Population genetic structure of *Ixodid* ticks and phylogenetic analysis of *Rickettsia* in Chongwe and Chisamba districts of Zambia

By

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A thesis submitted in fulfilment of the requirements for the award of the degree of Master of Science degree in Infectious diseases by The University of Zambia.

THE UNIVERSITY OF ZAMBIA

Lusaka

March 2024

DECLARATION

I, Cynthia Sipho Khumalo, hereby declare that the work presented in this thesis is my own original work. It is being submitted for the award of the Degree of Master of Science in Infectious diseases The University of Zambia, Lusaka. It has not been previously submitted for any degree at this or any other University.

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CERTIFICATE OF APPROVAL

This thesis of Cynthia Sipho Khumalo has been approved as fulfilling of the requirements for the degree of Master of Science in Infectious Diseases by Research at the University of Zambia.

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ABSTRACT

Ticks are known vectors of various disease-causing pathogens worldwide. The distribution of ticks, their genetic makeup and relations has not been fully explored. This study focused on the population genetics of Ixodid ticks and the molecular epidemiology of *Rickettsia* species infecting them. Population genetics is vital to understanding the vectorial capacity of the ubiquitous *Amblyomma, Hyalomma, and Rhipicephalus* tick species that can transmit various disease-causing organisms including *Rickettsia* which cause a group of re-emerging diseases known as Rickettsioses. This study was conducted on ticks that were collected from Chongwe and Chisamba districts of Zambia in November 2020 and June 2021 respectively. Ticks collected were morphologically identified, homogenised, DNA extracted and polymerase chain reaction (PCR) run using primers to amplify the cytochrome subunit I (*CO1*), *12SrDNA* and *16SmRNA* for tick species verification and population genetics as well as *omp*A, *omp*B and *glt*A genes for *Rickettsia* molecular epidemiology followed by sequencing. Sequences obtained were blasted then edited using ATGC plug in Genetyx ver.12 and aligned using clustalw. For *Rickettsia*, phylogeny was established using Mega version XI. Comparison of population genetics was by MEGA XI, DnaSP, Arlequin (Fst), NETWORK and GENAlex (to measure diversity). It was found that tick populations in Chongwe and Chisamba district were identified as belonging to *Amblyomma*, *Hyalomma* and *Rhipicephalus* genus. These tick populations were high in genetic diversity and low in genetic differentiation. The tick populations were observed to carry *Rickettsia* species with *Amblyomma* and *Hyalomma* carrying over 95% of the *Rickettsia* detected. *Rickettsia* species detected were *R. africae* and *R. aeschlimannii*-like are both zoonotic known to cause febrile diseases of varying morbidities and mortalities. The study accounts for the low genetic differentiation to the free movement of animals that act as carriers for the ticks across the two districts. Due to this effective vector control methods can be extrapolated between the two districts. The high genetic diversity is an indication of an expanding population and hence potential occurrence of diseases carried by the ticks such as *Rickettsia.* The *Rickettsia* species detected are all zoonotic species and hence pose a risk of the outbreak of Rickettsioses in the areas the vectors are expanding to. This warrants surveillance of Rickettsioses and further research on vector populations, factors attributed to their expansion and the pathogens they carry around the country.

DEDICATION

I dedicate this thesis to my family. My late father, Duncan S. Khumalo who always believed I could achieve this and so much more. My beloved mother, Theresa Kolala for all her support, emotionally, financially to mention but a few. My amazing sister Camilla Makhumalo Khumalo for all the moral support she consistently gave me through this journey.

ACKNOWLEDGEMENT

My heart is full of thanks to God, for everything I do is by his power, and I am thankful for his guidance, providence, and strength throughout this journey. To my Supervisor, Dr Walter Muleya, I am so indebted for the skills, training, time, and moral support during this journey. To my co-supervisors Prof Simbarashe Chitanga, Dr Katendi Changula, and Dr Edgar Simulundu all your efforts to mold my collective research skills are so generous, knowledgeable, and patient. I will always be grateful. To my colleague Malala Mulavu, your kindness, help, support is immeasurable, thank you.

I am appreciative to the National Institutes of Health (NIH) funding of the project 'Spatial Eco epidemiology of Tick-borne Rickettsial pathogens: Modelling the Impacts of Humans, Hosts and Ticks on the Move' (PI Gaff; NIH award 1R01AI136035-01; PI Chitanga; NIH Subaward 18-119-100684-010), that financially catered for my study. I am further obliged to the University of Zambia, School of Veterinary Medicine, Department of Disease Control, Science and Technology Research Partnership for Sustainable Development (SATREPS) and Hokkaido University Centre for Zoonosis Control (HUCZCZ) for allowing me to conduct my study in their Laboratories, including the use of their equipment and reagents for which without my project would not have been a success. I would also like to give special thanks to the laboratory technical team in the School of Veterinary Medicine (Dr. Joseph Ndebe, Mr. Andrew Mukubesa, Mr. Ladslav Moonga, and Mr. Patrick Katemangwe) who assisted me during my laboratory work. Many thanks also go to the Chongwe and Chisamba Veterinary District offices for granting me permission and helping to collect samples in their respective districts. I would love to thank my friends and colleagues, Chikwanda Chileshe and Danny Muzata for the moral support. To all other friends and colleagues who are too many to mention in the list, a heartfelt thanks to you all.

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ABBREVIATIONS AND ACRONYMS

ATBF	African tick bite fever
BIC	Bayesian information criterion
BLAST	Basic local alignment search tool.
Bp	base pair
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
***glt*A**	Citrate synthase
Km	Kilometre
MEGA	Molecular Evolutionary Genetics Analysis
***omp*A**	Outer membrane protein A
***omp*B**	Outer membrane protein B
PBS	Phosphate buffered saline.
PCR	Polymerase chain reaction
R_o	Basic reproduction number
RNA	Ribonucleic acid
Rpm	Revolutions per minute
r.t.p	Room temperature and pressure
Sca4	Surface cell antigen 4
SFG	Spotted fever group.

SNP	Single nucleotide polymorphism
spp.	Species
TBD	Tick borne disease.
TBF	Tick bite fever
TG	Typhus group
UNZAHSREC	University of Zambia Health Sciences Research Ethical Committee
vs	Versus

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Ticks are obligate parasites that are known to transmit disease-causing organisms from one animal to another, including humans (Verwoerd, 2015). Ticks from the families *Ixodidae* (hard ticks) and *Argasidae* (soft ticks) are known to transmit pathogens of both animal and human diseases (Spach et al., 1993). The family *Ixodidae* includes species such as *Hyalomma, Rhipicephalus*, *Boophilus, Dermacentor, Haemaphysalis*, *Ixodes ricinus* and *Amblyomma* (Bursali et al., 2014). Of these, members of the genus *Amblyomma*, *Rhipicephalus* and *Hyalomma* are of importance here in Zambia due to the disease they transmit (Simuunza et al., 2011).

Amblyomma species are haematophagous hard ticks found in the Afrotropics and parasitize several orders of Mammalia including cattle and humans, with humans as occasional hosts (Guglielmone et al., 2014, Socolovschi et al., 2009). Common *Amblyomma* species known in Zambia are *A. heberum* and *A. variegatum*. The latter two are known vectors of infectious pathogens such as *Anaplasma marginale, Ehrlichia ruminantium*, *Borriela* and *Rickettsias* that cause Anaplasmosis, Heartwater, Lyme disease and Rickettsiosis, respectively (Maphuti et al., 2022). Apart from their ability to introduce pathogens to their hosts, ticks cause damage to hides of cattle and loss of blood resulting in anaemia, weakness, toxicosis, and weight loss (Makala et al., 2003). Other *Amblyomma* species include *A. marmoreum*, *A. ovale*, *A. amricanum* (Walker 2003).

Ticks belonging to the genus *Rhipicephalus* are another common tick in Africa. The African blue tick, *Rhipicephalus decoloratus*, is a common tick in the southern Africa region and is known to be indigenous in some places (Nyangiwe et al., 2013). *R. decoloratus* was previously classified under the genus *Boophilus* and is known to parasitize cattle, goats, sheep, and wild animals (MacLeod et al., 1977). *R. appendiculatus* species have been known to transmit *Babesia* and *Theileria parva* in various animal species and lumpy skin disease virus in ruminants (Tuppurainen et al., 2011, Yamada et al., 2009). *Hyalomma* species are said to be the most important ixodid ticks economically because of the pathogens they transmit collectively (Bakheit et al., 2012). Common hosts of *Hyalomma* species include cattle, goats, sheep, horses, and

smaller mammals (Spengler et al., 2016). *Hyalomma rufipes*, common in Zambia and *Hyalomma marginatum* are two-tick host ticks which are known vectors of Crimean Congo Hemorrhagic fever (CCHF) and *R. aeschlimanni* (Chitima-Dobler et al., 2019).

Some common tick-borne diseases affecting livestock and pets in Zambia are listed in the table below (Table 1). Some are known to have caused disease in humans too (Mubemba et al., 2023)

Table 1: Table showing some common tick-borne diseases known to affect livestock in Zambia and their causative agents, common vectors, and distribution.

Tick pathogens	**Disease caused**	**Common tick vector**	**Distribution**	**Reference**
Crimean-Congo hemorrhagic fever virus	Crimean-Congo hemorrhagic fever	*Hyalomma*	Africa, Europe, and Asia. Zambia (Mwandi, Mumbwa, Mazabuka, Sesheke, Shibuyunji, Kazungula)	Kajihara et al., 2021
Rickettsia sp.	Rickettsioses	*Ambylomma, Hyalomma, Rhipicephalus*	Zambia (Lusaka, Namwala, Chirundu, Livingstone, Mphomwa, South Luangwa National Park)	Chitanga et al., 2021, Qiu et al., 2021, Chitimia-Dobler et al., 2017, Moonga et al., 2021, Chitanga et al., 2021
Anaplasma sp.	Anaplasmosis	*Dermacentor*	Zambia (Lusaka, Eastern and Central province, Shangombo)	Vlahakis et al., 2018, Simuunza et al., 2011, Qiu et al., 2018.
Ehrlichia sp.	Ehrlichiosis	*Amblyomma*	Zambia (Lusaka, Monze, Mazabuka, Shangobo, Eastern, Central and Western province)	Qiu et al., 2018, Malambwa et al., 2013,
Borrelia sp.	Borreliosis	*Ornithodoros faini*	Zambia	Qiu et al., 2021, Qiu et al., 2019
Coxiella sp.	Q-fever	*Rhipicephalus*, *Amblyomma* and	Worldwide and	Chitanga et

	Haemaphysalis	reported in Zambia (Chama, Chongwe, Petauke)	al., 2018, Qiu et al., 2013

The burden of Rickettsiosis in North America, Europe and Asia is caused by *Rickettsia* species transmitted by *Amblyomma* ticks (Bitencourth et al., 2017). Within Africa, the identified *Rickettsia* species include *Rickettsia felis, Rickettsia typhi, Rickettsia conorii* and *Rickettsia africae* (Mediannikov et al., 2013, Leulmi et al., 2014, Frean and Grayson, 2019) of which *Rickettsia africae* and *Rickettsia conorii* are transmitted by *Amblyomma* ticks. *Rickettsia africae, R. felis,* and *R. parkeri* have been identified in Zambia, (Chitanga et al., 2021). African tick bite fever caused by *Rickettsia africae* is transmitted by *Amblyomma*, *Rhipicephalus* and *Hyalomma* ticks (Frean and Grayson, 2019).

Variation in genetic structure has been observed amongst ticks of the same genus but from different geographical locations (Lampo et al., 1998), an observation that has been linked to change in habitat (Lado et al., 2020). The difference in genetic structure of a tick population becomes important as it has been observed that it has a linkage to ticks ability to their survival and/or vectorial capacity for different pathogens.

There has been tremendous advancement in the development and improvement of molecular approaches such as population genetics and molecular epidemiology with regards to the identification and characterization of pathogens and their vectors (Eremeeva, 2012, Okasha, 2006). Advancements in population genetics have helped predict incidences of diseases caused by tick-borne pathogens by providing information on the spread and establishment of new tick populations (Nadolny et al., 2015). In addition, population genetics has also revealed the influence of genetic diversity on the ticks' potential to adapt and establish new populations (Monzón et al., 2016). On the other hand, molecular epidemiology has been used in surveillance, diagnosis and determination and identification of both old and new pathogens circulating in an area (Batu et al., 2020).

Information derived from population genetics and molecular epidemiologic studies have significantly contributed to effective disease control. However, in Zambia, despite the presence of *Amblyomma*, *Rhipicephalus* and *Hyalomma* ticks, information on their population genetics and extensive data on the molecular epidemiology of the Rickettsias it transmits is still lacking.

Thus, this study was aimed at determining the genetic variation of *Amblyomma*, *Rhipicephalus*, and *Hyalomma* ticks within and between the study sites and determine the *Rickettsial* species they carried. Further, the study also provided information on the prevalence and the species of *Rickettsia* transmitted by the ticks in Chongwe and Chisamba districts of Zambia.

1.2 Statement of the problem

Ticks are vectors of various disease-causing organisms and their presence on a host also causes irritation. Their impact on the hosts is seen not only as morbidity and mortality but also as a reduction in products such as meat, milk, hides and draught power (Hurtado and Giraldo-Ríos, 2018, Makala et al., 2003). Tick borne diseases in Zambia affect up to 60% of farmers and result in losses of up to 364 million USD in income and 1.3 million in mortalities (Mumba et al., 2018) In the year 2000, it was estimated that tick borne diseases (TBD) caused mortalities of 2,021 cattle (Makala et al., 2003). Furthermore, all the tick species of interest in this study (*Amblyomma*, *Rhipicephalus*, and *Hyalomma*) are vectors of zoonotic pathogens such as *Rickettsia* which cause tick-bite fever (TBF) and African tick bite fever (ATBF), collectively called rickettsiosis in humans and animals. Rickettsioses are mostly experienced by people living in proximity with animals like herdsmen where the vector takes a blood meal from an infected animal and the human becomes an occasional host. Travel, migration and globalization have been implicated as causes of change in the geographical spread of Rickettsioses, with increased reports in endemic areas as well as new reports in areas that previously had no reports of cases. The increased incidence of Rickettsioses is attributed to the ability of the vector to establish new populations and adapt to transmitting the pathogen (Araya-Anchetta et al., 2015, Benham et al., 2021). Additionally, the disease is re-emerging in regions previously known to have eradicated it (Fang et al., 2017). The presence of *Rickettsia* infected ticks is a serious impediment to the growth and improvement of the livestock sector and the livelihood of the communities due to loss in revenue and manpower because of febrile diseases such as TBF and ATBF which have largely remained poorly understood and under reported. The lack of knowledge pertaining to the genetic diversity of ticks is a serious hindrance to developing molecular methods to control ticks and prediction of disease occurrence (Monzón et al., 2016). The *Amblyomma*, *Rhipicephalus*, and *Hyalomma* ticks present in Zambia may carry *Rickettsia* species which have remained largely undetected (Roux et al., 2000). Due to the lack of knowledge of the molecular epidemiology of Rickettsia in certain regions of the country, all causative agents of Rickettsioses

present in Zambia are not known and hence making prevention of disease difficult. Thus, this study seeks to assess the genetic structure of the *Amblyomma, Rhipicephalus*, and *Hyalomma* ticks and the *Rickettsia* species they harbour in Chongwe and Chisamba districts of Zambia bridging the gap in knowledge.

1.3 Justification of the study

In Zambia, *Amblyomma, Rhipicephalus*, and *Hyalomma* tick species are widespread, and their presence also indicates the possible presence of *Rickettsia*. However, studies on population genetics of these tick species and the molecular epidemiology of *Rickettsias* are not extensive. In addition, information on the population genetic structure of the tick and factors that may influence the spread and establishment of tick populations in new areas as well as extensive data on the species and distribution of *Rickettsia* species is also lacking. The information on the genetic structure of the tick population is key to development of molecular methods for tick control as well making predictions on potential pathogens in an area (Araya-Anchetta et. al. 2015). On the other hand, the knowledge of Rickettsial pathogens circulating in the country allows for prediction of rickettsioses hotspots (Schrodi et. al., 2014). The proposed study will therefore provide information on the distribution of the *Amblyomma, Rhipicephalus*, and *Hyalomma* tick populations as well as the epidemiology of *Rickettsias* in the study districts. This will significantly contribute to the knowledge gap that currently exists in Zambia.

1.4 Research questions

1. Are there significant differences in the genetic structure of *Ixodid* tick populations in Chongwe and Chisamba districts

2. What is the phylogeny of *Rickettsia* infecting *Ixodid* ticks in Chongwe and Chisamba districts?

1.4 Objectives

1.4.1 General objective

To determine the genetic variation in *Ixodid* tick populations and assess the molecular epidemiology of *Rickettsia* infecting *Ixodid* ticks in Chongwe, and Chisamba districts of Zambia.

