DIVERSITY OF TICKS AND TICK-BORNE PROTOZOAN PARASITES FROM LIVESTOCK AND WILDEBEESTS AT THE MAASAI-MARA WILDLIFE- LIVESTOCK INTERFACE, NAROK COUNTY, KENYA

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DECLARATION

This project is my original work and has not been submitted to any other University for any degree award

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LIST OF ABBREVATIONS

AMOVA	Analysis of molecular variance
BLAST	Basic local alignment search tool
bp	Base pairs
C1	Initial concentration
C2	Final concentration
CBPP	Contagious bovine pleuropneumonia
CCPP	Contagious caprine pleuropneumonia
CO1	Cytochrome oxidase subunit 1
DNA	Deoxyribonucleic acid
DNAsp	Deoxyribonucleic acid sequence polymorphism
DNTP	Deoxynucleotide triphosphates
ECF	East Coast fever
EDTA	Ethylene diamene tetraacetic acid
EtBr	Ethidium bromide
ETS	Externally transcribed spacer
FMD	Foot and mouth disease
GDP	Gross domestic product
HCl	Hydrochloric acid
ITS2	Internal Transcribed spacer 2
LSD	Lumpy skin disease
MEGA	Molecular evolutionary genetic analysis
MgCl2	Magnesium chloride
NaCl	Sodium chloride
NTS	Non transcribed spacer
PCR	Polymerase chain reaction
RLB	Reverse line blot
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SrDNA	Small subunit ribosomal deoxyribonucleic acid
SSU	Small subunit
TAE	Tris acetic acid Ethylene diamene tetraacetic acid

TBDs	Tick-borne diseases
UK	United Kingdom
USA	United States of America
V	Volts

ABSTRACT

Wildlife-livestock interfaces are hotspots for tick-borne livestock diseases, which cause high mortalities and morbidity. Wild bovines in these interfaces play a major role in maintaining tick vectors and tick-borne pathogens. This study explored the diversity of ticks infesting blue wildebeests (*Connochaetes taurinus*), cattle and sheep as well as the diversity of protozoan pathogens harboured by these ticks in the Maasai Mara which is one of the wildlife – livestock interfaces in Kenya. Ticks were collected from cattle, sheep and wildebeests, identified and genetic diversity determined by amplification of Cytochrome C Oxidase subunit 1 (COI), Internal Transcribed Spacer 2 (ITS2) and 12S ribosomal DNA (12SrDNA). Protozoan pathogen diversity was studied by amplification of 18S ribosomal DNA (18SrDNA). Of the 165 ticks collected, 74 (44.8%) were *Rhipicephalus evertsi* and 69 (41.8%) were *Rhipicephalus appendiculatus.* Other species collected were *Rhipicephalus pulchellus, Rhipicephalus Boophilus decoloratus* and *Amblyomma variegatum*. All the *R. appendiculatus* ticks were obtained from wildebeests while *R. evertsi* were collected from cattle, sheep and wildebeests. Eight of the *Rhipicephalus evertsi* ticks from wildebeest harboured *Theileria parva* parasite DNA, whereas two were found to be infected by *Theileria equi* and one harboured *Theileria ovis. Theileria parva* was found in ten of the *R. appendiculatus* ticks while *Theileria equi* was found in two *R. appendiculatus* ticks. Phylogenetic analysis showed that both *R. evertsi* and *R. appendiculatus* were significantly similar across domestic and wild hosts, suggesting that the vectors could be shared across both wild and domestic animals. *R. evertsi* was present across wild and the domestic species (wildebeest, cattle and sheep) in the Maasai Mara ecosystem. *Theileria* species responsible for bovine (*T. parva*), equine (*T. equi*) and ovine theileriosis (*T. ovis*) were found harboured by both *R. appendiculatus* and *R. evertsi* warranting further studies on the specific role of these tick species in transmission of these parasites within the Maasai Mara. Two strains of *Babesia* parasites, with genetic similarity to *B. microti* and *B. canis* were also identified in both *R. appendiculatus* and *R. evertsi* ticks. Protozoan pathogens of zoonotic importance were not detected in either of the analysed tick species.

CHAPTER ONE

INTRODUCTION

Ticks are obligate ectoparasites of vertebrates belonging to the order *Acari* and are second to mosquitoes as vectors of disease causing pathogens (Lutomiah *et al*., 2014). Approximately 840 species of ticks are known making up two families; the *Ixodidae* commonly referred to as hard ticks and the *Argasidae* or soft ticks (Jongejan and Uilenberg, 1994). The hard ticks comprise about 80% of all known tick species, including the species of greatest economic importance (Jongejan and Uilenberg, 1994). *Ixodidae* ticks of the *Ixodes* and *Haemaphysalis* genera are not found on the African continent while *Dermacentor* ticks are not implicated in any livestock diseases (Jongejan and Uilenberg, 1994). *Boophilus, Haemaphysalis, Hyalomma, Ixodes, Rhipicephalus* and *Amblyomma* account for the highest economic losses in livestock production (Jongejan and Uilenberg, 1994) as vectors of tick-borne diseases (TBDs) for example East Coast Fever (ECF), anaplasmosis, heart water and Lumpy Skin Disease (LSD).

