dogs in western Kenya (Knobel et al., 2013).

Prevalence of the pathogens detected in this study ranged from 0.7% in *Amblyomma* nymphs infected with *Rickettsia sp* to 5.5% in *R. maculatus* ticks infected with *Ehrlichia sp.* These were relatively low but comparable with tick-borne pathogen prevalence studies in questing ticks recorded elsewhere (Silaghi et al., 2012). Sprong et al. (2009) reported a prevalence of 6% of *R. helvetica* infection in *Ixodes ricinus* ticks collected by flagging in a two-year period whilst *A. phagocytophilum* prevalence of 1.1% to 7.4% across different sites and times have been reported in questing *Ixodes ricinus* ticks (Overzier et al., 2013).

Moreover, a tick-borne pathogen prevalence study on ticks collected from vegetation in Nigeria reported a prevalence of 0.1% for *Coxiella burnetii* to 3.1% for *Rickettsia sp.* (Reye et al., 2012). However in the same study, a prevalence as high as 12.5% for *Rickettsia* was recorded from feeding ticks collected from cattle. Pathogen prevalence between questing ticks and on-host ticks have generally recorded higher prevalence rates in on-host ticks (Ndip et al., 2004; Reye et al,. 2012; Knobel et al., 2013). This is expected particularly because the fed ticks could have obtained the infection from the animal host especially in endemic areas.

4.2 Conclusion

The continued threat of emerging vector-borne zoonoses necessitates focused systematic efforts in monitoring emerging animal pathogens to humans and novel pathogens that may become future threat. In this study, the recorded diversity of ticks and probable novel tick-borne pathogens within SHNR bear important implications on emerging zoonotic pathogens and ticks as one of their key vectors. Moreover, the diversity of ticks and pathogens found circulating in the SHNR in this study suggests the possible occurrence of many unidentified pathogens in wildlife that are potentially pathogenic. Periodic surveillance efforts that improve with availability of modern assays are therefore very important in elucidating the full spectrum of potential disease threats.

4.3 Recommendations

The increasing threat of tick-borne diseases needs to be countered with effective proactive measures. Better control and preventive measures for tick-borne diseases should proceed on the basis of information gained through systematic efforts such as surveillance studies. Preliminary steps in planning disease surveillance involve assessing the diversity of pathogens occurring in an area and establishing the epidemiological importance of the identified pathogens. Key outcomes of this study are the discovery of novel tick-borne pathogens and the potential role of the identified ticks as vectors. Nevertheless, to establish the epidemiological importance of the reported tick and pathogen diversity, extra effort is needed to:

1. Understand the risk of infections for the identified pathogens to humans and livestock, since they were identified from ticks in a wild habitat. This can be

done through carrying out investigations on the tick-borne pathogen seroprevalence in the surrounding human populations, livestock and wildlife.

2. Conduct investigations on ticks associated with specific hosts. It would be of great interest to establish the vectorial capacity of *R. maculatus* for the identified novel pathogens.
3. Characterize the novel *Rickettsia* and *Ehrlichia* species and establish their pathogenicity.
4. Replicate this study in other areas that have frequent interactions between humans, wildlife and livestock.

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APPENDICES

1-A: "Hot Shot" genomic DNA preparation buffers

Alkaline lysis reagent (pH 12)

Reagent	Final	Add
NaOH	25 mM	125 of 10N NaOH
Disodium EDTA	0.2 mM	20 of 0.5M EDTA
		50 ml of ddH2O

Neutralization buffer (pH 5)

Reagent	Final	Add
Tris-HCl	40mM	325 mg of Tris-HCl
	pH will be 5	50 ml of ddH2O

1-B: Multiplex PCR conditions for arboviruses screening

Cycle	Denaturation	Annealing	Extension
Initial Denaturation	95°C (5 min)		
1	94°C (20s)	63.5° (25s)	72° (5s)
2	94°C (20s)	62.5° (25s)	72° (5s)
3	94°C (20s)	61.5° (25s)	72° (10s)
4	94°C (20s)	60.5° (25s)	72° (11s)
5	94°C (20s)	59.5° (25s)	72° (12s)
6	94°C (20s)	58.5° (40s)	72°(15s)
7	94°C (20s)	57.5° (40s)	72°(15s)
8	94°C (20s)	56.5° (40s)	72°(20s)
9	94°C (20s)	55.5° (40s)	72°(25s)
10	94°C (20s)	54.5° (50s)	72°(30s)
11-15	94°C (20s)	53.5° (50s)	72°(30s)
16-20	94°C (20s)	52.5° (50s)	72°(30s)
21-25	94°C (20s)	51.5° (50s)	72°(30s)
26-30	94°C (20s)	50.5° (50s)	72°(30s)
31-40	94°C (20s)	49.5° (50s)	72°(30s)
41-50	94°C (20s)	47.5° (50s)	72°(30s)
Final Extension			72° (3 min)