1.4.2 Specific objectives

1. To determine the genetic structure, variation, and gene flow between and among *Ixodid* tick populations from Chongwe and Chisamba districts.
2. Determine the phylogenetic relationships of *Rickettsia* infecting *Ixodid* in the study sites.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Ticks

Ticks are arthropods and belong to the class Arachinida (Anderson and Magnarelli, 2008) which comprises three families namely: Ixodidae, Argasidae and Nuttillieladae (Spach et al., 1993). Ixodidae ticks contains 442 species in the genera *Amblyomma, Anomalohimalaya, Bothriocroton, Cosmiomma, Dermacentor, Haemaphysalis, Hyalomma, Margaropus, Nosomma, Rhipicentor* and *Rhipicephalus* in the family Ixodidae, with the genus *Boophilus* becoming a subgenus of the genus *Rhipicephalus* (Horak et al., 2003). Argasidae ticks commonly called the soft ticks contain 190 species in four genera: Argas, Carios, Ornithodoros and Otobius (Estrada Pena et al., 2010). The family Nuttilieladae has only one genus Nuttiliela (Horak et al., 2003). Ticks from the Ixodidae and Argasidae families are important vectors for pathogens with the Ixodidae family accounting for most of the transmission of pathogens among animals or from animals to humans and vice versa (Bursali et al., 2014). Ticks of importance in the Ixodidae family include *Amblyomma*, *Hyalomma*, and *Rhipicephalus*.

Second to mosquitos, ticks are considered first as vectors of disease-causing pathogens in animals and in humans and transmit various pathogens, including bacteria, protozoa, viruses, and helminths (de la Fuente et al., 2008). Ticks acquire these pathogens either through feeding on an infected host or through co-feeding (Cutler et al., 2021). Pathogen acquisition during co-feeding is important as it allows for transmission of pathogens between infected and uninfected ticks, even when they are feeding on immune hosts, incapable of establishing an active infection/parasitaemia (Voordouw, 2015). Another mode of pathogen transmission between ticks is sexual transmission, as has been demonstrated for *Rickettsia massiliae* in *Rhipicephalus* tick species (Tomassone et al., 2018; Socolovschi et al., 2009). However, in ticks which acquire pathogens through co-feeding and/or sexual transmission, it has been observed that there is no subsequent transovarial transmission of the pathogens to the next generation (Wilson et al., 2017, Tomassone et al., 2018).

2.2 Life cycle of Ixodid ticks

Ixodid tick species includes several hard ticks of the family Ixodidae including *Amblyomma, Hyalomma and Rhipicephalus* and these species are considered the common and important cattle

ticks in Zambia (Pegram and Banda, 1990). An ixodid tick life cycle consists of four stages. The first is an inactive egg and thereafter the parasitic of larvae, nymph, and adult follow. The three parasitic stages feed on a host before moulting to another stage (Apanaskevich et al., 2013). Variation in the length of developmental stages brings about the variations in life cycle durations. The number of hosts required for the life cycle of a tick differs (Apanaskevich et al., 2013). Most Ixodid ticks are either three-host or two-host ticks where the parasitic stagstages fed on a host drop and moult or the larvae feeds, moults into a nymph which also feeds then drops to moult into an adult (Figure 1) (Bursali et al., 2014). Ixodid ticks must mate to produce eggs. The female will feed to full capacity before it mates and after which it will drop off the host and lay eggs in suitable environments of optimal temperature and humidity (Pappas and Oliver Jr., 1971). Ixodid ticks compared to hematophagous insects require feeding for a few days before pathogen transmission can occur (Troughton and Levin, 2007). *Amblyomma* species have been reported to produce large egg batch numbers while *Hyalomma* species produce moderate egg batch numbers (Balashov, 2012). This also depends on the amount of blood the female dhad taken which could be up to 100 times its body weight. After the laid eggs hatch into larvae, they get on.

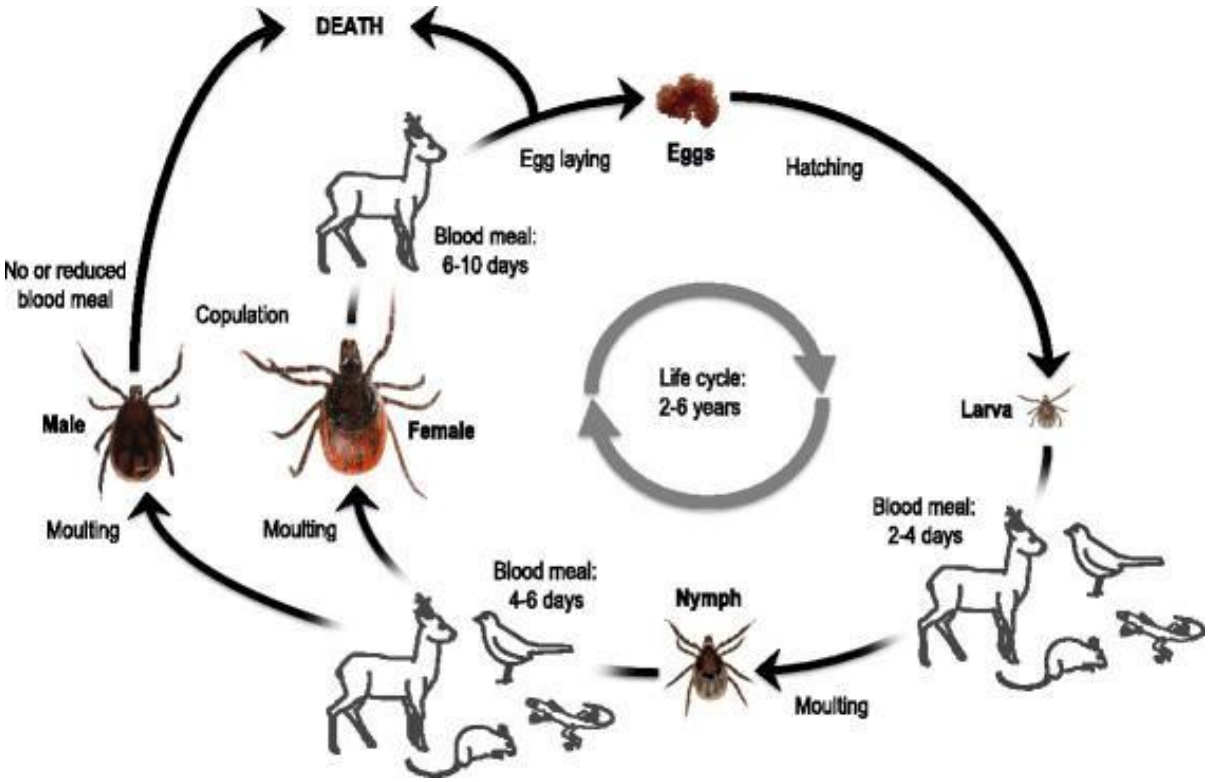

Figure 1: General life cycle of Ixodid ticks (Herrmann and Gern, 2015)

2.2.1 Life cycle of *Amblyomma* ticks

Hard ticks including *Amblyomma* species typically require one to two years to complete their life cycle (Figure 2) (Spach et al., 1993). *Amblyomma* larvae require a blood meal to moult into nymph and so does the nymph to moult into a reproductive adult (Socolovschi et al., 2009). The engorged reproductive female lays its eggs and the preoviposition period is between one and two weeks, with the mean egg incubation period being six weeks (Labruna et al., 2003). When the eggs hatch into larvae they remain on the ground in a phase called diapause. For most, this period can range between two and six months and notably has an impact on the total period of the life cycle (Belozerov, 1982). After diapause, the larvae then migrate from the ground onto vegetation. The period for the tick to moult from larvae to nymph and nymph to adult is approximately eight weeks for *Amblyomma* (Labruna et al., 2003). The life cycle of *Amblyomma* can be completed in six months in the laboratory compared to one year in the field (Labruna et al., 2003), suggesting that environmental factors such as the temperature and photoperiod affect the life cycle of ticks (Belozerov, 1982) by increasing the pre-molt interval at different life stages and inducing diapause induction in the next stage, respectively. When photoperiods reduce, it leads to the termination of diapause and continuation with the life cycle (Labruna et al., 2003).

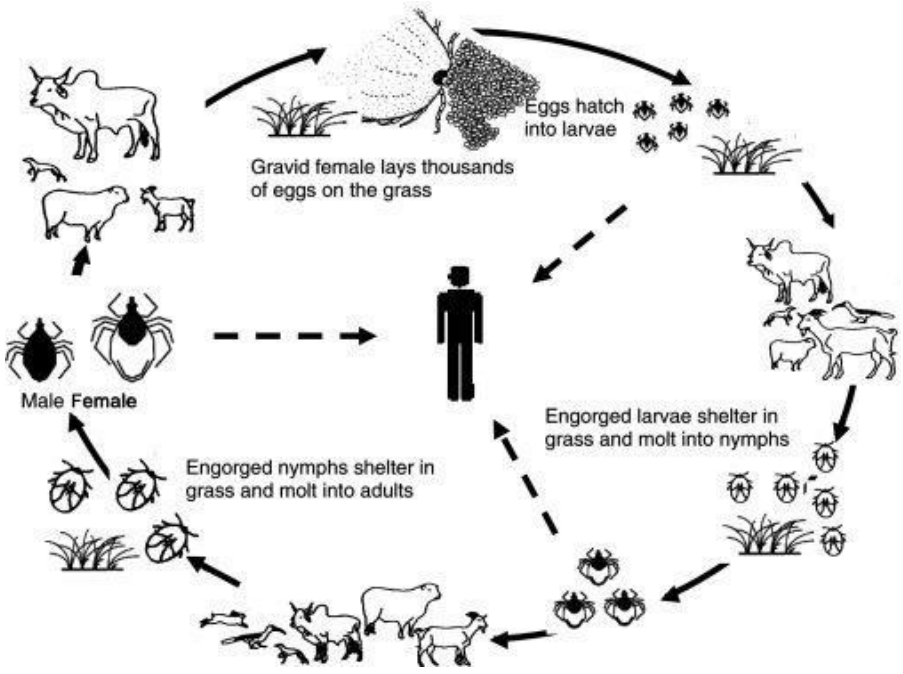

Figure 2: Life cycle of Amblyomma species (Socolovschi et al., 2009)

2.2.2 Life cycle of *Rhipicephalus decoloratus*

Rhipicephalus decoloratus is a one host tick most dominated by larvae stages over the adult stage (Horak et al., 2006). This is because most of the larvae that moult to nymph and drop either after engorgement or by mechanical mechanisms of the host have very low survival rates. The nymph is more likely to survive if it stays on the host and feeds again (Londt and Van der Bijl, 1977). The same is true for a nymph that drops before moulting into an adult (Figure 3). *Rhipicephalus* ticks are said to be good at adapting to different environmental conditions and resist chemical acaricides especially in the case of *R. micrplus*. These are among the reasons for *R. microplus* displacing *R. decoloratus* in places it was initially known to be indigenous to.

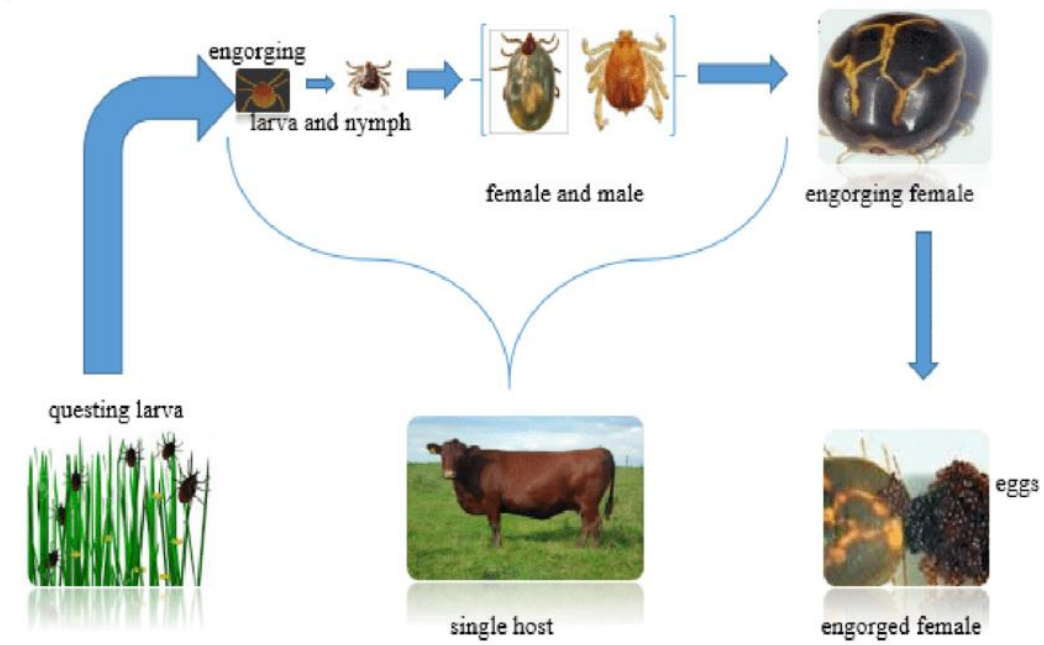

Figure 3: Life cycle of *Rhipicephalus decoloratus* (Nyangiwe et al., 2018)

2.2.3 Life cycle of *Hyalomma* species

Hyalomma species are known to parasitize many animal hosts and *Hyalomma rufipes* is especially known to parasitize humans. Its distribution is mainly dependent on humidity therefore it is mostly found in warm climates like arid, semi-arid, desert, or semi-desert areas. *Hyalomma rufipes* is the primary vector of Crimean-Congo Haemorrhagic fever and transmits various *Rickettsia* species including *R. africae* and *R. aeschlimannii*, *Ehrlichia*, *Borrelia* and *Coxiella* (Vatansever, 2017).

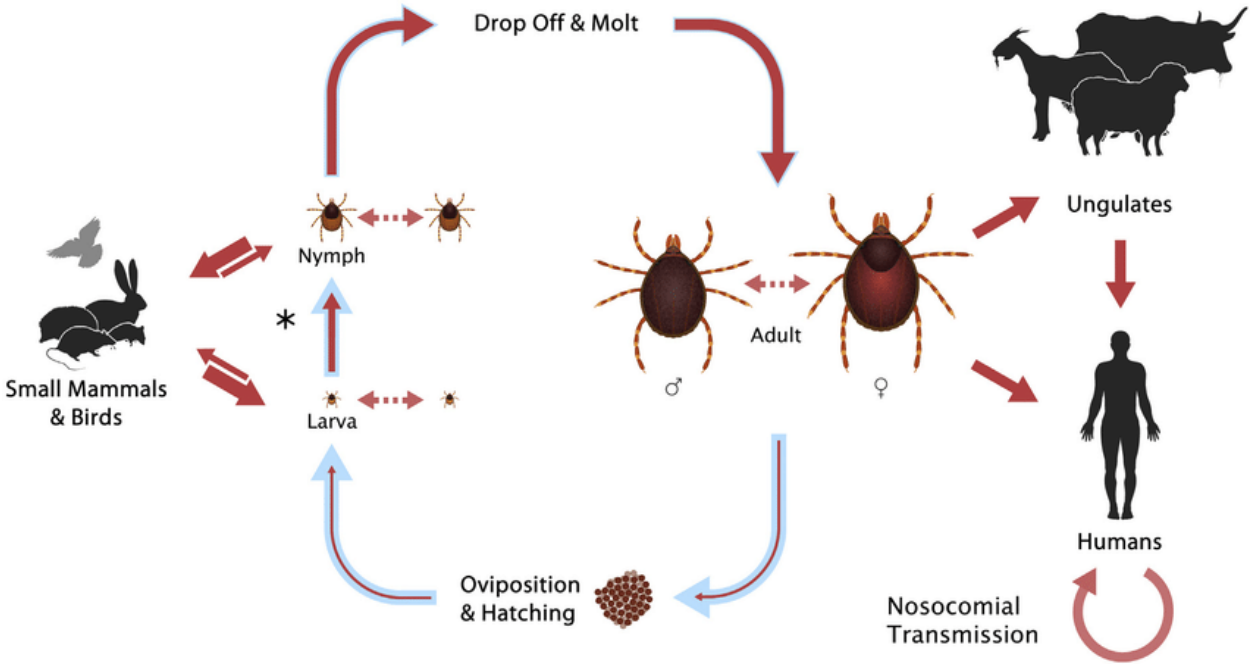

Figure 4: Life cycle of Hyalomma species (Spengler et al., 2016)

2.3 Population genetics of ticks

Population genetics is defined as the study of the genetic structure within, between or among biological populations (Okasha, 2006) and has been utilised to study various ubiquitous pathogens and vector populations (Amzati et al., 2018, Lado et al., 2020, Muleya et al., 2012). This is used to establish relationships among species populations by comparing DNA (or other genomic data) of the same species. The difference in these species is the polymorphisms that are inherited as a set termed as haplotypes. The most common polymorphism is the single nucleotide polymorphism (SNP) or microsatellites (Haidong, 2005).