Livestock production is an important economic and social activity in Kenya contributing close to 10% of the country's Gross Domestic Product (GDP) and accounts for more than 30% of farm gate value of agricultural produce (Kiptarus, 2005). Beef cattle production in Kenya is practiced in a number of rangelands including Kajiado, Laikipia, Narok, and Taita Taveta (Kiptarus, 2005) where livestock farmers practice nomadic pastoralism thus competing for scarce land, pastures and water resources with wildlife creating wildlife-livestock interfaces that are hotspots for disease transmission (Wambwa, 2005). The interfaces may be linear if falling along a fence, patchy if reflecting host preference for certain habitats, focal where a water point is shared or diffuse where range and resources are shared (Bengis *et al.*, 2002). The Maasai Mara reserve in Kenya forms one of the major wildlife-livestock interfaces. It lies within the coordinates 1°05S, 35°13E. and covers nearly 1,672 kilometers squares ranging in elevation from 1,500 meters to 2,100 meters above sea level (Macaluso *et al*., 2003). The region boasts of abundant wildlife and vast plains where livestock production is a

major economic activity. This ecosystem experiences seasonal interactions between wild herbivores such as wildebeests and cattle thus creating disease transmission sites between the two species. The role of wildebeests as hosts of tick species infesting livestock remains largely unexplored. This study aimed at determining the role played by ticks in the interactions between livestock and wildebeests in the Maasai Mara as a model to provide insights on the importance of this and other similar wildlife-livestock interfaces which have been identified as hotspots for transmission of both livestock diseases and zoonoses.

1.1 Problem statement

Tick-borne diseases have been a major challenge to livestock farming in Kenya causing diseases and death with resultant decreases livestock productivity (Simuunza *et al*., 2011). Currently there is inadequate knowledge of the tick species found within the wildlife-livestock interfaces in Kenya, their distribution as well as the role they play in the epidemiology of tick-borne diseases within these interfaces which are hotspots for disease transmission. The present study was located in the Maasai Mara comprising the protected national reserve and some adjoining community-based reserves. In this locality, wild ungulates and livestock species interact while sharing water and pasture resources. These interactions peak during dry seasons as well as during the annual seasonal migration of the blue wildebeests (*Connochaetes taurinus*) across the Maasai Mara and Serengeti corridor. Given the paucity of informative literature on the role played by wildlife in transmission of ticks and tick-borne pathogens in the Maasai Mara, this study determined the diversity of ticks infesting cattle, sheep and wildebeests as well as the tick-borne protozoan disease causing pathogens harboured by these ticks. The study gives insights on the potential consequences of wildlife-livestock interactions in relation to transmission of livestock diseases and zoonoses. It is envisaged that the findings will provide useful data for risk analysis and designing future interventions or prevention and control strategies for ticks and tickborne diseases in much of Kenya's savannah ecosystems.

1.2 Rationale and justification

1.2.1 Livestock and wildlife interactions

In Kenya, wildlife-livestock interfaces are distributed throughout the country including areas such as Nairobi, Machakos, Samburu, Kitui and the rangeland areas of Kajiado, Laikipia, Narok, and Taita Taveta, which border wildlife reserves. Recurrent droughts and human population increase allow unavoidable contact between domestic and wild animals in grazing fields and watering points. Seasonal movement of wildlife exemplified by wildebeests in the Maasai Mara also results in frequent interactions with livestock increasing the possibility of disease transmission. Transmission of infections from the wild to livestock can decimate livestock economies and increase problems of rural poverty while equally generating human-wildlife conflict. This study set in Maasai Mara and its environs in Narok County, therefore investigated the genetic similarity between the ticks infesting wildebeest and livestock in the study area and tick-borne protozoan parasites harboured by these ticks. Worldwide, a number of human infections caused by viruses, bacteria or parasites are tick-transmitted zoonoses. Such infections include Lyme borreliosis, tick-borne encephalitis, ehrlichiosis and babesiosis, Q fever, tularemia and Mediterranean spotted fever (Granström, 1997). This study sought to identify tick-borne zoonotic pathogens that could possibly be circulating in ticks between the wildebeests and cattle since surveillance data for zoonoses derived from the livestock-wildlife interfaces remains inadequate.

1.3 Research questions

This study aimed at addressing the following questions;

1. Which are the tick species infesting wild ungulates and livestock in the Mara ecosystem? To what extent are the tick species genetically similar of diverse?
2. Which species of tick-borne protozoan parasites are present in ticks feeding on wild ungulates and livestock in the at the Mara ecosystem? To what extent are the tick-borne pathogens genetically similar of diverse?
3. Do wild ungulates and livestock share ticks and tick-borne protozoan pathogens?

1.4 Study objectives

1.4.1 Main objective

To determine the genetic diversity of ticks and the tick-borne protozoan parasites in wildebeest (*Connochaetes taurinus*), cattle (*Bos indicus*) and sheep in the wildlife-livestock interface at the Maasai Mara ecosystem.

1.4.2 Specific objectives

1. To investigate the species and genetic diversity of ticks infesting wildebeest (*Connochaetes taurinus*) in Maasai Mara ecosystem.

2. To investigate the species and genetic diversity of ticks infesting livestock in the Maasai Mara ecosystem

3. To assess the diversity of protozoan parasite species harboured by ticks infesting wildebeests and cattle in the Maasai Mara wildlife-livestock interface.

CHAPTER TWO

LITERATURE REVIEW

2.1 Importance of livestock in Kenya

Livestock production is one of the most important economic and social activities in Kenya contributing close 10% of our Gross Domestic Product (GDP) and accounting for more 30% of farm gate value of agricultural produce (Kiptarus, 2005). Millions of people depend on livestock-based activities especially in the arid and semi-arid areas. The role of livestock extends beyond the supply of meat and milk to encompass capital, traction power, hides, fuel and fertilizer. Cattle production in Kenya entails both the dairy and the beef sub-sectors. The dairy industry is a mainstay of about 600,000 - 800,000 small-scale farmers and employs players along the milk market chain. Kenya has one of the largest dairy industries in sub-Saharan Africa (Wambugu *et al*., 2011) which constitutes the largest contribution of livestock to the country's GDP which is 3.5% of the total GDP. Smallholder dairy farmers produce over 70% of Kenya's milk (Kiptarus, 2005). Beef production is practiced primarily in the Arid and Semi-arid areas of the country. The Zebu cattle in these areas make up most of the beef herds with a significant percentage of beef also coming from dairy bull calves and culled cows. The beef industry in Kenya is made up of three livestock production systems which are Extensive pastoral beef, dairy bull calves and commercial beef production System (Kiptarus, 2005). Kenya's population of beef cattle as per the livestock census in 2005 stood at over 9 million heads 80% of which were kept in rangelands including Kajiado, Laikipia, Narok, and Taita Taveta (Kiptarus, 2005).

2.2 Challenges faced by livestock farmers in Kenya

Livestock farmers in Northern and North Eastern Kenya as well as in the rangeland districts mainly practice nomadic pastoralism. This causes frequent competition for scarce land, pastures and water resources amongst communities. Scarcity of these resources also drives pastoralist communities into wildlife reserves thereby creating wildlife-livestock interfaces

which becomes a hotspot for pathogen transmission across species (Wambwa, 2005). The major constraints faced by pastoralists in Kenya include diseases, lack of proper artificial insemination services and lack of feeds. Cattle diseases are however significant with variations in prevalence and severity across the country (Emongor *et al*, 2000). Tick-borne diseases such as East Coast Fever (ECF), Anaplasmosis and Heart water are also among the challenges faced by pastoral communities (Emongor *et al*., 2000).

2.3 General structure and life cycle of ticks

Hard ticks have a single upper plate to the posterior that provides protection while also serving as attachment site for muscles of the *capitulum*. The scutum of some ticks shows various patches of color as seen on *R. pulchellus*. Male hard ticks can be distinguished from female hard ticks by the size of the scutum with males having a larger scutum covering the upper body surface while in females the scutum is smaller and covers less than half the upper body to allow for engorgement. (Houseman, 2013).

The bodies of ixodid ticks consist of two regions, the mouthparts referred to as the *capitulum* and the body also known as the *idiosoma* with mouthparts protruding in front of the body and are visible from above. Ticks have eyes located near the front corners of the scutum and legs with six segments. The legs have claws at the end used for clinging and moving around on the host and spines that aid in attachment. The tips of the first pair of legs carry the haller's organ, a sensory organ appearing as a small capsule with receptors for heat, moisture and chemicals (Jongejan and Uilenberg, 1994). The mouthparts consist of three structures called palps, chelicerae and a hypostome attached to a basis capitulum. These three structures function in allowing penetration of the host skin for a blood meal. The palps are leg like structures that serve as sensory organs. The chelicerae are cutting organs with cutting edges facing outward (Jongejan and Uilenberg, 1994). To attach to a host, the chelicerae are inserted into the skin and pushed apart opening a hole through which the hypostome is inserted. The digestive tract

opening is at the tip of the hypostome (Houseman, 2013). The cuticle of hard ticks has numerous internal folds appearing like grooves on the body surface. Festoons are uniform rectangular folds located on the rounded posterior end of the scutum that unfold and stretch allowing ticks to take large blood meals and engorge (Houseman, 2013).

Spiracles are located on the sides of the body after the last pair of legs and are used for breathing. The anus is toward the posterior of the body with reproductive opening between the anus and the capitulum (Houseman, 2013). The main key features of ixodid tick morphology are as illustrated in figure 2.1.