In population genetic studies of ticks, the environment includes the host which the tick parasitizes and any change in the physiology of the host which exerts pressure on the ticks and thus forces them to evolve (Randolph, 2004). The effects of adverse habitat conditions of an organism and that of random and non-random mating are important aspects when studying a population at molecular level (Muleya et al., 2012). Population genetics can differ from species to species despite being in the same family and in the case of ticks the same host preference. Important factors that govern these differences are host resilience and habitat changes (Nadolny et al., 2015). Because the ticks do not spend their entire lifetime on the host, they are also subject to abiotic conditions of their environment such as climate. With the aid of PCR, studies such as

population genetics and phylogenetics have become easier and have provided information on genetic divergence and recombination. Before the year 2000 the emergence of tick-borne pathogens is said to have been at a rather constant rate. But in the last two decades pathogens in ticks have begun to emerge rapidly and hosts are succumbing to the infections more and more (Adem, 2019). The geographical expansion of ticks in North America has been attributed to reforestation and globally to climate change (Adem, 2019, Troughton and Levin, 2007).

Population genetic studies of the ubiquitous *Amblyomma* tick species in areas such as the state of Veracruz in Mexico where results showed no separation of tick populations with respect to geography (Aguilar-Domínguez et al., 2019) and in America, on the contrary the tick species maintained independent patterns of population establishment (Nadolny et al., 2015) providing valuable information on the spread of the ticks with respect to host movement. In Sothern Africa reviews of tick species including *Amblyomma*, *Hyalomma* and *Rhipicephalus* have showed that these tick populations were likely to establish new population in place where they were currently not endemic and hence the spread of the diseases that they carry (Makwarela et al., 2023). In South Africa a study established to separate clusters of *Rhipicephalus* populations that intersected owing to the free movement of cattle between the two geographical regions (Baron et al., 2018).

Many populations genetic studies of ticks have employed the use of the mitochondrial *16SmRNA* gene owing to its high mutation rate, maternal heredity, low recombination rates and (Aguilar-Domínguez et al., 2019, Bitencourth et al., 2019, de Lima et al., 2017, Lampo et al., 1998). *16SmRNA* is used to compare populations across biogeographical and phylogenetics at species level because it is conserved and changes slowly over time as opposed to intrapopulation analysis because of the low intraspecific variability (Araya-Anchetta et al., 2015, Bitencourth et al., 2019). In contrast, *12SrDNA* is used for intra population studies due to its sensitivity (Norris et al., 1996). In addition, the cytochrome oxidase subunit 1(*CO1*) gene has also been utilised for species identification in population genetic studies due to its high mutation rate and genetic divergence.

Various measures of population differences due to their genetic structure are used and a common measure is F_{st} (Frankham et al., 2002). F_{st} summarizes the coefficient of inbreeding as genetic differences between and among populations (Beaumont, 2005).

Another measure of population differences is how many haplotypes are contained and how they relate. One study identified up to 33 haplotypes of *Amblyomma ovale* that had clustered into two, one in a settlement intersecting the Atlantic rainforest and the other in the rainforest only (Bitencourth et al., 2019). The two areas are of different biomes hence the distinct clusters. Prior to this, Bitencourth et al. (2017) had investigated the diversity of *A. sculptum* in just one biome and found haplotypes of low and high frequency. Those of low frequency indicated the expansion of the species population.

Amblyomma, Hyalomma, and Rhipicephalus species are widespread in Southern Africa and Zambia to be particular and as such variations in adaptation, speciation and vectorial capacity should be considered hence the undertaking of the proposed study (Makwarela et al., 2023, MacLeod et al., 1977).

2.4 Ticks as vectors of *Rickettsia*

Ticks are well known vectors of diseases. Some examples of vector borne diseases include Rift Valley fever, Lumpy skin, Crimean-Congo Haemorrhagic Fever, West Nile Anaplasmosis, Ehrlichiosis, East Coast Fever, Trypanosomiasis and Rickettsioses (Kilpatrick and Randolph, 2012). In Africa, the species of the genus *Amblyomma* often includes *A herebram* and *A variegatum* (Guglielmone et al., 2014) and both are known to transmit *Rickettsia*. The southern African indigenous *Rhipicephalus decoloratus* and *Hyalomma* species are also known to transmit various species of *Rickettsia* (Portillo et al., 2007; Halajian et al., 2016). When the tick is taking a blood meal, there are factors that will affect acquisition of the pathogen. These include the host skin, salivary proteins, defensins and availability of pathogen in host blood. The host skin is meant to protect the host as a barrier for the host, but this is overcome by the ticks due to their probiosis and proteins. Proteins key to pathogen acquisition are found in the salivary glands and midgut. Defensins are protein part of the tick's immunity that protect them from bacteria. This factor is very valuable for *Rickettsia* and other bacteria. These proteins have been shown to favour acquisition of certain pathogens over others. The host must have blood rich in the pathogen and secondly, the tick must take enough blood. Once the tick has acquired the pathogen by ingestion, the pathogen must overcome tick gut barriers to have initial contact with tick cells (Socolovschi et al., 2009). *Rickettsia* interacts with cellular receptors that are not yet known to evade the tick immune response (Ceraul et al., 2007). Once inside the host, the *Rickettsias* infects

the endothelial cells of small and medium vessels (Sahni et al., 2013) facilitating its dissemination to various organs. Epithelial cells in the tick midgut support the multiplication of Rickettsial pathogens and are an important stage for which the vector is required. After entry into the abdominal cavity the pathogens then replicate and invade hemocytes thereby infecting any tissue including salivary glands and ovaries in females. The pathogen can be maintained transstadially for as long as the vector lives. Exclusive to Ixodidae, the maintenance of the pathogen is not only through the life stages but also from one generation to the next known as transovarial transmission. This entails that *Amblyomma* ticks are reservoirs of *Rickettsias* and that the presence of the vector is very vital to disease occurrence. It has been demonstrated that *R. decoloratus* and *R. appendiculatus* are able to transmit pathogens transovarially hence making them effective reservoir hosts even of *Rickettsia* (Lubinga et al., 2014). The transstadial and transovarial methods of transmission are the most common but Rickettsias develop different strategies to adapt to change in the vector (Socolovschi et al., 2009).

2.5 Rickettsias and their distribution

Rickettsia are obligate intracellular bacteria of the spotted fever group under the genus *Rickettsia* and cause a group of neglected tropical diseases collectively called Rickettsioses (Parola et al., 2013, Frean and Grayson, 2019, Jongejan et al., 2020). *Rickettsias* are divided into the spotted fever group (SFG) and the typhus group (TG). The scrub typhus group has since been reclassified under the genus *Orientia* following DNA sequencing (Weinert et al., 2009). *Rickettsias* are widespread, but certain species are restricted to certain geographical locations due to the vectors that transmit them. Rickettsioses have been reported world-wide with the most pathogenic species being from the SFG. These include Mediterranean spotted fever caused by *Rickettsia conorii conorii,* Rocky Mountain spotted fever caused by *R. rickettsia,* Israeli spotted fever caused by *R. conorii israelensis* and Astrakhan spotted fever caused by *R. conorii caspia* of the Mediterranean group. In Asia, Indian tick typhus, Far-eastern tick-borne rickettsioses, North Asian tick typhus, lymphangitis-associated rickettsioses and the Japanese spotted fever are caused by *R. conorii indica, R. heilongjiangesis, R. sibirica sibirica, R. sibirica mongolitimonae and R. japonica,* respectively (Socolovschi et al., 2009). In Central America *Rickettsia species* that have been isolated include *R. africae, R. amblyommatis, R. asembonensis, R. bellii, R. felis, R. parkeri, R. rhipicephali,* and *R. rickettsia* (Bermúdez et al., 2018). In southern Africa, the two common rickettsioses are boutonneuse fever like tick bite fever (BFL-TBF) and ATBF

caused by *Rickettsia conorii* and *R. africae,* respectively (Frean and Grayson, 2019). Other *Rickettsia* species include *R. australis*, *R. slovaca*, *R. aeschilemannii*, *R. massiliae*, *R. raoultii,* and *R. honei* found in Australia, Asia, Africa, and Europe (Guccione et al., 2023).

2.6 Transmission of *Rickettsia*

Transmission of *Rickettsia* is mostly by ticks and other vectors are fleas and lice (Azad and Beard, 1998). SFG *Rickettsia* are transmitted by ticks which also maintain them in the environment and those of the TG are transmitted by other blood sucking parasites, namely fleas and lice. Some ticks known to transmit *Rickettsia* include *Rhipicephalus*, *Dermacentor* and *Amblyomma*. Certain *Rickettsia* species can be transmitted by more than one vector like *R. africae* which is transmitted by *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *A. hebraeum* of which the latter two (*A. variegatum* and *A. hebraeum*) are of the same species (Yssouf et al., 2014). In the case of *Rickettsia prowazekii* (Reháçek, 1989), and *R. typhi* (Fang et al., 2017), transmission occurs via lice and fleas as they bite the host. *Rickettsia* is transmitted through saliva when the tick takes a blood meal from a host. This is made possible due to the feeding pattern of the tick which involves the puncturing of the epidermis and dermis with its mouth parts followed by the insertion of the hypostome into a pool of blood formed under the skin. Using the hypostome blood is ingested while releasing saliva containing anaesthetic, anticoagulants, host defence inhibiting factors and infectious agents such as *Rickettsia* (Fang et al., 2017).

2.7 Diagnosis of Rickettsiosis

After the discovery of *Rickettsia* in 1876, diagnosis of *Rickettsia* was by clinical signs of fever, rash, headache, and a history of recent exposure to ticks (Fang et al., 2017). As earlier alluded to, diagnosis based on clinical signs is not reliable or definitive. This is because of the similarity of symptoms with other febrile diseases (Adem et al 2019, Chitanga et al 2021). In addition to clinical signs, other tests such as the Weil-Felix proteus vulgaris OX-2 serological test have been used and have led to the discovery of *Rickettsia japonica*. Other tests used in the diagnosis of Rickettsiosis include immunohistochemical staining based on the detection of antigens from skin biopsy (Fang et al., 2017), enzyme-linked immunosorbent assay (ELISA) and western blotting. The downside of immunohistochemical staining is that it is not reliable after 48 hours of infection and when antimicrobial treatment has commenced. Currently serological tests are not

favoured because of the number of false positives because of cross reaction with species closely related to *Rickettsia* such as *Orentia* (Fenollar et al., 2007). Thus, in recent times, molecular approaches that are highly specific and selective such as PCR (Joshi and Deshpande, 2010), have significantly improved and are utilised in the diagnosis of *Rickettsia*. Use of molecular methods in identifying species has become a gold standard as it is not disputed compared to immune assays i.e. western blotting (Roux et al., 2000) and bacterial culture. PCR diagnosis of *Rickettsia* is based on the detection of rickettsial genes such as the outer membrane protein A (*omp*A), outer membrane protein B (*omp*B), citrate synthase (*glt*A) genes, heme-regulated transporter A (*hrtA*) and surface cell antigen (*sca4*) (Bitencourth et al., 2019, Loyola et al., 2018, Moreira-Soto et al., 2017). The sample of choice includes blood collected from a suspected patient/host and the biting tick if available.

2.8 Molecular epidemiology of *Rickettsia*

Molecular epidemiology of infectious diseases employs molecular biology and adds epidemiology to study the effect of genetics on cause and spread of disease. Molecular epidemiology has been used to characterise disease pathogens and discriminate known species among groups and identify new isolates (Eremeeva, 2012).

Molecular epidemiology of *Rickettsia* transmitted by *Amblyomma* ticks has mainly focused on the analysis of the outer membrane protein A (*omp*A), outer membrane protein B (*omp*B) and citrate synthase (*glt*A) genes encoding the proteins that express surface based high molecular mass epitopes (Philip et al., 1978) to identify Rickettsial pathogens (Moonga et al., 2019). Other genes that have been utilised include the 16S ribosomal RNA (rRNA) and gene D 192 (sca4) (Abdad et al., 2018, Roux et al., 2000).

Molecular epidemiology of *Rickettsia* and Rickettsioses studies have helped establish the presence of various Rickettsial pathogens around the world including *R. felis, R. africae, R. monacesis, R. aeschlimannii* (Eremeeva, 2012). In Zambia species that have been identified include *R. felis*, *R. conorii*, *R. africae*, and *R. aeschlimannii* (Chitanga et al., 2021, Chitimia-Dobler et al., 2017, Moonga et al., 2019).

Owing to the lack of information on the genetic structure of the *Amblyomma, Hyalomma, and Rhipicephalus* and the non-extensive molecular epidemiology of *Rickettsia* species in all regions of Zambia, the current study has been proposed to generate data on the population structure,

genetic variation and spread patterns of ticks. The study also aimed to identify and determine the proportions of *Rickettsia* species infecting ticks in the study sites. The information obtained from this study will give insight and guidance on public health policy formulation in Zambia.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study sites

This study was conducted in Chongwe and Chisamba districts of Lusaka province and Central province, as shown in Figure 5. These study sites were chosen because of their vast livestock farming. Central and Lusaka Provinces rank third and nineth as provinces with livestock-raising households with cattle population of 57,834 and 9634 respectively (Ministry of Livestock and Fisheries – Zambia, 2022). Other animals reared in the two districts include sheep, goats, fish, and chickens. Chongwe district is located 40.5 km east of Lusaka at 15.35º south latitude and 28.7º east longitude. Chongwe is situated 1069m above sea level and covers an area of 8669km^2 (Kuntashula et al., 2018). Within Chongwe district the veterinary camps where samples were collected from are Kapete, Kanakantapa, and Palabana. Chisamba is located north of Lusaka district between latitude 14º 30’ and 15º 00’ south and between longitude 28º 00’ and 28º 30’ east. The main vegetation in Chongwe district is Brachystegia, Colophospermu mmopane and Acacia. Chisamba district is 50km from Lusaka the capital of Zambia, covers an area of 2978.5km^2 and sits 1138m above sea level (Chirwa et al., 2015). Chisamba district is fed with three rivers and hence has prolific vegetation of Brachystegia. For Chisamba district the veterinary camps where samples were collected are Mwomboshi, Chipembi and Lufwambula. Both districts are of humid subtropical, dry winter climate.

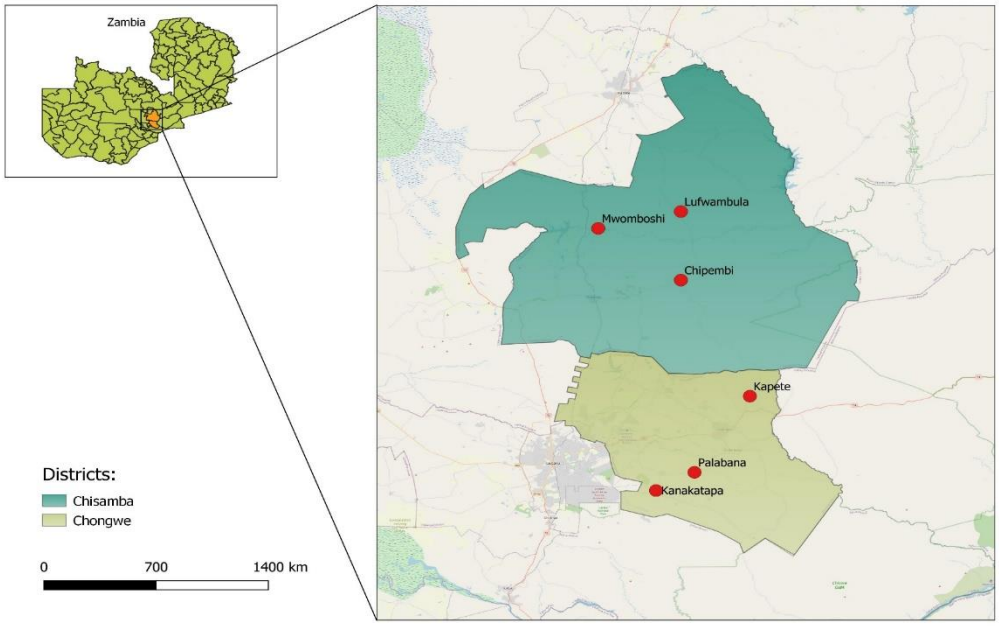

Figure 5: Sample collection sites within Chongwe and Chisamba districts of Zambia. The two districts share a border and are divided into veterinary camps. Map of study sites constructed in GIS (Steiniger et al., 2010).

3.2 Study design

This was a cross sectional study, in which samples were collected at two different times; November 2020 from Chongwe and June 2021 from Chisamba and then collectively processed and analysed.

3.3 Sample size

Sample size formula for a population genetic study that should capture genotypes that occur at 5% frequency (rare genotypes) factoring in bottle neck effect.

$$E(k) = n(M1 \div M)$$

Where:

E(k) is the average number of desired genotypes.

M finite population

M1 Desired genotypes

n is sample size.

In an estimated sample size of 100 the desired genotypes will occur at an average of 5 (Crossa, 1989). Samples were randomly selected categorically to ensure a good representation of each district, veterinary camp, farmer, life stage (adult, nymph, larva) and sex. (male and female).

3.4 Experimental approach

3.4.1 Sample collection

Ticks of all life stages were collected from the environment by dragging and flagging and from cattle using forceps in each respective district. Collected ticks were stored in perforated falcon tubes containing leaves and transported to the School of Veterinary Medicine and stored in storage containers between -20℃ and -24℃.