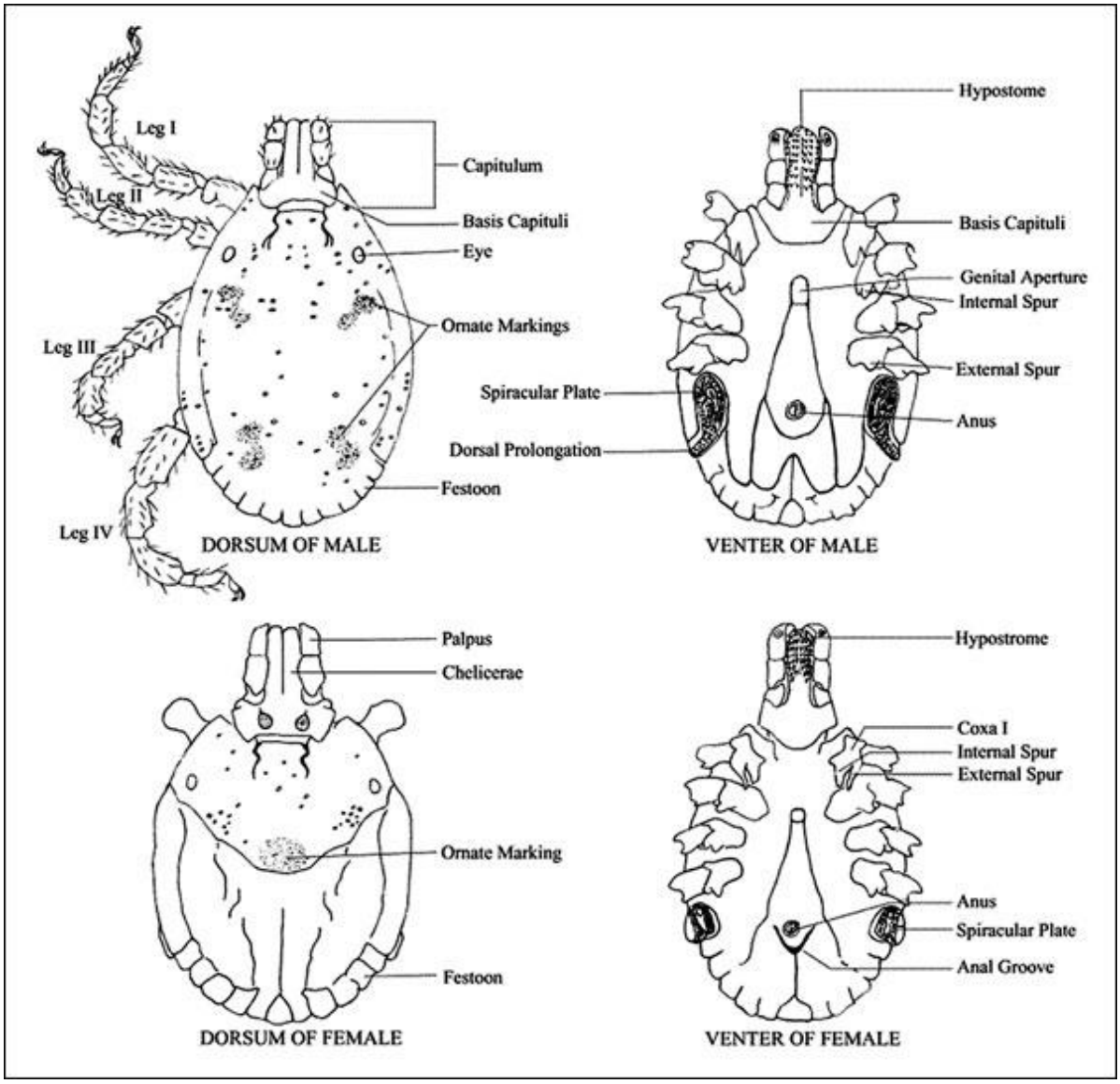

Figure 2. 1: Key physical features of male and female hard ticks (Houseman, 2013)

Most Ixodid ticks are three host ticks meaning each instar detaches from its host after feeding, drops off, moults and waits for another host. Some are two host ticks e.g. *Rhipicephalus evertsi* and some *Hyalomma* species whose larvae remain on the same host and moult rapidly into nymphs which continue feeding then drop off as engorged nymphs to moult into adults and quest for the second host. One host ticks e.g. the *Boophilus* species and *Hyalomma scupense* spend all their active stages on the same host with engorged females falling off to oviposit (Jongejan and Uilenberg, 1994).

2.4 Ixodid ticks classification

Approximately 840 species of ticks are known to occur in the world and these are classified into the families *Ixodidae* and *Argasidae* (Jongejan and Uilenberg, 1994). Ixodid ticks comprise about 80% of known tick species, including the species of greatest economic importance (Jongejan and Uilenberg, 1994).

2.4.1 Genus *Rhipicephalus*

Rhipicephalus ticks are vectors of East Coast Fever (ECF) in cattle and Nairobi Sheep disease. They are usually not ornamented and have festoons on their posterior end. Most are three host ticks found on mammals but some are two-host ticks such as *R. evertsi* (Jongejan and Uilenberg, 2004). *R. appendiculatus* is the most important rhipicephaline tick in East Africa and infests both wild and domestic ruminants (Jongejan and Uilenberg, 2004).

The genus *Rhipicephalu*s is also composed of *Boophilus* ticks a group that is composed of many species with different geographical distribution. Also known as the blue tick, they transmit both Anaplasmosis and Babesiosis to cattle in East Africa (Jongejan and Uilenberg, 2004). They are one-host ticks with life cycles that take three weeks on the host preferably cattle with *Boophilus microplus* being the most important species. As one-host ticks, they often attain high numbers on cattle herds causing considerable damage. These ticks are the main vectors of bovine babesiosis whose causative agents are the moderately pathogenic *Babesia bigemina* and the highly pathogenic *Babesia bovis* (Jongejan and Uilenberg, 1994).

2.4.2 Genus *Amblyomma*

The genus *Amblyomma* consist of several species which include *A. variegatum, A. gemma, A. lipidum,* which are fairly large ticks. The most important species in this genus is *A. variegatum* which transmits *Ehrlichia ruminantum* which causes heart water disease in sheep and goats. The tick can take up 5ml of blood leading to significant blood loss to the host. On average one *A. variegatum* female tick lays 20,000 eggs ensuring a significant percentage survival of the

larvae. Its long mouthparts leave large wounds which get secondarily infected by *Dermatophilus congolensis* and screw worms. Their legs are striped and their scuta are distinctly ornate. These three-host ticks are present in the tropics and subtropics zones parasitizing mammals, reptiles and amphibians with stages of some *Amblyomma* ticks infesting birds that play a role in their dispersal (Jongejan and Uilenberg, 1994).

2.4.3 Genus *Hyalomma*

Hyalomma ticks have palps that are longer than they are wide and they look hexagonal dorsally. Their anal groove may be distinct and never extends anteriorly around the anus. They have festoons and the spiracular plates have tail-like protrusions. They also have ventral plates (Okelo-Onen *et al*., 1999). Often their legs are striped like the *Amblyomma* but their scuta lack ornamentation. They transfer sweating sickness of cattle which is not an infection but caused by toxins secreted by female ticks on to the host's skin. *Hyalomma* ticks have long hypostomes and eyes that are located in sockets. They infest both domestic and wild mammals as well as birds, and prefer semi-arid zones. The genus is made up of up to 30 species which are three-host ticks. Some of them however undergo a two-host cycle (Jongejan and Uilenberg, 2004). *Hyalomma* differ in their questing behaviour in that as opposed to other hard ticks which perch on vegetation awaiting a passing host, they hide and actively run out from their dwellings when hosts approach. Many *Hyalomma* ticks transmit *Theileria annulata* with all stages of these ticks being adapted to cattle. *Hyalomma* ticks also transmit Crimean Congo hemorrhagic fever a zoonosis (Jongejan and Uilenberg, 1994).

2.5 Ixodid tick species found in Kenya

Rhipicephalus appendiculatus is a species of key importance in Kenya since it is a principle vector of *Theileria parva*. Other *Rhipicephalus* species in Kenya include *R. evertsi,* and *R. pulchellus* (Macaluso *et al*., 2003). Also in Kenya are *Amblyomma* ticks such as *Amblyomma variegatum* and *Amblyomma gemma* vectors of *cowdria ruminantum* that causes heart water disease. *Boophilus* ticks present in Kenya are *Boophilus decoloratus* vectors of *Babesia* and

Anaplasma. In a study carried out in the Maasai Mara National Reserve, Kenya (Macaluso *et al*., 2003), focusing on spotted fever group rickettsial organisms, *Amblyomma variegatum, Rhipicephalus appendiculatus, Rhipicephalus evertsi*, *Rhipicephalus pulchellus* and *Boophilus microplus* were identified.

2.6. Impact of climate change on ticks and tick-borne diseases

Climate change has had significant effects on key habitat elements that are critical to wildlife, domestic animals, and humans (Hofmeister *et al*., 2010). Rising temperatures and sea levels, changing rainfall patterns and droughts combined with expanding human populations have significantly impacted on the contact between wildlife and domestic animals. Climate change also leads to the introduction of exotic and invasive species including pathogens of emerging zoonotic diseases (Hofmeister *et al*., 2010) which are attributed to contact between wildlife and domestic animals and humans as they encroach onto previously uninhabited regions for pastoralism and agriculture (Hofmeister *et al*., 2010). Rises in temperature accelerate vector metabolism and biting rates and this increases the frequency of blood feeding leading to enhanced egg production and vector population sizes (Hofmeister *et al*., 2010). Higher temperature have also been shown to increase the geographical range and distribution of vectors which tends to be limited by temperature and humidity (Mellor and Leake, 2000). Temperature rises are also predicted over most of tropical Africa which may eventually increase the suitability of *R. appendiculatus* to these regions by shortening generation time leading to higher populations of this tick species (Olwoch *et al*, 2008).

Changes in precipitation patterns also significantly impacts epidemiology of diseases through survival of disease causing pathogens or vectors (Hofmeister *et al*., 2010). An example of effect of climate change on tick borne diseases is the predicted reduction in the distribution range of *R. appendiculatus* in the arid regions of western Africa following predicted rises in temperature in the already hot and dry areas (Olwoch *et al*., 2008). On the other hand, Olwoch *et al* in 2008

postulated that under future climate scenarios, the northern and Eastern Cape provinces in South Africa alongside Botswana, Zambia and Eastern DRC that were formerly unsuitable to ticks are becoming suitable for survival of *R. appendiculatus* (Olwoch *et al*, 2008).

2.7 Tick control methods and their shortcomings

2.7.1 Chemical control

This has been the main method used for tick control (Jongejan and Uilenberg, 1994). Acaricides are synthetic chemicals used to kill ticks on livestock or in the environment. Some of the acaricides that have been used include synthetic pyrethroids, organophosphates, carbamates and amidines. Chemical control is considered as one of the best methods, but it has its demerits. Some of the acaricides are expensive for ordinary smallholder framers and in addition dips and spray races are expensive to construct and maintain thus many smallholder farmers with few head of cattle find it uneconomical to operate unless under communal ownership and maintenance. When using hand sprays, acaricides drip from the animal and is wasted and causes environmental pollution. Handlers are also exposed to the harmful effects of acaricides if they lack protective clothing. There also exist fake, ineffective and adulterated acaricides in the markets. There are numerous recent reports on increasing resistance of ticks to many newer classes of acaricides currently in use. This problem is aggregated by use of low dosages by farmers to save on the cost as well as influx of ineffective poor quality chemicals in the markets. Water scarcity in pastoral areas also hinders proper use of acaricides. In addition, residues and remnants of the chemicals are known to persist in milk and meat making then unsuitable for consumption.

2.7.2 Mechanical control

This is done by handpicking where animals are restrained and ticks are picked and either burned or buried. This can also be done during milking and cleaning of livestock sheds. It is a

traditional control technique that was done as a communal cultural practice. However, this method is tedious, time consuming and is not sustainable for a large herds of cattle.

2.7.3 Use of anti-tick vaccines

Vaccination of livestock with defined protein antigens can induce significant immunity to tick infestation. Examples of commercially available tick vaccines include Gavac TM, and Tick Gard PLUS, (Andreotti, 2006) that are being used to control *B. microplus* and field studies have shown that these can make a valuable contribution to an integrated approach in control of ticks (Andreotti, 2006). Recently, there have been numerous efforts to develop other anti-tick vaccines targeting other tick species since the available vaccines are not effective in preventing infestation by certain tick species (Imamura *et al*., 2008).

2.7.4 Biological control

This involves use of the predators of ticks, which include birds, parasitoid wasps, certain species of nematodes, the bacteria *Bacillus thuringiensis* and entomopathogenic fungi of the genus *deuteromycete* (Samish *et al*., 2004) which reduces the density of the target population or even eliminates it. Studies by Kaaya *et al*., (2000) have found engorged *B. decoloratus* females, *R. evertsi* and *R. appendiculatus* susceptible to specific nematode strains such as *S. carpocapsae* and *H. bacterio-Phora* while nymphs, larvae and unfed adults were found to be resistant. *A. variegatum* and *A. gemma* were found to be resistant to the nematodes whether fed or unfed and despite their stage in development. Chicken are also natural predators of livestock ticks and can therefore be used in integrated tick management (Sahito *et al*., 2013). Biological control of ticks is a safe, effective and cheap way encouraged for control of ticks.