3.4.2 Morphological identification of ticks

Ticks were identified morphologically using the guide provided by Walker (2003) and all tick species collected were used for further analysis. Ticks collected as samples for this research were each assigned a label containing a letter denoting the site then followed by the animal identification with numbers running chronologically from one (1). The next three characters denoted the tick species, sex, and life stage. The ticks identified were then stored separately in storage tubes and frozen at -20℃ and -24℃.

3.4.3 Tick preparation and Extraction of Deoxyribonucleic acid (DNA)

Ticks were individually washed in Phosphate buffered saline (PBS), followed by 70% ethanol and then PBS again. These were then placed in smasher tubes containing 200µl Dulbecco's modified eagle's medium (DMEM) and then smashed at 4000rpm for two minutes with 10 seconds between each minute. The resulting homogenate was then used to extract total DNA using NucleoSpin®.

To each homogenized tick sample, 180µl of buffer T1 and 25µl of proteinase K were added. This was then vortexed and incubated at 56 ºC overnight. The samples were then vortexed and 200µl of lysis buffer B3 was added. The samples were then vortexed vigorously and incubated at 70 ºC for 10 minutes after which they were vortexed. The samples were then centrifuged at 11000g, at 4ºC for five minutes and the supernatant transferred to a micro centrifuge tube. To this supernatant 96 % molecular grade ethanol was added and the sample was vortexed. This solution

was then loaded into a NucleoSpin® Tissue column with a collection tube attached. This was centrifuged at 11000g, at 4ºC for one minute. The flow through was discarded. The remaining sample supernatant was added, and the centrifugation repeated. After discarding the flow through 500µl of wash buffer BW was added to the NucloeSpin® Tissue column and centrifuged for one minute at 11000g, at 4ºC. The flow through was discarded and 600µl of buffer B5 was added to the NucleoSpin® Tissue column. The sample was then centrifuged at 11000g, 4ºC for 1 minute and the flow through was discarded. The Nucleospin® Tissue column was centrifuged for 1 minute at 11000g to remove residual ethanol. The NucleoSpin® Tissue column was then transferred to a new micro centrifuge tube. One hundred microliters of elution buffer BE was added to the column and incubated at room temperature and pressure (r.t.p) for one minute. The column was then discarded, and the extracted DNA stored at -30 ºC ready for molecular analyses.

3.4.4. PCR amplification, DNA purification, cycle sequencing, ethanol precipitation and capillary electrophoresis of *12smRNA* and *16SrDNA*

Extracted DNA was used for amplification. PCR was used to amplify segments of the genome for *12SmRNA* and *16SrDNA* genes using primers listed in Table 1. This was a 20µl reaction consisting of 10µl 2 X one-taq master-mix, 0.48µM of the forward and reverse primers, 5.08µl of nuclease free water and 3µl of the template. Conditions used were initial denaturation at 95ºC for 1 minute, denaturation at 95ºC for 30 seconds, annealing for 30 seconds, extension at 72ºC for 1-minute, final extension at 72ºC for 10 minutes run for 35 cycles and held at 4ºC till further processing. Amplicons were visualised by agarose gel electrophoresis pre-stained with ethidium bromide with a ladder to estimate band size. Expected amplicon size was 355bp and 454bp for *12SrDNA* and *16SmRNA* respectively.

Table 2: Primers for Ixodid tick species identification and population genetics

Gene	**Forward 5'→ 3′**	**Reverse 5' → 3'**	**Annealing temperature**	**Reference**
12SrDNA	AAACTAGGGATTAGATACCCT	AATGAGAGCGACGGGCGATGT	50	(Beati and Keirans, 2001)

16SmRNA	CTGCTCAATGATTTTTTAAATTGCTGT	GTCTGAACTCAGATCAAGT	48°C	(Nadolny et al., 2011)

The PCR products were then purified. The Wizard Promega™ Gel DNA recovery kit was used for the purpose of purification of *Ixodid* tick *16SmRNA*, and *12SrDNA* PCR products according to the manufacturer's instructions. A volume equal to the PCR product of membrane binding solution was added to the product and this was then transferred to the minicolumn assembly, and this was incubated at r.t.p for one minute. This was centrifuged at 16000G for one minute and the flow-through was then discarded and 700µl of membrane wash solution was added and this was again centrifuged at 16000G for one minute. The flow-through was then discarded and 500µl of membrane wash solution was added and centrifuged at 16000G for 5 minutes. The flow-through was discarded and then the minicolumn was centrifuged again at 16000G for one minute. Then the collection tube was changed for a clean microcentrifuge tube. Nuclease-free water was then added to the minicolumn and incubated for one minute. This was then centrifuged at 16000G for a minute and then the minicolumn was discarded.

The brilliant dye terminator cycle sequencing kit (Nimagen® Netherlands) was used to sequence the purified PCR product. The reaction volumes were as follows; 0.5µl brilliant dye, 3.5µl brilliant dye buffer, 0.5µM primer (Table 2), 10.5µl nuclease free water and 5µl template purified DNA. The thermal cycler conditions were as follows; initial denaturation at 96ºC for 45 seconds, followed by 28 cycles of denaturation at 96ºC for 10 seconds, annealing at 50ºC for 5 seconds, and extension at 60ºC for four minutes.

Excess buffers and dNTPs were removed from the cycle sequencing products using the ethanol precipitation method. Briefly, for each sample, 2µl of EDTA, 2µl of acetate and 90µl of absolute alcohol were added and then incubated for 10 minutes in the dark. The mixture was centrifuged at 15000 rpm for 20 minutes and the supernatant pipetted out. 200µl of 70% alcohol was then added and then centrifuged at the same speed for 10 minutes and the resulting supernatant pipetted out. After this, the samples were wrapped in aluminium foil and vacuum dried for 10 minutes and then dissolved in 20µl of highly ionised formaldehyde and finally denatured at 95ºC for five minutes. All purified and denatured samples were then subjected to capillary electrophoresis using the SeqStudio genetic analyzer (Applied Biosystems).

3.4.5 PCR amplification, DNA purification, cycle sequencing, ethanol precipitation and capillary electrophoresis of *Rickettsia ompB, ompA* and *gltA*

3.4.5.1 Screening for *Rickettsia* in sample pools

Samples were pooled as follows: 1µl of each sample was used and 10 samples constituted a pool. Pools were screened for *Rickettsia* using PCR targeting the *omp*B gene (Roux et al., 2000). This involved a 20µl reaction consisting of 10µl 2X one-taq master mix, 0.48µM forward and reverse primers (Table 2), 5.08µl of nuclease free water and 3µl of the template. The PCR conditions were as follows; initial denaturation at 95ºC for one minute followed by 35 cycles of denaturation 95ºC for 30 seconds, annealing for 30 seconds, extension at 72ºC for 1-minute and a final extension step at 72ºC for 10 minutes (Chitimia et al., 2010). Expected amplicons were 532bp, 420bp and 589bp for *omp*A, *omp*B and *glt*A respectively. Amplicons were visualised by gel electrophoresis with ethidium bromide staining.

3.4.5.2 Un-pooled samples

For all *Rickettsia* positive pooled samples, samples were split, and individual samples tested by PCR. PCR was used to amplify *ompA, ompB* and *gltA* genes using the primers (Table 3) and condition described in the section 3.4.4 for PCR. Amplicons were visualised by agarose gel electrophoresis pre-stained with ethidium bromide and a 100bp ladder to estimate band size. Thereafter the PCR products were purified then subjected to cycle sequencing, precipitated by ethanol and loaded into the sequencer for capillary electrophoresis.

Table 3: Primers used for *Rickettsia* PCR and sequencing.

Gene	Forward 5'→ 3′	Reverse 5' → 3'	Annealing temperature	Reference
Rickettsia ompA	ATGGCGAATATTTCTCCAAAA	AGTGCAGCATTCGCTCCCCCT	48°C	(Regnery et al., 1991)
Rickettsia ompB	GTAACCGGAAGTAATCGTTTCGTAA	GCTTTATAACCAGCTAAACCACC	46°C	(Choi et al., 2005)

Rickettsia gltA 1	TGCGGAAGCCGATTG CTTTA	TCTTGCTCATGATC GGCATGT	47°C	(Chitanga et al., 2021)
Rickettsia gltA 2	TGTCGGTTCTCTTTCG GCAT	GCGGATTGTTGTCT AGCTGC	47°C	(Chitanga et al., 2021)
Rickettsia gltA 3	GCCGATCATGAGCAG AATGC	ATCCAGCCTACGGT TCTTGC	47°C	(Chitanga et al., 2021)

3.5 Data analysis

Nucleotide sequences obtained in this study were first subjected to NCBI BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST), then edited and finally assembled using the ATGC software plug-in in Genetyx ver. 12 (Genetyx Co., Tokyo, Japan).

3.5.1. Population genetics

The *12SrDNA* and *16SmRNA* gene sequences were used for identification, phylogenetic and population genetic analysis. To determine the species of ticks, reference sequences were downloaded from the GenBank and together with the nucleotide sequences obtained in this study were used to generate multiple sequence alignment using Clustal W1.6 (GENETYX Corporation, Tokyo, Japan). Afterwards, a fasta file of the multiple sequence alignment file was converted to a mega file and utilised to generate a maximum likelihood phylogenetic tree based on a suitable model with a confidence level of 1000 bootstrap (Tamura, 1992) replicates using MEGA XI (Tamura et al., 2021) computer software.

Haplotypes and nucleotide diversity were calculated using Network ver. 3.5 (Excoffier et al., 2005) and DnaSP ver. 6.0 (Rozas et al., 2017), respectively. Further, the haplotype diversity was verified with Arlequin ver. 3.5.2.2. (Excoffier and Lischer, 2010). Arlequin was also used to calculate the pairwise genetic differentiation between populations. The scale used to score the differentiation is zero to one with 0.0-0.3 been low, 0.4-0.7 moderate and 0.8 to 1 been high (Muleya et al., 2022).

3.5.2 Molecular epidemiology of *Rickettsia*

Proportion of *Rickettsia* was determined by calculating the number of positive samples versus the total number of samples collected for each study site. Furthermore, the sequences obtained from *Rickettsia* positive samples were assembled by Genetyx (GENETYX Corporation, Tokyo, Japan) and blasted on http://www.ncbi.nlm.nih.gov/BLAST website for verification of *Rickettsias* and then together with reference sequences downloaded from GenBank, a multiple sequence alignment was generated using ClustalW1.6 incorporated in Genetyx ver. 12 (GENETYX Corporation, Tokyo, Japan). A fasta file of this alignment converted to MEGA file format was then used to generate phylogenetic trees based on the maximum likelihood model and a suitable model determined by MEGA XI (Tamura et al., 2021) computer software.

3.6 Ethical considerations

Consent to conduct this study was obtained from the University of Zambia Health Sciences Research Ethics Committee (UNZAHSREC) with approval reference 202211230190. In addition, verbal consent of local leaders and the owners of the animals were sought before collection of samples.

CHAPTER FOUR

4.0 RESULTS

4.1 Morphological identification of ticks

The total number of ticks collected was 168. Ticks were morphologically identified only to genus level, sex and life stage as shown in Table 4 below. Of the 168, two were environmental samples and the rest were from cattle.

Table 4: Morphological identification of Tick genera

Tick genus	**Sex**		**Life stage**			**Total**
	Male	**Female**	**Adult**	**Nymph**	**Larvae**	
Amblyomma	19	57	48	28	0	76
Hyalomma	14	33	15	31	1	47
Rhipicephalus	20	24	21	23	1	45
Total	53	115	84	82	2	168

4.2 Molecular identification of ticks and their distribution

4.2.1 Molecular identification of ticks

To confirm the morphological identity of the ticks; mitochondrial *12SrDNA* and *16SmRNA* genes were amplified and sequenced. Sample gel electrophoresis pictures are in figure 6. Of the 168 samples collected only 111 sequences were obtained. The total number of sequences obtained in this study from *12SrDNA* and *16SmRNA* were (n=61) and (n=50) respectively (Table 4). Blast analysis segregated these sequences as *Amblyomma* (n=37), *Hyalomma* (n=47) and *Rhipicephalus* (n=27) (Table 4). Table 5 shows the sequences obtained on 12SrDNA and 16SmRNA. All the sequences obtained in this study have been deposited in the DNA Database of Japan with accession *12SrDNA Amblyomma* (LC795422-LC795451), *12SrDNA Hyalomma* (LC795686-LC795715), *12SrDNA Rhipicephalus* (LC795752-LC795774), *16SmRNA Amblyomma* (LC796238-LC796260), *16SmRNA Hyalomma* (LC7966-20-22,24,25,27-29, 31-39 & LC796641), and *16SmRNA Rhipicephalus* (LC7966-42-45, 47, 49-55, 58-63).

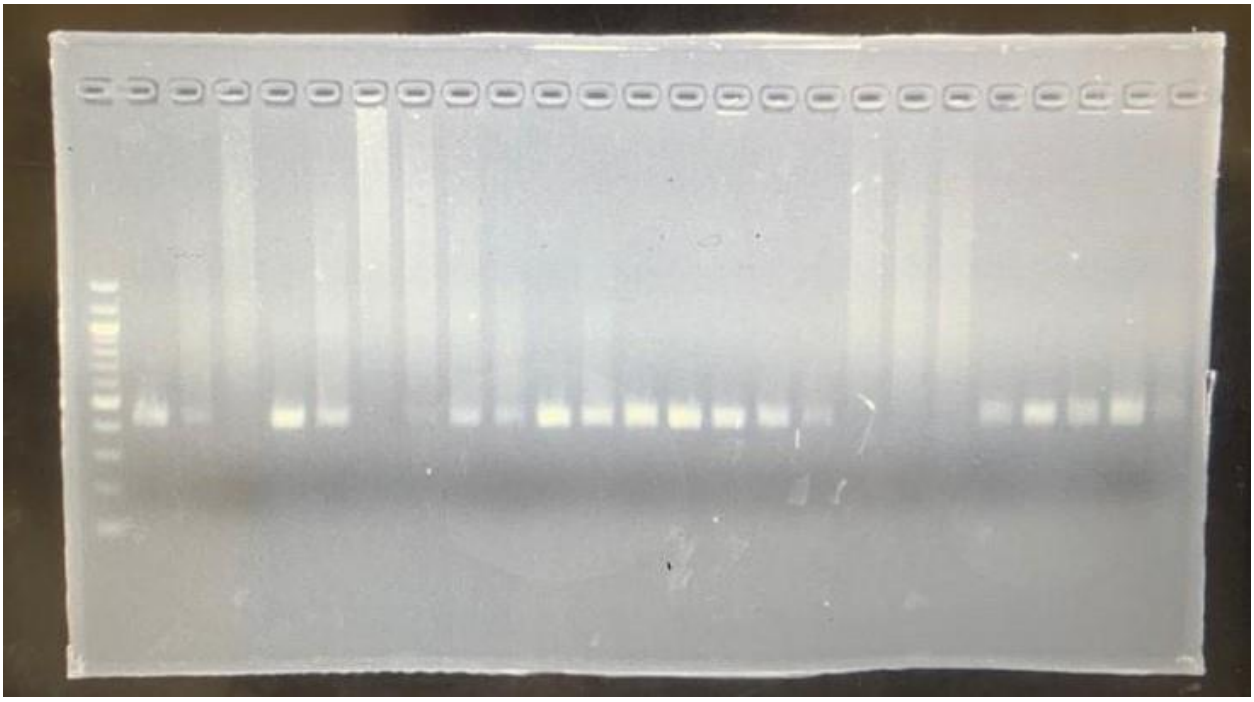

Figure 6: gel electrophoresis of 24 samples amplified on *16SmRNA.*

Table 5: Sequences obtained for the *12SrDNA* and *16SmRNA* from ticks from Chongwe and Chisamba districts.

	12SrDNA	***16SmRNA***	**Total**
Amblyomma	28	9	37
Hyalomma	32	15	47
Rhipicephalus	1	26	27
Total	61	50	111

4.2.2 Tick distribution according to districts

There were more *Amblyomma* (n=28) and *Hyalomma* (n=32) ticks collected in Chongwe district in comparison to Chisamba district, whilst more *Rhipicephalus* ticks (n=26) were collected from Chisamba (Table 6).

Table 6: Distribution of *Amblyomma, Hyalomma* and *Rhipicephalus* ticks among veterinary camps and between Chongwe and Chisamba districts

	Chongwe district				Chisamba district				Overall Total
	Kanakatapa	**Kapete**	**Palabana**	**Total**	**Chipembi**	**Mwomboshi**	**Lufwambula**	**Total**	
Amblyomma	18	6	4	28	1	1	7	9	37
Hyalomma	17	4	11	32	1	0	14	15	47
Rhipicephalus	1	0	0	1	10	9	7	26	27
Total	36	10	15	61	12	10	28	50	**111**

4.3 Phylogenetic and Population genetic structure of tick populations

4.3.1 Phylogenetic analysis of tick populations

Further, the identity of the species of the obtained sequences was confirmed using phylogenetic analysis of *16SmRNA* and *12SrDNA* genes (Figs. 7 and 8). In this analysis, all sequences clustered according to their specific species.