2.7.5 Use of resistant breeds of livestock

Tick resistance is an acquired characteristic that enables an animal to limit the establishment, growth and persistence of tick populations. Each animal develops some level of resistance upon tick infestation which may be high or low. For example, indigenous breeds the Zebu (*B.*

indicus) and Sanga (*B. taurus and B. indicus* cross breed) cattle carry significantly fewer ixodid ticks than exotic European (*B. taurus*) breeds of cattle (Brossard, 1998). A comparison of tick resistance in three cattle breeds in Kenya was done in the *Abernossa* ranch. These were the *Arsi, Boran* and *Boran*-Friesian crossbreed, it was established that the *Arsi* breed was the most resistant followed by the *Boran* with the cross breed showing the least resistance (Solomon and Kaaya, 1996). The local *Bos Taurus* in North Africa and the *N'Dama* in west Africa are also resistant to tick borne East Coast Fever (Uilenberg, 1995).

2.8 Vertebrate tick hosts: Role of wildlife and livestock

Wild ruminants are implicated as reservoirs of tick-borne pathogens. For instance, the African buffalo (*Syncerus c affer*), the gemsbock (*Oryx gazelle gazelle*), the eland (*taurotragus oryx*), the antelope (*Hippotragus equines*), the Springbok (*Antodorcas marsupialis*), the red hartebeest (*Alcelaphus buselaphuscaama*), the blesbok (*Damaliscus dorcasphillipsi*), the greater kudu (*Tragelaphus strepsiceros*) and black wildebeest (*Connochaetes ghou*) are some of the wildlife species known to serve as reservoirs for pathogenic protozoa (Tonetti *et al*., 2009). Most of these ruminants serve as reservoirs of ticks and tick borne pathogens and are sources of infection to livestock in the wildlife-livestock interfaces. The transmission of these pathogens may be vector borne with ticks serving as the major vectors especially during wildlife-livestock interactions. The contact between humans and domestic animals exposes them to zoonotic diseases (Jones *et al*., 2011). The mixing of animals from the wild and the domestic ecosystems is a route of introducing diseases into the latter thus exposing domestic animals and humans to parasitic infections (Munang'andu *et al*., 2012).

In a study carried out in Narok County (Nyariki *et al*., 2009) it was established that transmission of certain livestock diseases is largely aided by wildlife since the county borders the Maasai Mara National reserve. Control of diseases in the area is thus made difficult because wildlife cannot be effectively vaccinated or quarantined. Consequently, livestock health is affected and

veterinary care costs are escalated. According to Nyariki *et al*, (2009), the most common livestock diseases in Narok county include Foot and Mouth Disease (FMD), trypanosomosis, anaplasmosis, lumpy skin disease (LSD), malignant catarrh fever, contagious bovine-pleuropneumonia (CBPP), contagious caprine pleuropneumonia (CCPP), Nairobi sheep disease, East coast fever (ECF), and helminthiosis. Some of these are TBDs such as such as East Coast Fever (ECF), anaplasmosis, heart water and Lumpy Skin Disease (LSD) and can be controlled by effective integrated management strategies of the relevant tick vectors. The Blue Wildebeest (*Connochaetes taurinus*) and the Eland (*Taurotragus oryx*) have previously been identified as natural hosts of *Theileria parva* and *T. mutans* which are known to be infective to cattle (Burridge, 1975).

Tick-borne diseases are a major constraint to raising productive cattle herds in Kenya. The major pathogens transmitted by ticks include protozoan pathogens such as *Theileria* and *Babesia*, rickettsial pathogens which include *anaplasma* and bacteria e.g. *Cowdria ruminantum*, These pathogens cause animal diseases with the major ones being theileriosis in cattle; heart water in cattle, sheep and goats; babesiosis in cattle, horses, sheep and goats; anaplasmosis in cattle and Nairobi sheep disease.

Babesiosis is commonly referred to as red water and is caused by tick-borne hemoparasites of genus *Babesia*. *Babesia* parasites are divided into the small babesias which include *Babesia gibsoni, B. microti*, and *B. rodhaini* and large babesias including *Babesia bovis, B. caballi*, *B. canis* and *B. bigemina* (Homer *et al*, 2000). These classifications based on morphology are consistent with the classification based on phylogeny and also on the 18S ribosomal DNA sequences that show the large and small *Babesia* falling into two different phylogenetic groups with the later appearing to be closer to *Theileria* species (Homer *et al*., 2000). *Babesia* species

infect wild and domestic animals with only a few such as *B. duncani*, *B. microti* and *B. divergens* being documented to infect humans (Homer *et al*, 2000). *Rhipicephalus* ticks innoculate *Babesia* sporozoites into host's blood when feeding, these invade the red blood cells and grow into trophozoites that multiply through binary fission producing merozoites that continue infecting red blood cells and replicating within the host. Some trophozoites produce gametocytes which can infect feeding ticks. Once picked by the tick gametocytes fuse within the tick gut to produce a zygote that develops into a kinete. Kinetes get to the tick hemolymph and replicate upon which they invade different organs of the tick. Sporogony starts when kinetes get to the salivary glands. New sporozoites are injected into a host (Hunfeld *et al*., 2008).

Theileriosis on the other hand is caused by infection with protozoa of the genus *Theileria* sub order *piroplasmiidea*. *Theileria* infection results in the different Theilerioses. The two important *Theileria* species that infect cattle are *T. parva* the causative agent of ECF, and *T. annulata* the causative agent of tropical theileriosis in Nothern Africa (Norval *et al*., 1992). Others include *T mutans, T buffeli, T. velifera, T. taurotragi* and *T. sergenti* (Norval *et al*., 1992). *Theileria* sporozoites are injected into the host blood in tick saliva where they invade the lymphocytes developing into macroschizonts. Nuclear division takes place producing numerous piroplasms that escape the lymphocytes to the erythrocytes where they are ingested by feeding *R. appendiculatus* ticks. In the gut of the tick, the parasites differentiate to produce male and female gamonts after which fertilization occurs to produce zygotes. The zygotes enter the cells lining the gut and differentiate to form kinetes which cross the gut wall into haemolymph. They then move to the salivary glands and form intracellular sporoblasts that divides by fission to produce many sporozoites. The sporozoites are then introduced with saliva into a new mammalian host.

Bovine anaplasmosis, also known as gall sickness is a rickettsial disease caused by a rickettsial pathogen that invades the erythrocytes known as *Anaplasma marginale* which is biologically transmitted by infected ticks or mechanically by biting flies (Aubry and Geale, 2011). The genus *Anaplasma* also includes *A. centrali* and *A. bovis*, formerly *Ehrlichia bovis* (Dumler *et al*., 2001).

2.9 Techniques for identification of ticks and tick-borne pathogens

Effective control of tick borne diseases calls for proper identification of the tick species that transmit these diseases. This has traditionally been done morphologically by using published keys (Walker, 2003). It is however difficult to achieve accurate identification especially where the ticks are immature, when they are engorged with blood or are physically damaged (Lv *et al*, 2014). Different genetic markers have therefore been identified for molecular identification and phylogenetic studies of ticks (Dantas-Torres *et al*., 2012). The markers have to be flanked by regions of conserved DNA; they should be short and should have no heterozygosity and thus give unambiguous results (Lv *et al*, 2014). Tick morphology is therefore used only in preliminary tick identification while molecular techniques are useful in confirming the species identities.

The molecular markers amplified by PCR that can be used for tick species differentiation. These include both ribosomal and nuclear fragments. The Internal Transcribed spacer 2 gene (ITS2) and the CO1 gene are mitochondrial markers used in diversity studies. Other ribosomal genes 18S rDNA and 28S rDNA have also proved to be useful tools for phylogenetic studies within the order Acari (Cruickshank, 2002). Cytochrome Oxidase Subunit 1 (CO1), 12S ribosomal DNA, 16S mitochondrial DNA have been used in the construction of a DNA database from ticks collected in Japan and South Korea (Takano *et al.*, 2014). The ITS2 has

been used for identification of *Hyalomma, Rhipicephalus*, *Dermacenter* and *Boophilus* ticks in Iran (Abdigoudarzi *et al*., 2011).

Detection of protozoan parasites in ticks is important in epidemiological studies. The techniques range from using conventional staining methods and microscopy through the more specific use of DNA probes following cloning to the very sensitive means of DNA amplification using PCR (Jongejan and Uilenberg, 1994). Definitive identification of parasites is difficult when parasites are morphologically identical, are small in size or are few in numbers (Jeneby, 2011). PCR makes use of protozoan specific universal primers and *Taq* DNA polymerase to synthesize a large number of copies from a single protozoan DNA template. PCR is more specific in differentiating between closely related parasites within ticks when compared to microscopy (Jongejan and Uilenberg, 1994). PCR can also be effectively performed on ethanol preserved specimens but should be used with care due to the danger of introducing extraneous DNA leading to contamination (Jongejan and Uilenberg 1994). To overcome the problem of contamination, the surface of the ticks can be sterilized to reduce false positive results. This can be done by washing the ticks in 70% ethanol and rinsing with sterilized water (Sparagano *et al*., 1999). Tick DNA extraction is often difficult because ticks have a hard chitin exoskeleton. DNA from ticks has also been found to be highly susceptible to degradation (Halos *et al*., 2004). There are different protocols employed in extraction of DNA from ticks. Halos *et al*., (2004) conducted a study to compare three protocols.

The first method made use of the Qiamp DNA extraction kit. (Halos *et al*., 2004). In the second method the whole ticks were crushed by use of mortar and pestle in 1ml of lysis buffer composed of NaCl 0.1M, Tris HCl 0.21M, PH 8 EDTA 0.05M and 0.5% SDS. This was followed by enzymatic digestion with proteinase K for 16 hours at 56 degrees Celsius.

Extraction was then carried out using the phenol-chloroform method and the DNA precipitated with absolute ethanol (Halos *et al*., 2004). This method has been used in a study to detect *Theileria ovis* in *Rhipicephalus bursa* adult ticks in which individual tick salivary glands were extracted (Aktas *et al*., 2006). In the third method, samples were collected in a 2ml sterile micro tubes with sterile micro beads that were 1mm in diameter and one steel micro-bead that was 4 mm diameter. They were then cooled in liquid nitrogen for one minute and crushing was done by vigorous shaking with a bead beater for two cycles of one minute thirty seconds. This was followed by brief centrifugation at a speed of 10,000g. The samples were then extracted using the Qiamp DNA extraction kit following the manufacturer's protocol. This method has been used by (Ica *et al*., 2007) in a study to detect haemoprotozoan pathogen species in ticks obtained from cattle. In another study ticks were frozen and then crushed into a powder. Genomic DNA was then extracted using a commercial Qiagen Tissue Kit (Swai *et al*., 2006) extracted DNA of questing brown ear ticks (*R. appendiculatus*) by adding proteinase-K to macerated ticks which was followed by DNA isolation using the phenol-chloroform technique and ethanol precipitation. Another method involved briefly boiling crushed ticks in ammonium hydroxide at a concentration of 0.7M for 15 minutes at 100°C which then underwent evaporation to a final volume of 50 microliters and centrifuged for 10 minutes at 10,000g. The supernatants were stored at -20°C before PCR amplification.