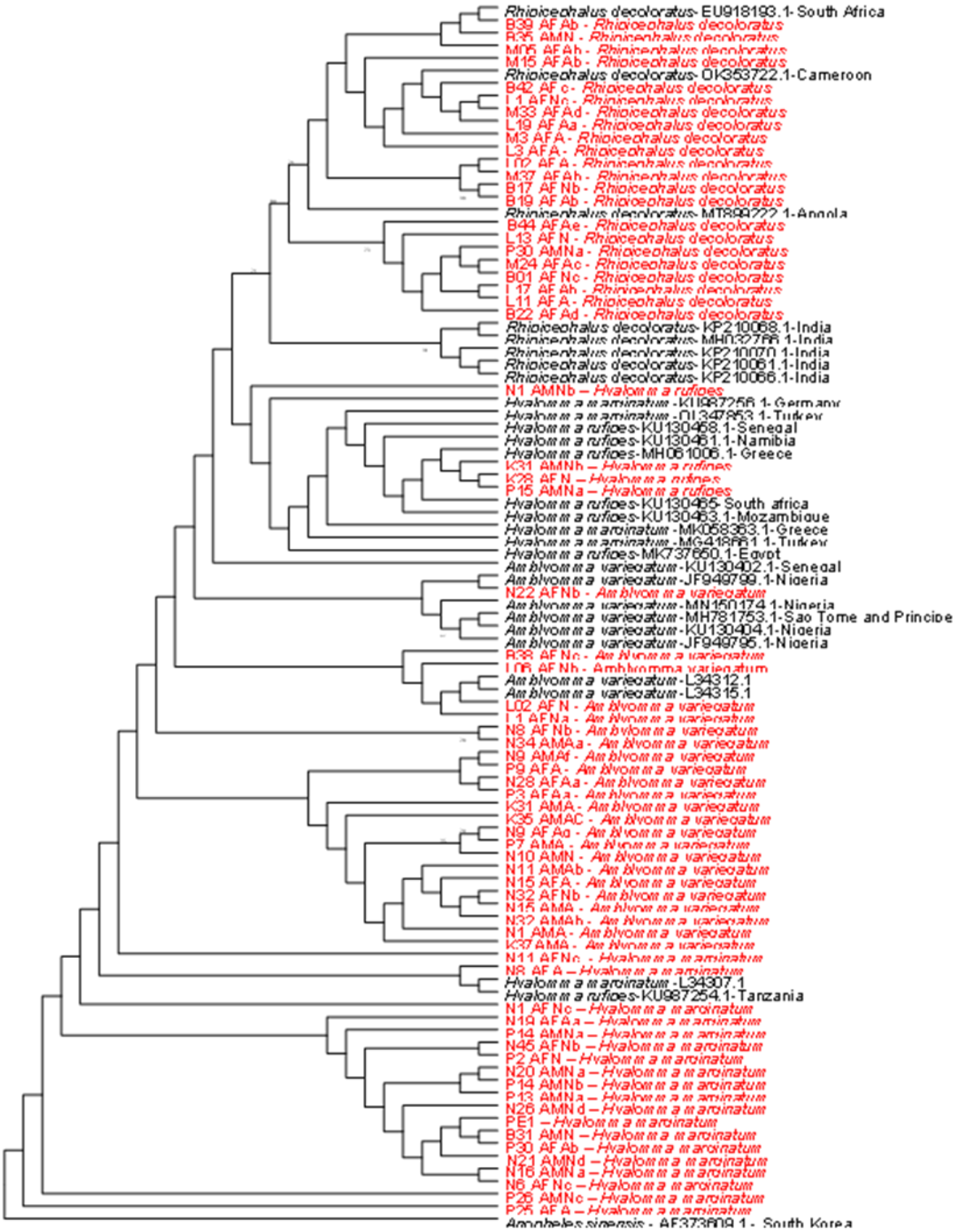

Figure 7: Phylogenetic analysis of 430bp mitochondrial *16SmRNA* amplified in ticks of Cattle and environment from Chongwe and Chisamba. Bootstrap values of ≥70 are indicated at branch nodes. References sequences used are shown by their GenBank accession numbers and country of origin. The sequences from this study are in red text.

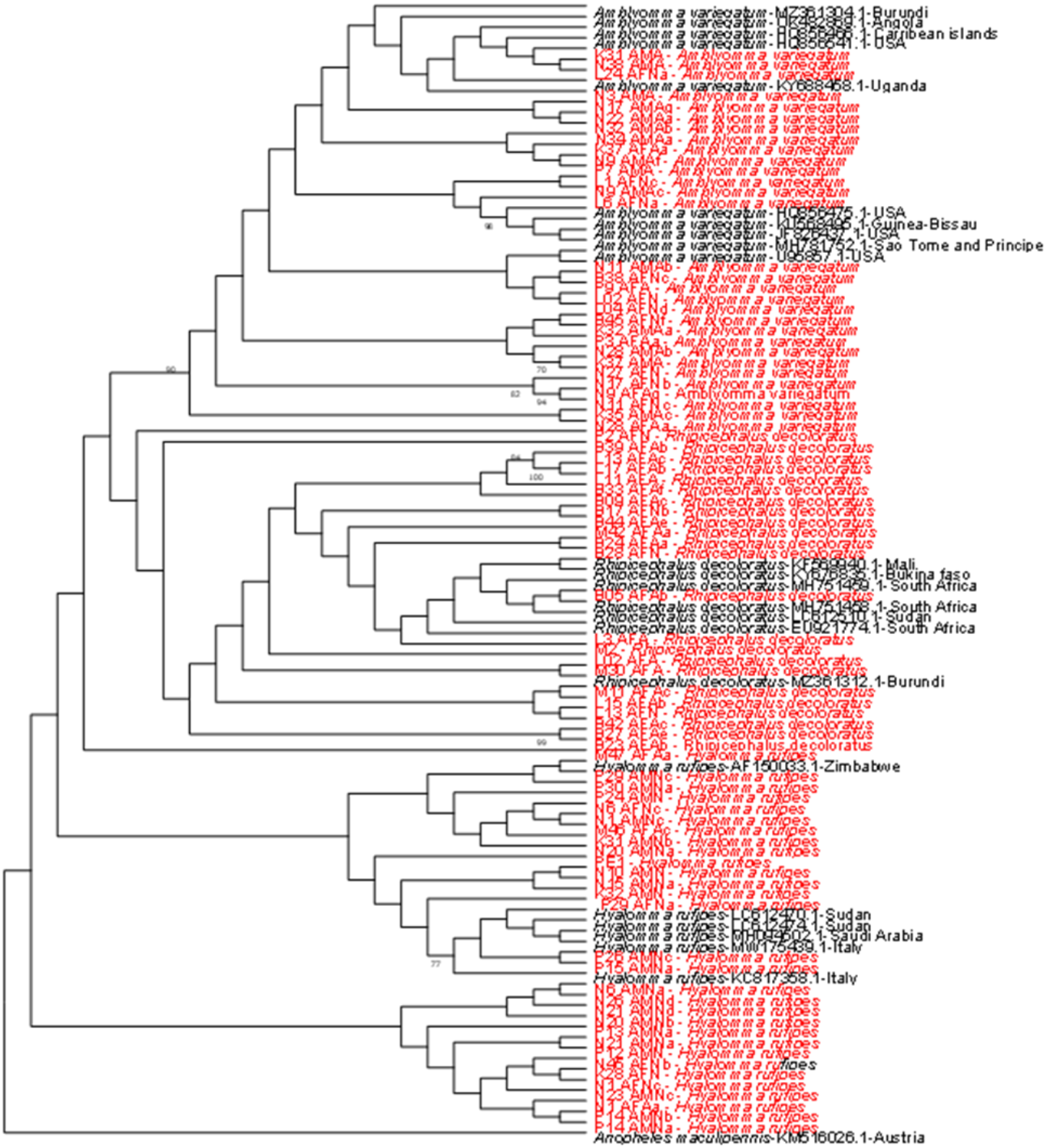

Figure 8: Phylogenetic analysis of 333bp mitochondrial *12SrDNA* amplified in ticks of Cattle from Chongwe and Chisamba. Bootstrap values of ≥70 are indicated at branch node. References sequences used are shown by their GenBank accession numbers and country of origin. The sequences from this study are in red text.

4.3.2 Population genetic analysis

4.3.2.1 Inter population analysis - between Districts.

To compare between the two districts that share a significant border by distance, the *16SmRNA* gene was used for its benefits of making better geographical inferences at species level.

Amblyomma tick populations between Chongwe and Chisamba districts showed low genetic differentiation with Fst value of 0.01635 and p value of 0.8. Between the two districts, a total of 13 haplotypes were observed with haplotype diversity of 0.783 and nucleotide diversity of 0.03 indicating high diversity and recent expansion (Figure 9). With regards to *Hyalomma* the Fst value of 0.156 and a p-value of 0.315 were obtained, indicative of moderate genetic differentiation. Genetic differentiation of the *Rhipicephalus* population between Chisamba and Chongwe was not assessed due to the low number of sequences (n=1) from Chongwe.

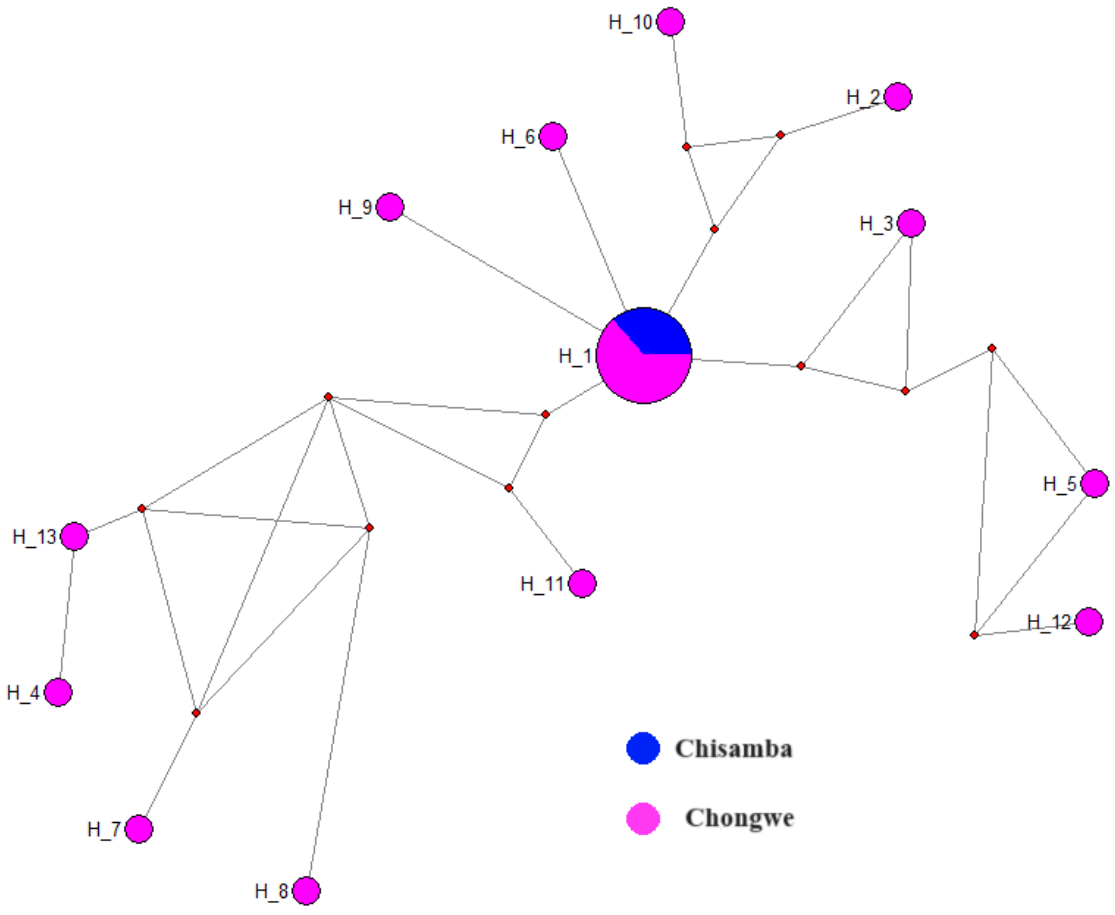

Figure 9: Chongwe and Chisamba *Amblyomma 16SmRNA* network.

4.3.2.2 Intra population analysis - within district

To assess the intra population genetics, *12SrDNA* was used because of its variability at genus level.

4.3.2.2.1 *Amblyomma* populations

Owing to the low number of sequences of *Amblyomma* from Chisamba district, intra population analysis was not conducted. Nevertheless, the *Amblyomma* population in Chongwe consisted of 21 haplotypes (Fig. 9) with the major haplotype being H1 and consisting of sequences from Kapete (n=1) and Kanakatapa (n=2) veterinary camps. Haplotype H1 was the anchor of the network with the rest of the haplotypes radiating from it (Fig. 10). The rest of the sequences didn't share any haplotypes.

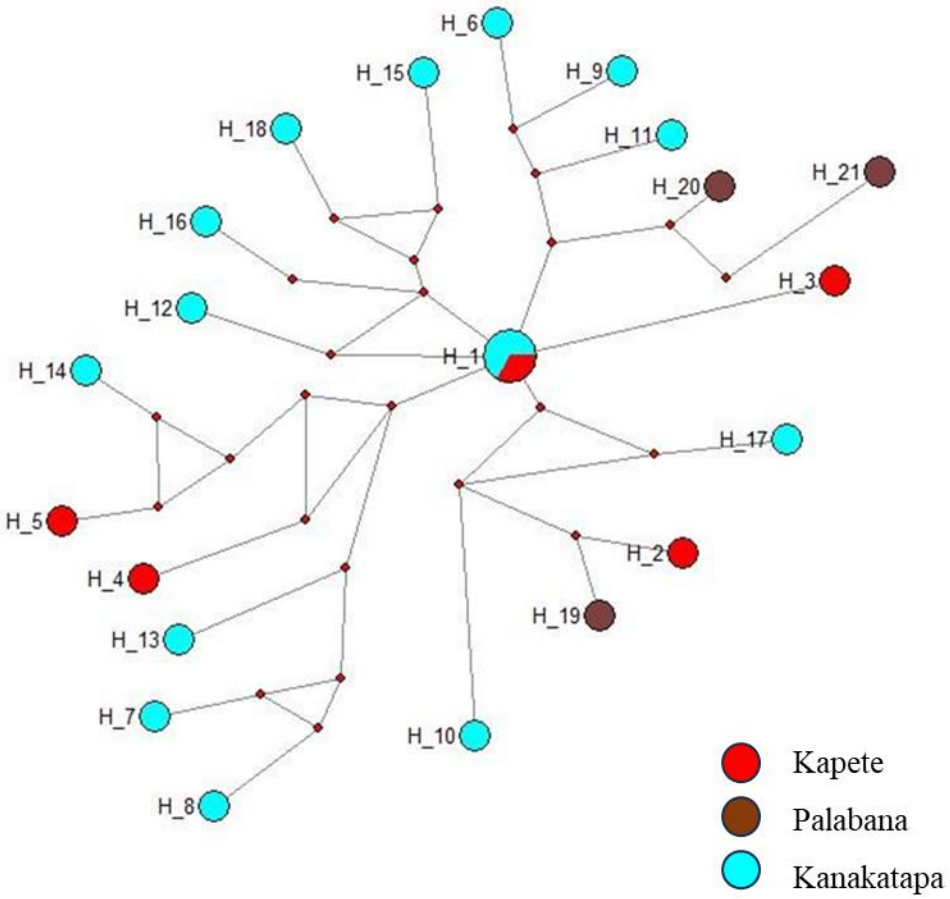

Figure 10: Chongwe *Amblyomma* network *12SrDNA.*

Further, the *Amblyomma* population had a nucleotide and haplotype diversity of 0.111 (11%) and 0.98, respectively, indicative of high polymorphism and diversity (Table 7). In addition, Tajima's test of neutrality as well as the Fst and corresponding p values were supporting the score of low genetic differentiation. The results show that the *Amblyomma* population in Chongwe district are similar and are expanding geographically.

When the district was divided into veterinary camps, pairwise comparison showed that variations were insignificant except between Kapete and Palabana (Table 8).

Table 7: *Amblyomma* intrapopulation DNA polymorphisms and Tajima's test of neutrality

Number of Haplotypes (h)	**Number of polymorphic sites (S)**	**Average number of nucleotide differences (k)**	**Nucleotide diversity (Pi)**	**Haplotype diversity (hd)**	**Tajima's test for neutrality**
21	161	27.585	0.11123	0.988	-2.24975, $p<0.01$

Table 8: Kanakatapa, Kapete, and Palabana *Amblyomma* population pairwise genetic differences.

	Kanakatapa	**Kapete**	**Palabana**
Kanakatapa	0	0.06253	0.09573
Kapete	0.06253, p = 0.48649	0	0.14306
Palabana	0.09573, p = 0.41441	0.14306, p = 0.53153	0

4.3.2.2.2 *Hyalomma* population

The *Hyalomma* population from Chongwe presented a total of 26 haplotypes based on the median joining network (Figure 10). All the haplotypes were not shared among the study sites apart from Haplotype H2, which was shared by sequences from Kapete and Kanakantapa, and also formed the anchor of the network. Haplotypes H2 and H21 were the most represented

haplotypes, and each consisted of two sequences with H21 exclusively consisting of only sequences from Palabana (Figure 11). Haplotypes from Palabana, despite not being shared, were directly related to H2 and other haplotypes such as H6 and H13 (Figure 11). With regards to Chisamba district, *Hyalomma* sequences after initial analysis were not sufficient for downstream analysis.

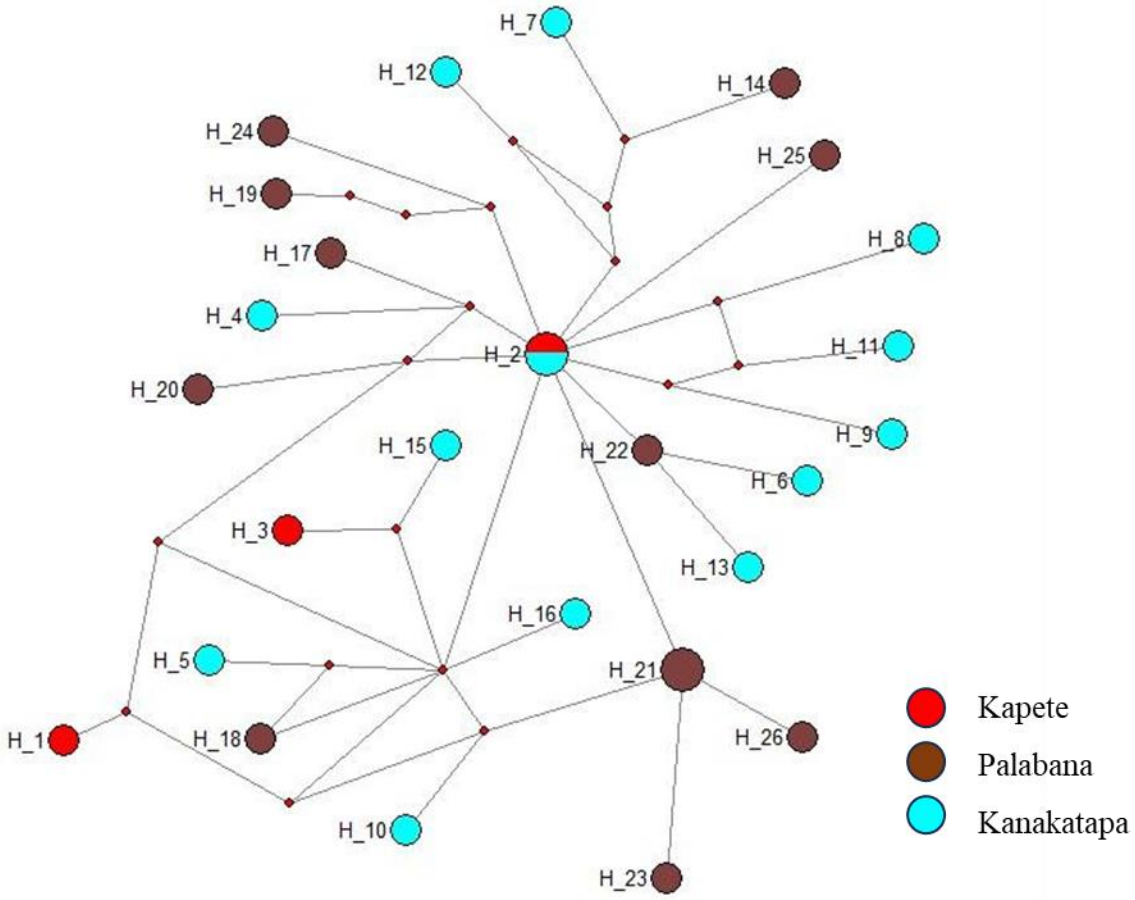

Figure 11: Chongwe *Hyalomma* network *12SrDNA.*

Within the *Hyalomma* population, moderate nucleotide diversity was observed as well as high haplotype diversity (Table 9). In addition, Tajima's test of neutrality (-2.25, $p < 0.01$) indicated the presence of recently emerging haplotypes (Table 9). The Fst values among the three study sites indicated moderate to low genetic differentiation but with insignificant p values (Table 10).

Table 9: *Hyalomma* intrapopulation DNA polymorphisms and Tajima's test of neutrality

Number of Haplotypes (h)	**Number of polymorphic sites (S)**	**Average number of nucleotide differences (k)**	**Nucleotide diversity (Pi)**	**Haplotype diversity (hd)**	**Tajima's test for neutrality**
26	130	19.193	0.07708	0.995	-2.25759, $p<0.01$

Table 10: Kanakatapa, Kapete, and Palabana *Hyalomma* population pairwise genetic differences.

	Kanakatapa	**Kapete**	**Palabana**
Kanakatapa	0	0.12288	0.07248
Kapete	0.12288, p = 0.35135	0	0.19517
Palabana	0.07248, p = 0.02703	0.19517, p = 0.17117	0

4.3.2.2.3 *Rhipicephalus* population

Owing to the reduced number of sequences from Chongwe, intrapopulation analysis for *Rhipicephalus* was not conducted. The major haplotype H1 consisted of sequences from all the veterinary camps namely, Mwomboshi, Chipembi, and Lifwambula (Figure 12). The total number of haplotypes identified by the MJ network was seventeen (17), with Chipembi veterinary camp providing the most haplotypes. Haplotype H1 formed the anchor of the network, and all the other haplotypes were closely related to H1 either directly or via one median vector.

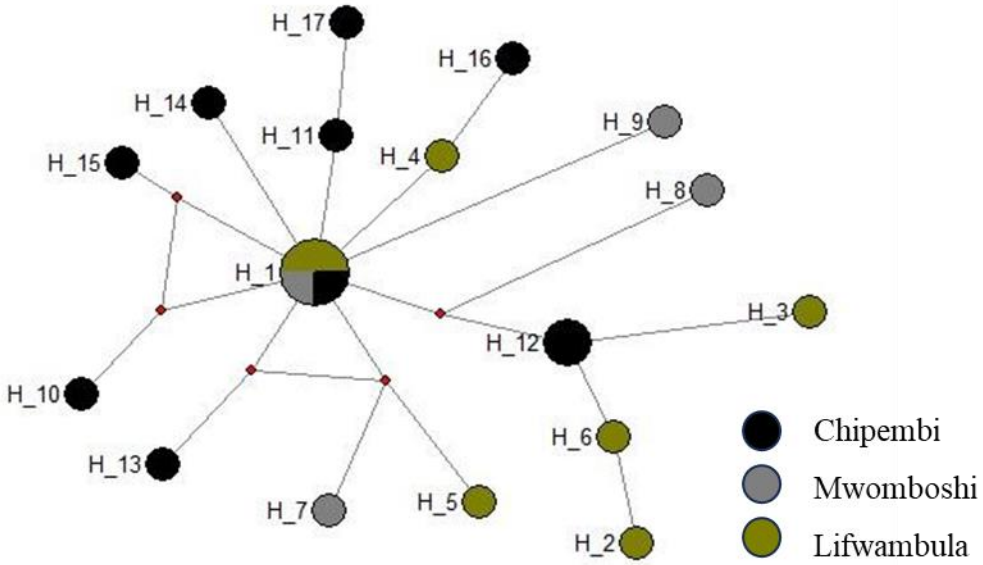

Figure 12: Chisamba *Rhipicephalus* network *12SrDNA.*

Moderate nucleotide diversity and high haplotype diversity were observed (Table 11). Tajima's test of neutrality (-2.29, $p < 0.01$) also indicated the presence of recent haplotypes (Table 11) and the Fst values indicated moderate genetic differentiation except for Mwobmoshi and Chipembi which indicated low genetic differentiation (Table 12).

Table 11: *Rhipicephalus* intrapopulation DNA polymorphisms and tajima's test of neutrality

Number of Haplotypes (h)	**Number of polymorphic sites (S)**	**Average number of nucleotide differences (k)**	**Nucleotide diversity (Pi)**	**Haplotype diversity (hd)**	**Tajima's test for neutrality**
17	81	11.224	0.05939	0.967	-2.29829, p<0.01

Table 12: *Rhipicephalus* intrapopulation pairwise genetic differentiation

	Chipembi	**Lufwambula**	**Mwomboshi**
Chipembi	0	0.17452	0.01959
Lufwambula	0.17452, p = 0.04505	0	0.18168
Mwomboshi	0.01959, p = 0.92793	0.18168, p = 0.24324	0

4.4 Molecular epidemiology of *Rickettsia*

4.4.1 Prevalence and distribution of *Rickettsia* (in ticks and in districts)

A total of 168 tick samples (*Amblyomma*, n=51, *Hyalomma*, n=52 and *Rhipicephalus*, n=65) were screened for *Rickettsia* using the *omp*B gene. An overall proportion of 51.2% (86/168) was observed based on the amplification of a fragment of the *omp*B gene. For all samples detected as positive on the *omp*B gene, further amplification of the *omp*A and *glt*A genes was done to allow for species identification.

Of the positive samples, 30 (*omp*B), 25 (*omp*A) and 43 (*glt*A) were successfully sequenced and utilised for phylogenetic analysis of *Rickettsia*. Table 13 shows the sequence identities of *Rickettsia* species (as a consensus) and by individual genes. All the sequences obtained in this study have been deposited in the genebank with accession numbers *ompA* (LC790077-LC790096), *ompB* (LC791413-LC791455), and *gltA* (LC791456-LC791468).

Table 13: Samples collected from Chongwe and Chisamba used in phylogenetic analysis.

S/N	**Sample ID**	**Tick host**	***Rickettsia* species**	**Sequence identity**		
				***omp*A**	***omp*B**	***gltA***
1.	P30 AFAa	*Hyalomma rufipes*	*Rickettsia sp.*	*R. aeschlimannii*	*R. aeschlimannii*	
2.	N20 AMNb	*Ixodid sp.*	*Rickettsia sp.*	*R. aeschlimannii*		*R. aeschlimannii*
3.	N21	*Ixodid sp.*	*Rickettsia sp.*	*R.*	*R.*	

	AMNa			*aeschlimanni i*	*aeschliman nii*	
4.	N8 AFA	*Hyalomma rufipes*	*Rickettsia sp.*	*R. aeschlimanni i*	*R. aeschliman nii*	
5.	N19 AFAa	*Hyalomma rufipes*	*Rickettsia sp.*	*R. aeschlimanni i*	*R. aeschliman nii*	
6.	N6 AMNa	*Hyalomma rufipes*	*Rickettsia sp.*	*R. aeschlimanni i*		*R. aeschli mannii*
7.	P29 AFAa	*Amblyomma variegatum*	*Rickettsia sp.*	*R. aeschlimanni i*		*R. aeschli mannii*
8.	N1 AMNb	*Hyalomma rufipes*	*Rickettsia sp.*	*R. aeschlimanni i*	*Rickettsia sp.*	
9.	N20 AMNa	*Hyalomma rufipes*	*Rickettsia sp.*	*R. aeschlimanni i*	*R. aeschliman nii*	
10.	N26 AMNa	*Hyalomma rufipes*	*Rickettsia sp.*	*Rickettsia sp.*	*R, aeschliman nii*	*R. aeschli mannii*
11.	K31 AMA	*Amblyomma variegatum*	*Rickettsia sp.*	*Rickettsia sp.*		
12.	P7 AMA	*Amblyomma variegatum*	*Rickettsia sp.*	*Rickettsia sp.*		*Rickett sia sp.*
13.	K37 AFAa	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*	*R. africae*	
14.	N7 AMAc	*Amblyomma variegatum*	*Rickettsia sp..*	*R. africae*	*R. africae*	*R. aeschli mannii*
15.	K28 AFN	*Hyalomma rufipes*	*Rickettsia sp.*	*R. africae*	*R. aeschliman nii*	*R. aeschli mannii*
16.	N11 AMAb	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*		*Rickett sia sp.*
17.	K30 AMA	*Amblyomma*	*Rickettsia sp.*	*R. africae*	*R. africae*	

		variegatum				
18.	P9 AFA	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*		*Rickettsia sp.*
19.	N22 AMAa	*Amblyomma variegatum*	*R. africae*	*R. africae*	*R. africae*	*Rickettsia sp.*
20.	LO2 AFNb	*Amblyomma variegatum*	*R. africae*	*R. africae*	*R. africae*	
21.	N1 AMA	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*		*Rickettsia sp.*
22.	K37 AMA	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*		*Rickettsia sp.*
23.	L1 AFNa	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*		
24.	N31 AMNb	*Ixodid sp.*	*Rickettsia sp.*		*R. aeschlimannii*	*R. aeschlimannii*
25.	N1 AFNc	*Hyalomma rufipes*	*Rickettsia sp.*		*R. aeschlimannii*	*R. aeschlimannii*
26.	P29 AFNa	*Hyalomma rufipes*	*Rickettsia sp.*		*R. aeschlimannii*	*R. aeschlimannii*
27.	P26 AMNc	*Hyalomma rufipes*	*Rickettsia sp.*		*R. aeschlimannii*	*R. aeschlimannii*
28.	N10 AMN	*Hyalomma rufipes*	*Rickettsia sp.*		*R. aeschlimannii*	*R. aeschlimannii*
29.	N23 AMNc	*Hyalomma rufipes*	*Rickettsia sp.*		*R. aeschlimannii*	
30.	P18 AFN	*Amblyomma variegatum*	*Rickettsia sp.*		*R. aesch;imannii*	
31.	N28 AFAa	*Amblyomma variegatum*	*Rickettsia sp.*		*Rickettsia sp.*	*R. africae*
32.	N22 AFNb	*Amblyomma*	*Rickettsia sp.*		*Rickettsia*	*R.*

		variegatum		*sp.*	*africae*
33.	N32 AMAb	*Amblyomma variegatum*	*Rickettsia sp.*	*Rickettsia sp.*	
34.	N1 AMA	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*	*Rickettsia sp.*
35.	N38 AMA	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*	
36.	N9 AMAc	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*	*R. africae*
37.	N15 AMA	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*	*Rickettsia sp.*
38.	N17 AFNb	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*	*Rickettsia sp.*
39.	N34 AMAa	*Amblyomma variegatum*	*Rickettsia sp.*	*R. africae*	
40.	N29 AFAb	*Amblyomma variegatum*	*Rickettsia sp.*		*R. aeschlimannii*
41.	L02 AFA	*Rhipicephalus decolaratus*	*Rickettsia sp.*		*R. aeschlimannii*
42.	P14 AMNb	*Hyalomma Rufipes*	*Rickettsia sp.*	*R. aeschlimannii*	*R. aeschlimannii*

4.4.2 Phylogenetic relationships of *Rickettsia*

Phylogenetic analysis of *omp*A based on 437 bp nucleotide sequences showed that nine (09) sequences (P30 AFAa, N20 AMNb, N21 AMNa, N8 AFA, N19 AFAa, N6 AMNa, P29 AFAa, N1 AMNb and N20 AMNa) under study closely clustered with *R. aeschilimani* from Zambia while other study sequences (n=11, 5 *R. africae* and 6 *Rickettsia sp*.) formed a separate cluster distantly related to the rest of the study sequences (Figure 13). One study sequence (P7 AMA) closely clustered with *R. africae* sequences from Zambia, Kenya, Antigua, and South Africa. In addition, other study sequences, N26 AMNa and K31 AMA closely clustered with Rickettsia sp. from Zambia and *R. africae* from South Africa, respectively (Figure 13). The sequences under study did not show evidence of clustering according to geographical origin.

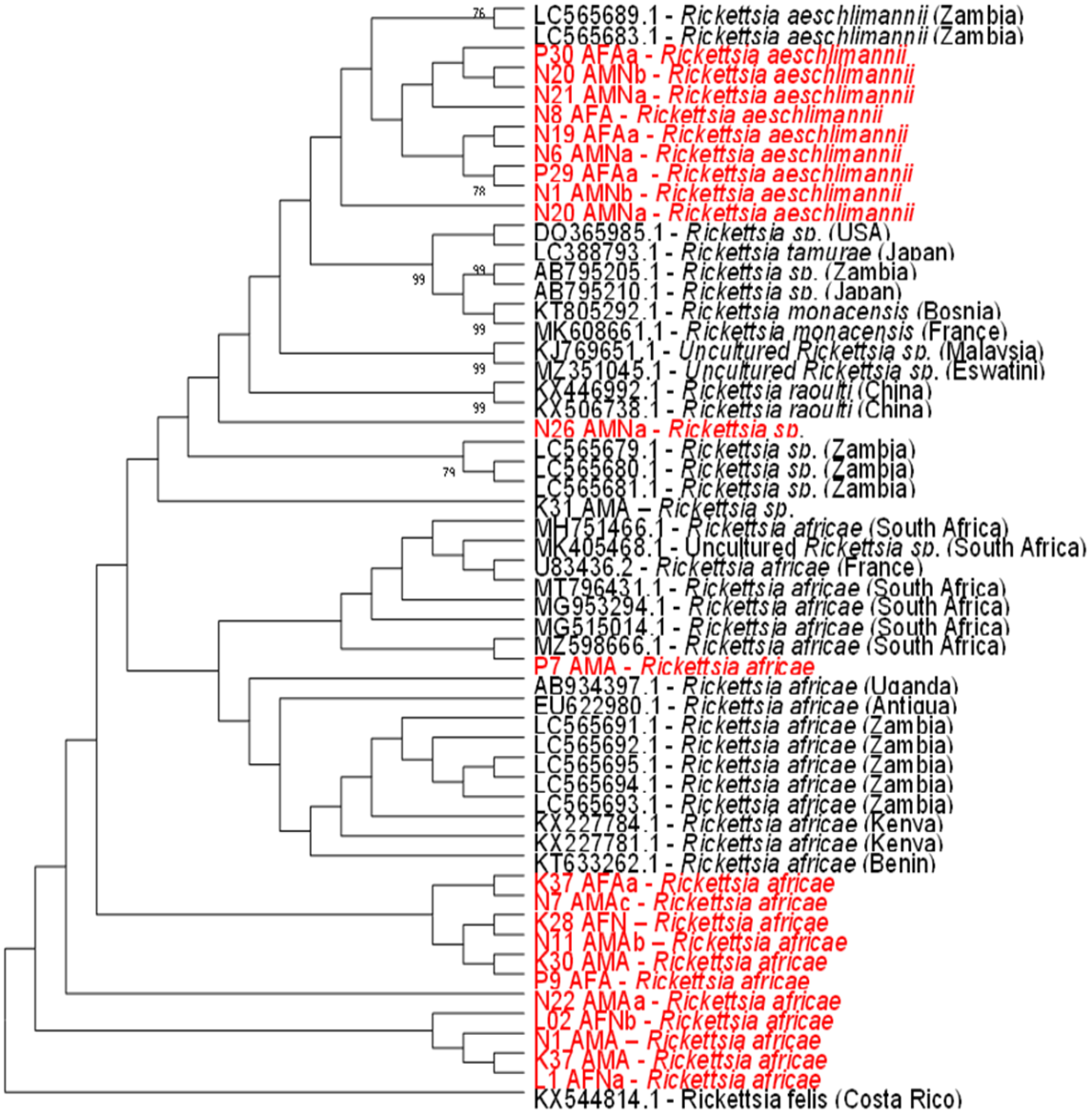

Figure 13: Phylogenetic analysis of 437bp *Rickettsia ompA* detected in ticks of Cattle from Chongwe and Chisamba. Bootstrap values of ≥75 are indicated at branch nodes. Reference sequences used are shown by their GenBank accession numbers and country of origin. The Rickettsiae from this study are in red text.

Phylogenetic analysis based on 423 bp nucleotide sequences of the *omp*B gene showed that 15 study sequences closely clustered with *R. aeschlimannii* from Zambia (Figure 14). Of the remaining fifteen (15) sequences, one sequence (N28 AFAa) closely clustered with *R. africae* sequences from Kenya and Ethiopia while the remaining fourteen formed a separate cluster (Figure 14). There was no evidence of clustering according to geographical origin.

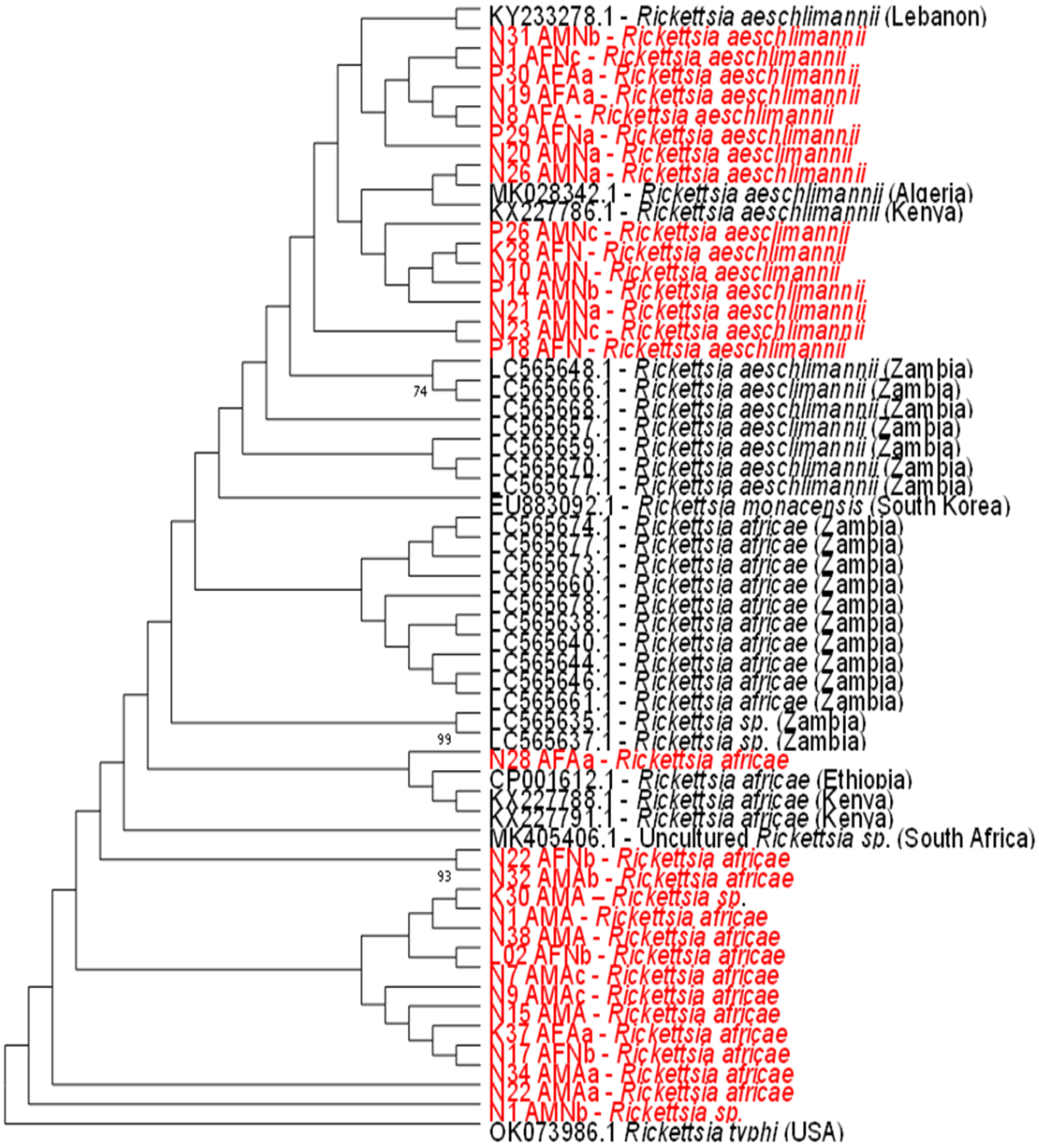

Figure 14: Phylogenetic analysis of 423bp *Rickettsia ompB* detected in ticks of Cattle from Chongwe and Chisamba. Bootstrap values of ≥70 are indicated at branch nodes. Reference sequences used are shown by their GenBank accession numbersand country of origin. The Rickettsiae from this study are in red text.

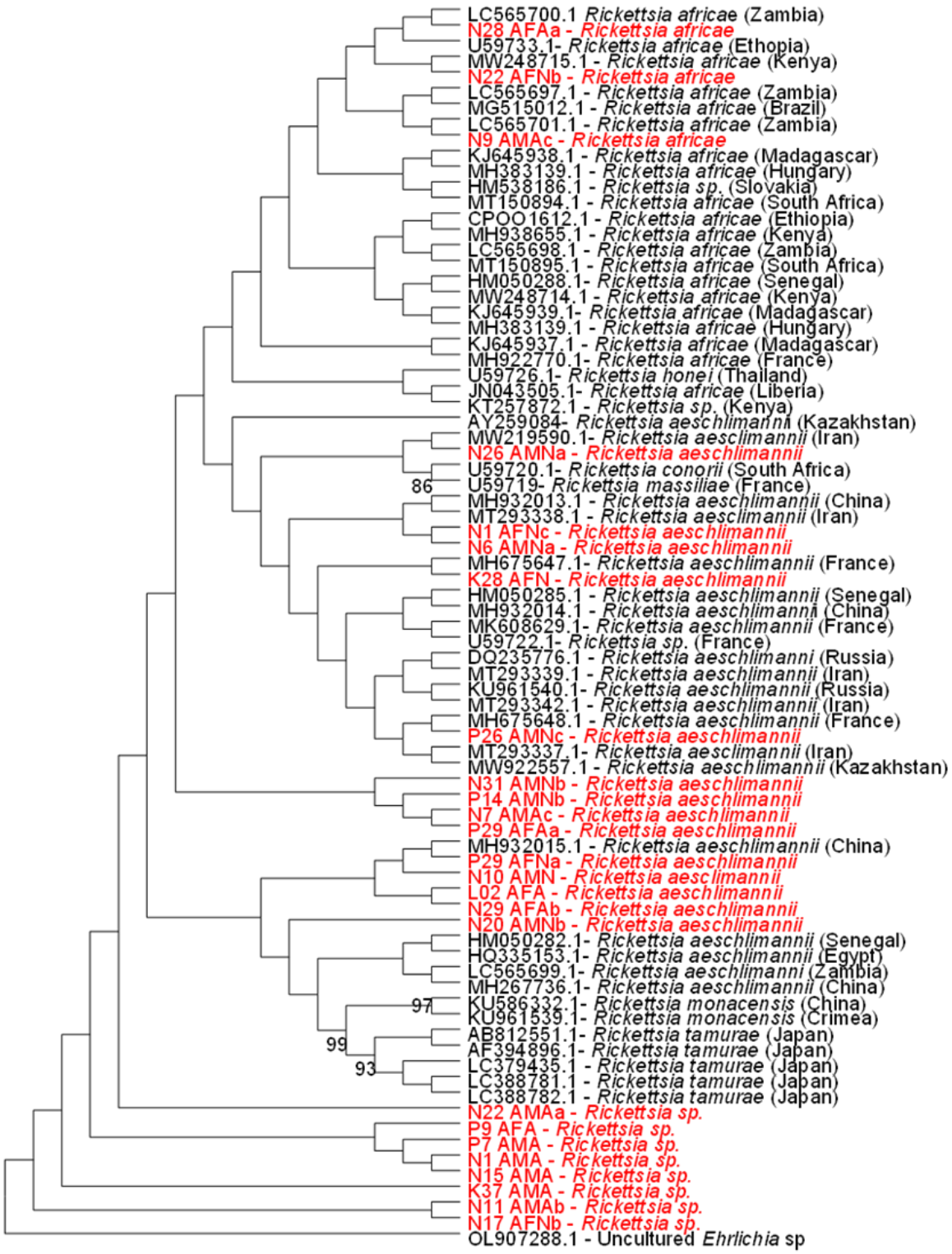

Figure 15: Phylogenetic analysis of 543bp *Rickettsia glt*A detected in ticks of Cattle from Chongwe and Chisamba. Bootstrap values of ≥75 are indicated at branch nodes. Reference sequences used are shown by their GenBank accession numbers and country of origin. The Rickettsiae from this study are in red text.

Phylogenetic analysis based on 543 bp nucleotide sequences of the *glt*A gene showed that study sequences n=14 closely clustered with *R. aeschlimannii* from Zambia, South Africa, Senegal, Egypt, Iran, Iraq, France, Russia, China, and Russia (Figure 15). Of the remaining fifteen (11) sequences, three sequences (N28 AFAa, N22 AFNb, and N9 AMAc) closely clustered with *R. africae* sequences from Kenya and Ethiopia while the remaining eight formed a separate cluster (Figure 15).

CHAPTER FIVE

5.0 DISCUSSION

Chongwe and Chisamba districts of Zambia are districts known to have numerous farming entities, both commercial and subsistence and the presence of ticks in these areas is a serious impediment to livestock development. In addition to the morbidity and mortalities caused by tick borne diseases, ticks cause mechanical harm to animals thus reducing live weights and increases treatment costs against the pathogens they carry. This negatively affecting the productivity of the livestock industry (Mumba et al., 2018). In both Chongwe and Chisamba districts, ticks are controlled using acaricides (Luguru et al., 2009). This method is both expensive and when not properly implemented, can result in ticks developing resistance to the chemicals used (Muyobela et al., 2015). The tick species in areas surrounding Chongwe and Chisamba districts are known vectors of several pathogens, rickettsiales inclusive (Moonga et al., 2019), however, information on their distribution, population genetics as well as the extent to which they transmit pathogens such as rickettsiales is not fully understood (Mubemba et al., 2022). Therefore, this study, for the first time, using molecular methods, provides information on the tick population genetic structure and the phylogenetic analysis of *Rickettsia* in ticks in Chongwe and Chisamba districts.

In the recent past molecular identification is highly recommended to remove all doubt and wrong classification that was observed when tick identity was based on morphological identity only. This was mainly due to the similarities of ticks within and among species (Abdullah et al., 2016). On morphological identification *Amblyomma* was 53.6% but on molecular identification it was only 33.3%. This shows why molecular identification is preferred to definitively identify ticks. Kapete, Chipembi and Mwomboshi veterinary camps were places where most animals had few or little ticks because they had effective tick control facilities like dip tanks and regular extension services from professionals on importance of these measures.

Phylogenetic analysis of *16SmRNA* and *12SrDNA* gene sequences from both districts revealed three distinct populations of *Amblyomma*, *Hyalomma* and *Rhipicephalus* tick species. This is in agreement with previous studies that have identified *Ambylomma variegatum*, *Rhipicephalus appendiculus*, *R. decoloratus*, *Hyalomma and Heamaphysalis* species (Beati et al., 2012). This study detected a higher infestation of *Hyalomma* compared to *Amblyomma* and *Rhipicephalus*.

The 16S *mRNA* gene has been utilised in most population genetic studies of ticks to compare populations across biogeographical areas due to its high maternal heredity, low recombination rates and highly conserved structure (Aguilar-Domínguez et al., 2019, Bitencourth et al., 2019, de Lima et al., 2017, Lampo et al., 1998). Based on this gene, low genetic differentiation and high haplotype diversity, suggestive of recent population expansion was observed within the *Amblyomma* tick population when Chongwe and Chisamba districts were treated as a single population. On the other hand, within the *Hyalomma* tick population, moderate genetic differentiation and high haplotype diversity was observed with a value of 0.995. This could also be due to the low number of samples used in the study. In a study by Benham et al, (2021), sample representation of less than five was excluded from the study as was the case with the samples from Kapete and Palabana because this can show bias. Chisamba and Chongwe districts are separated by a substantial area, however, there is a lack of geographical barriers between the two districts and the closest veterinary camps, Kanakantapa in Chongwe, and Chipembi in Chisamba, are approximately 23 km apart. This is suggestive of free movement of animals between the two districts or an overlap of grazing areas, factors which would facilitate movement of ticks between the two districts. This similarity in population of ticks in the two districts could lead to suggestion of similar disease risk in the two, and thus presence of disease transmitted by same tick in one area could be suggestive of presence of same disease in the adjacent area.

With regards to the population dynamics prevailing within each district in each respective species, individual tick species within each district were treated as single populations and analysed using the *12SrDNA* gene owing to its sensitivity to genetic differentiation as it varies even within individuals of the same species (Norris et al., 1996). The population of *Amblyomma* in Chongwe showed low to moderate genetic differentiation and high haplotype and nucleotide diversity coupled with a negative Tajima's test of neutrality score indicative of true presence of recent haplotypes and population expansion. Further, phylogenetic analysis showed sequences clustering irrespective of their districts of origin. In Chongwe district, the *Hyalomma* species population showed low to moderate genetic differentiation, moderate nucleotide diversity, high haplotype diversity and a significant negative Tajima's test of neutrality score indicative of true presence of emerging haplotypes. Further, the *Hyalomma* sequences (n=17) clustered closer to the sequence KU987254 from Tanzania than the sequence L34307.1 from Zambia (Black and

Piesman, 1994, Chitimia-Dobler et al., 2016). This shows that the genetic makeup of the ticks has evolved over the years. With selection pressure that could have been caused by the introduction of acaricide use to combat tick borne diseases. On the other hand, *Hyalomma rufipes* species identified is known to be host specific and to have genetic differences between South African and/or Namibian populations when compared to eastern African populations (Cangi et al., 2013) thus this could have also been the reason for the clustering pattern observed. Nevertheless, moderate nucleotide diversity, high haplotype diversity, moderate to low genetic differentiation and indicative of presence of recent haplotypes was observed within the *Rhipicephalus* species population in Chisamba district.

Within our sample size, *Rhipicephalus* ticks were generally more represented in Chisamba district than Chongwe. In the other tick species this was observed in the difference of numbers of life stages. This difference could be attributed to the time of sampling which was at onset of the rains in Chongwe when hence good representation of all life stages. This is as opposed to the cold dry season for Chisamba when proliferation of ticks is not as high.

Overall, within each district, *Amblyomma*, *Hyalomma* and *Rhipicephalus* populations showed the presence of similar and closely related individuals in all veterinary camps because of the evident population expansion prevailing (Figures10, 11 and 12). Within each district, high diversity as well as circulation of similar haplotypes was also observed, and this can be attributed to free mixing of ticks from different veterinary camps likely due to an ineffective tick control system and the practice of free-range grazing where animals are allowed to freely roam beyond their respective farms within each district. Expansion of these tick populations is an indication of the expansion of tickborne diseases such as Anaplasmosis, Ehrlichiosis and Rickettsioses (Goddad, 2009) and no doubt pose a serious risk to both livestock and human populations.

To determine the public health risk on humans presented by the expansion of tick populations in Chongwe and Chisamba districts, the phylogenetic analysis of *Rickettsia* was conducted. An overall proportion of 51.2% (86/168) was obtained from ticks collected from cattle in Chongwe and Chisamba districts using PCR targeting the *omp*A, *omp*B and *glt*A *Rickettsia* genes. This prevalence is significantly higher than 18.6% detected from ticks in the southern province (Chitanga et al., 2021). Other studies have reported *Rickettsia* prevalence of 9.3% in South

Africa and 35.67% in Egypt (Allam et al., 2018, Halajian et al., 2016). *Rickettsia* was detected in all tick species, with the infection rate in *Amblyomma* and *Hyalomma* being 75.9% and 78.6% respectively. By comparison, Allam et al., had the highest infection rate by species in Hyalomma. *Rhipicephalus* ticks in the sample pool were seven and only two were positive for *Rickettsia* giving an infection rate of 28.6%. The higher infection rate in *Amblyomma* and *Hyalomma* ticks can be attributed to the transovarian transmission of pathogens, which in tells that one infected adult female will produce infected larva, nymph and adult and the cycle will continue.

Blast and phylogenetic analysis (Figures 13, 14, and 15) identified the detected *Rickettsia* as *R. africae* and other non-specific *Rickettsia* sp., in all tick species. The non-specific *Rickettsia* sp. was due to failure of consensus of sequences of all three genes or that a sample did not have a representative sequence on one or more of the genes. *Amblyomma* and *Hyalomma* ticks had most and only one *Rhipicephalus* tick had *R. aeschlimannii* on *glt*A. It is of note that despite lack of consensus on all three genes, a few samples were very closely related to *R. aeschlimannii*. Some sample only fell short on one gene. This also applies to *R. africae*. All *Rickettsia sp*. were from ticks from all veterinary camps within Chongwe and Chisamba districts except Chipembi veterinary camp in Chisamba district. *R. africae* causes a febrile illness known as African tick bite fever and is second to malaria as a cause of febrile diseases (Moonga, 2021). In Zambia, few studies have reported species of *Rickettsia* circulating. In the southern province, Chitanga et al, (2021) reported *Rickettsia africae* in ticks collected from cattle. Apart from this, there are no other reports of *Rickettsia* species in general in other parts of the country. *R. africae* has been reported not only in Zambia but across the African continent. On the other hand, *R. aeschlimannii*, a zoonotic pathogen and a ubiquitous member of the SFG, as well as *R. aeschlimannii*-like species have been reported previously in Zambia (Qiu et al., 2022) (Chitanga et al., 2021). To the best of our knowledge, this study is the first to report both *R. africae* and *R. aeschlimannii* in Chongwe and Chisamba districts of Zambia. The first report of *R. aeschlimannii* was from Morocco in 1997 and thereafter in 2002, while the first documented case of human infection was reported from France where the patient had recently travelled to Morocco (Beati et al., 1997, Raoult et al., 2002). Thus far, none of the study sites have reported any human rickettsiosis and this could be attributed to misdiagnosis or under reporting and the

detection of these pathogens in these areas entails the need to sensitize the community and health care providers on the presence of *Rickettsia*.

The current study demonstrated a highly diverse and expanding population of ticks infected with zoonotic *Rickettsia* within both districts, posing a serious public health risk and warrants the improvement of tick control measures. The study was however limited in the sample size which reduced the representation of each population as could be observed from the insignificant p values and failure to conduct some analyses. Nevertheless, the study produced new information especially on the population genetics of ticks in Zambia which has not been attempted until now. Further, the study detected and identified *R. africae* for the first time in a naïve area of Zambia.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

1. It was found that the tick populations in Chongwe and Chisamba district comprised of three populations namely: *Amblyomma*, *Hyalomma*, and *Rhipicephalus* and showed low to moderate genetic variation between the two districts due to the free-range grazing of tick hosts and the movement of these hosts.
2. The tick populations in the study sites were positive for *Rickettsia* and phylogenetic analysis confirmed that they carried *R. africae* and *R. aeschlimannii-like* with *Amblyomma* and *Hyalomma* carrying the most pathogens.

6.2 Recommendations

1. The expansion of tick populations should be monitored to predict disease occurrence caused by the pathogens ticks carry.

2. Both *Rickettsia* species reported in the study sites are pathogenic and zoonotic and studies to assess the extent of infections in both cattle and humans in these communities are warranted.

3. Molecular epidemiology of *Rickettsia* in other districts of Zambia is recommended to assess the country wide burden of Rickettsioses. Rickettsioses should be considered a differential diagnosis in both animals and humans presenting with high fever.

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APPENDICES

Appendix A

INFORMATION SHEET

Study title – Population genetics of *Amblyomma* ticks and molecular epidemiology of *Rickettsia* in selected districts of Zambia.

This is a cordial request for you to allow your animals to take part in this study. Please read the following information clearly and where not clear please feel free to ask. Use the information below to decide if you would like to take part in this study or not.

WHO I AM AND WHAT THE STUDY IS ABOUT

My names are Khumalo Cynthia Sipho. I am a full-time postgraduate student of the University of Zambia undertaking a study on the population genetics of *Amblyomma* ticks and molecular epidemiology of *Rickettsia*. *Amblyomma* ticks are vectors of various disease causing organisms. Understanding genetic patterns will assist in implementation of effective control measures of the ticks and furthermore the diseases they cause. Rickettsioses is a group of diseases caused by bacteria of the family Rickettsiaceae of which *Amblyomma* species are vectors. It is a neglected tropical disease and this status also stands in our country Zambia.

WHAT WILL TAKING PART INVOLVE?

Taking part in the study, if you agree to do so, will involve you providing information about your animals, restraining of your cattle, collecting ticks off the animal and collecting blood.

DO YOU HAVE TO TAKE PART?

This is entirely up to you. No one should coarse you into participating and you will not be discriminated against for not participating. If at any point you wish to withdraw, inform the principle investigator even without giving reason.

WHAT ARE THE POSSIBLE RISKS AND BENEFITS OF PARTICIPATING?

Risks and corrective measures

Your animal will resist restraint hence only knowledgeable people should assist in restraint. Animal may bleed a little longer at needle puncture site hence disinfectant and sterile cotton

wool to hold pressure and stop bleeding. Animals more at risk for this are those that might have an underlying hemolytic fever diseases. Needles can break in animals hence only veterinarians with current registration in the Veterinary Council of Zambia are allowed to draw the blood.

Benefits

The animals will get relief after ticks are picked off them. This study also allows for the screening of your herd for *Rickettsia* and other tick borne diseases of interest free of charge.

WILL PARTICIPATING BE CONFIDENTIAL?

The data collected in this study will be strictly confidential and restricted only to authorized personnel on the research team. When and if the information is published the names and any identifiers will be withheld and all disposed at the end of this study.

WHO SHOULD YOU CONTACT FOR FURTHER INFORMATION?

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Appendix B

Informed Concert:

You are been asked to participate in the prevalence of Rickettsioses in Chongwe district study.

I …… has been explained to and fully understand the nature of this study. I am hereby volunteering to participate in this study under no mandate. I understand that all information I provide will be strictly confidential. I am aware that I may discontinue participation at any point and I am not mandated to answer any question. In case of any dissatisfaction or conflict I am at liberty to report the matter to the ethical committee mentioned above or/and the university of Zambia.

Sign: …….. Date: ……..

Witness signature: …… Date: …….

Researcher signature: ……. Date: …….

Translation to Ichitonga

SONGA YABUVWUNTAUZI:

Bube bwa nzengele ziluma muzyooko zisalesa zyamu Zambia

Ooku nkulomba kwabunyina kuli ndinywe kuti muzumizye zinyama zyenu kuti zyitole lubazu mubuvwuntauzi obu. Ndalomba amubale kuzibisigwa ooku kutobela cakupakamana, mpomutakotelesesia mulinvwe kwaanguluka kubuzya. Amubelesye kuzibisigwa kuli ansi kuti musale kutola lubazu mubuvwwuntauzi obu na pe.

INO NDIME NI? ALIMWI INO OBU BUVWUNTAUZI BUJATIKIZYA NZI?

Izina lwangu ndime Khumalo Cynthia Sipho. Ndisicikolo uzwide utola pepa lisumpukide lyabili kuzwa kucikolo cipati ca University of Zambia. Eeli pepa lyabili ndyakulanga-langa nzengele zyacikwa ciyumu ziitwa kuti amblyomma ticks. Alimwi lilalanga-langa amalwazi a tuuka tushoonto tujanwa kuzyuuka ziluma mbuli nzengele alumoma (rickettsia) mbuli mbwayambukila ambwakoonzya kwiimikwa. Nzengele zyacikwa ciyumu zileta malwazi aindene-indene. Kuzibisisia mbozipangidwa kuleta zila zibotu zyakulwana nzengele eezi amalwazi ngozileta. Rickettsioses, nkabunga kamalwazi aletwa amukwashi watuuka tushoonto shoonto tubweezega a

nzengele zyacikwa ciyumu. Aya malwazi tabikilidwa maanu kapati muzibela zinjaanji kujatikizya a Zambia.

INO KUTOLA LUBAZU KUNOJATIKIZYA NZI?

Kutola lubazu mubuvwuntauzi nawazumina kucita boobu, ciyoyandika kuti upa lizibo lujatikizya banyama bako, kweendelezya ng'ombe zyako, akubweza nzengene amalowa kuzya kubanyama.

SENA MULEELEDE KUTOLA LUBAZU?

Eeci cili kuli ndinwe. Kunyina weleede kukusungilizya kutola lubazu pe, alimwi kunyina utikamudyakaizye kuti mwatatola lubazu. Kuti mwayanda kuleka akatikati, nkumuzibya mweendelizi wakusepasepa kakunyina akupa muzeezo ncolekela.

INO ZILIJAZYO NZI? ALIMWI MBUBOTU NZI BULI MUKUTOLA LUBAZU?

Zilijazyo abukwabilizi bwazyo.

Zinyama zyanu ziyokaka kwendelezegwa,nkakaako kuyandika biyo bantu balaluzibo kuti bakagwasye mukweedelezya zinyama. Inga zinyama kaziyakuzwa malowa kwakayindi kalamfumfu awo mpoziyoyaswa ndonga kwanogwishigwa bulowa, aboobo musamu abutonje bula musamu buya kubelesegwa kajata kuti bulowa buleke kuzwa. Zinyama zili acilijazyo kapati zizyezyo zila amalwazi akutafwambana kuleka bulowa kuzwa ayo ayitwa kuti hemolytic fever disease. Ndonga inga zyatyokala mucinyama aboobo balike basiabupampu muzinyama balo balilembeshede mukabunga kalanganya zinyama mu Zambia mbobazumizidye kugwisha bulowa.

Bubotu

Zinyama ziyojana kupumula zyakugwishigwa nzengele. Alimwi ooku kulanga-langa kuzumizya kuvwuntauzya malwazi aletwa atuuka tushoonto tubwezegwa anzengele, alimwi amalwazi aambi aletwa anzengele kakunyina kubbadela.

SENA KUTOLA LUBAZU KUNIKULI KWAMASESEKA?

Twaambo tuyakubwezelelwa mubuvwuntauzi oobu tunakuli twamaseseka, alimwi toyozubulidwa biyo bantu bazumizidye kubwezelela twaambo mubuvwuntauzi obu. kuti na

twaambo tukalembwe, mazyina anu aceeco cikozya kumuzubulula ciyosiswa akusoogwa kumamanino abuvwuntauzi.

NGUNI NGOMUYELEDE KUTUMINA KUTI NAMUYANDA KUZIBA TWAAMBO TUNJI?

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Sikutola lubazu

Mebo nde_____, ndapandulwidwa akutelesesya bube bwabuvwuntauzi obu. Ndalyaba kutola lubazu mubuvwuntauzi obu kakunyina kusinikizigwa. Ndatelela kwamba kuti twaambo toonse tweendipa tunoli twamaseseke. Ndilizi kuti ndilakozya kuleka fumbwa ciindi alimwi sesinikizidwa kuvwila mubuzyo uli oonse. Kuti na kwaba kutakkutila nokuba kwiimpana, ndili angulukide kutola twaambo ootu kukabunga kalanganya bwendelezi bwabuvwuntauzi nokuba kucikolo ca university of Zambia.

Sikutola lubazu…… Buzuba……..

Sikupa Bumboni……. Buzuba……..

Muvwuntauzi…… Buzuba……..

Translation to Nyanja

INFORMATION SHEET

Dzina langa ndine **KHUMALO CYNTHIA SIPHO**. Ndikupemphani kuti mulore ziweto zanu kutengako mbali mu kafuku- fuku komwe nizaona pa nthata zomwe zipezeka pa ng'ombe. Nthatazi zimapangisa matenda osiyana-siyana mu anthu monga Rickettsiosis. Chonde werengani zarembedwa ndipo pambuyo pake mupange cosankha mwa ufulu ngati kuti ziweto zanu zitengeko mbali kapena yai.

ZA INE KOMANSO KAFUKUFUKU UMENE NDIKUCITA.

Ine ndikucita ma phunziro a pa mwamba a 'masters' pa University Of Zambia. Kafuku-fuku umeneyu ndiwa 'population genetics of *Amblyomma* ticks and molecular epidemiology of *Rickettsia"*. Nthata zimenezi zimanyamura tudoyo tomwe tupangisa matenda osiyana-siyana mu matupi a anthu. Ndipo Kufufuza kumeneku pa nthata zimenezi kuzathandiza kupeza njira zocepestera nthata zimenezi komanso matenda amene zimapangisa.

KODI KUTENGAKO MBALI KUZAPHATIKIZAPO CANI?

Ngati mu bvomekeza kuti ng'ombe zanu zitengeko mbali, Muzafunsidwa za kasungidwe ka ng'ombe zanu, tizatengako nthata zina pa ng'ombe zanu komanso magazi ocepacabe mosewenzetsa nsingano.

KODI NG'OMBE ZANU ZIZAFUNIKA KU TENGAKO MBALI?

Kutengako mbali mu kafuku-fuku uyu ndi cosankha canu inu eni aziweto ndipo musakakamidzidwe ndi aliyense. Mulinso ndi ufulu oleka ngakhale nchitoyi itayamba kale.

ZOWOPSA NDI MAPHINDU NG'OMBE ZANU ZIKATENGAKO MBALI.

Zowopsa.

Palibe zobvuta zomwe ng'ombe zanu zizakumane nazo pambuyo po thodora nthata ndi kutengako magazi. Ogwira nchito imeneyi ndi a ku ofesi ya Verterinary pa boma lanu.

Maphindu.

Kucosedwa kwa nthata pa ng'ombe zanu kuteteza matenda omwe angayambisike mu ng'ombe komanso anthu. Kuzakhalanso kothandiza cifukwaka titha kupeza tudoyo tumene tupangisa matenda mu nthatazo ndikupereka cithandizo coyenera.

KUTENGAKO MBALI KWANU KUKHALA KWA CHISINSI.

Tizacita zonse zothekera pa kuonesesa kuti zomwe tipeze pa kafuku-fuku uyu pa ng'ombe zanu kuti zasungidwa mwa chisinsi. Kulibe wina oposa ise amene azadziwa za ziweto zanu. Pambuyo pomaliza kafuku-fuku umeneyo, dzina lanu silizalembedwa mu zofalisa za sayansi.

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Appendix B

KUBVOMEKEZA KWANU.

Mupemphedwa kutengako mbali mu kufufuza kwa nthata za Ng'ombe muno mu boma ya Chongwe.

Ine [Lembani dzina lanu]……. Ndanvetsetsa cholinga ndi maphindu a kafuku-fuku kameneka. Ndidziwa za ufulu wa ng'ombe zanga ndipo ndabvomekeza kuti zitengeko mbali mu kafuku-fuku uyu. Ndinesnso ozindikira kuti ndigaletse zoweta zanga kutengako mbali pa kufuku-fuku umeneyu nthawi ina iliyonse pa Zifukwa za ine mwini. Motero ndibvomera kutengako mbali mu kafuku-fuku kameneka.

Signature yanu …… Date: ……..

Signature ya mboni……..… Date: …….

Researchers' signature……. Date: …….