Amplification of protozoan DNA from the total genomic DNA is normally based on the parasite 18SrDNA which is a part of the ribosomal gene operon that codes for ribosomal RNAs. Ribosomes are made up of ribosomal RNAs and proteins and they translate messenger RNA molecules into proteins. The ribosomal gene operon includes a non-transcribed spacer (NTS), an externally transcribed spacer (ETS), the 18S gene, an Internal Transcribed spacer1 (ITS1),

a 5.8S gene, an ITS2 and a 28S gene This operon is usually repeated multiple times in the eukaryotic genomes.

Ribosomal genes are highly expressed because of the critical role ribosomes play in assembling proteins. The 18SrRNA gene includes highly conserved and variable nucleotide sequence regions and can be useful in phylogenetic reconstruction and organism recognition at different taxonomic levels (Majaneva *et al*., 2013) Amplification of *Babesia* species DNA has been done using conventional PCR and 18S primers to detect multispecies DNA sequences of 18SrRNA gene fragment (Fyumagwa *et al.*, 2011). Protozoan specific primers are used to target the protozoan DNA in the total extracted DNA for amplification. In a study to detect *Theileria ovis* in *R. bursa* ticks, a pair of primers; 5-TCGAGACCTTCGGGT-3 (forward) and the reverse primer 5-TCCGGACATTGTAAAACAAA-3 were used to amplify 520 base pair fragment of the small sub unit ribosomal RNA gene of *T. ovis* (Aktas *et al*, 2006). Nested PCR can also be used in amplification of the 18SRNA gene of *Babesia, Theileria* and piroplasms of the genera *Entopolypoides* and *Cytauxzoon* (Jeneby, 2011). It is a modification of PCR designed to improve sensitivity and specificity and involves use of two primer sets and two successive PCR reaction. This has been used in a study to detect haemoparasites infecting non-human primates in Kenya (Jeneby, 2011). The primary amplification was performed using the forward primer, IL0-9029 (5-AACCTGGTTGATCTTGCCAG-3) an 18S primer which non-specifically targets eukaryotes and prokaryotes and reverse primer, ILO-9030 (5-GAACCTGCG GAAG GATCATTA-3) that is most similar to 18S of possibly all *Theileria, Babesia* and *Cytauxzoon*. Secondary amplification utilized forward primer ILO-MWG 4/70 (5-TGGTTGATCTTGCCAGTA-3) most similar to 18S of all *Theileria, Babesia* and *Cytauxzoon* and reverse primer ILO-7782 (5-GAACCTGCG GAAG GATC ATTA-3) which is also similar to 18S of all *Theileria, Babesia* and *Cytauxzoon* (Jeneby, 2011).

The reverse line blot technique (RLB) has also been used for detection of protozoans in ticks. This technique is useful for detection of *Babesia* and *Theileria* (Gubbels *et al*., 1999). Here, amplification of protozoan 18SrRNA has been done by using the primers RLB F2 and RLB R2 (Namavari *et al*., 2011). The same primers have been used to amplify a fragment of approximately 400 base pairs of the 18S SSU rRNA gene which spans the V4 region of *Babesia* species and *Theileria* species (Tonetti *et al*, 2009). The first step in the RLB technique is amplification of a variable region in the18Sr RNA gene of protozoans within its conserved parts. The primers are designed to target specifically the protozoan 18SrRNA gene. The PCR products are then hybridized onto a blot to which specific oligonucleotide probes for each targeted organism has been covalently linked. The pathogen species amplified by PCR hybridize specifically at their oligonucleotide probe. Washing is done to remove unbound PCR product and the hybridized PCR products are visualized using a biotin label attached to the PCR primer.

Detection of *A. marginale* in tick can be done by PCR using primers to amplify msp5 gene, The primers used are; Forward 5'-GCA TAG CCT CCG CGTCTT TC-3' and reverse 5'-TCC TCG CCT TGGCCC TCA GA-3' with a product size of 457bp (Nair *et al*., 2013). For *Anaplasma bovis*, nested PCR can be employed using the forward primer 5'-TAC CTT GTTACG ACT T 3'and 5'-AGA GTT TGATCM TGG 3' (reverse) (Ikwap *et al*., 2010). The product from this primary reaction is then used as the template DNA for amplification of *A. bovis* 16SrRNA gene using the specific primers (Ikwap *et al*., 2010)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area and tick sampling

Adult and nymph stage ticks were obtained by purposive sampling of cattle and sheep and convenience sampling from wildebeest from four locations in Maasai Mara shown in Figure 3.1 in August 2012. Two of the sites were in the Mara triangle within the National park where migratory wildebeest were sampled, whereas the other two sites (Aitong and Talek) were outside the national reserve where cattle and non-migratory wildebeest were sampled. Ethical clearance for sampling wildebeest was obtained from the Scientific Research Committee of the Kenya Wildlife Service. 30 wildebeests were immobilized by using darts impregnated with a combination of Etorphine hydrochloride (M99®) and Xylazine hydrochloride (Norvatis Pty Ltd, South Africa). Ticks were sampled from ears, underside, anal, udder and scrotal regions. The animals were reversed using a combination of Diprenophine and Atipamazole. Ticks were also sampled from 30 cattle and 1 sheep in *Talek* and *Aitong* villages outside the MNR, from farms with the consent and involvement of individual farmers. Cattle were immobilized in crushes, while sheep were hand held, following which the ticks were sampled from similar body parts as in wildebeests plucking them off gently using a pair of serrated forceps. The forceps were used to hold ticks firmly over their scuta and mouthparts close to the host skin before pulling strongly out from the animal skin. The ticks were placed in a bottle containing absolute ethanol and transported back to the laboratory where they were stored at room temperature for further analysis.

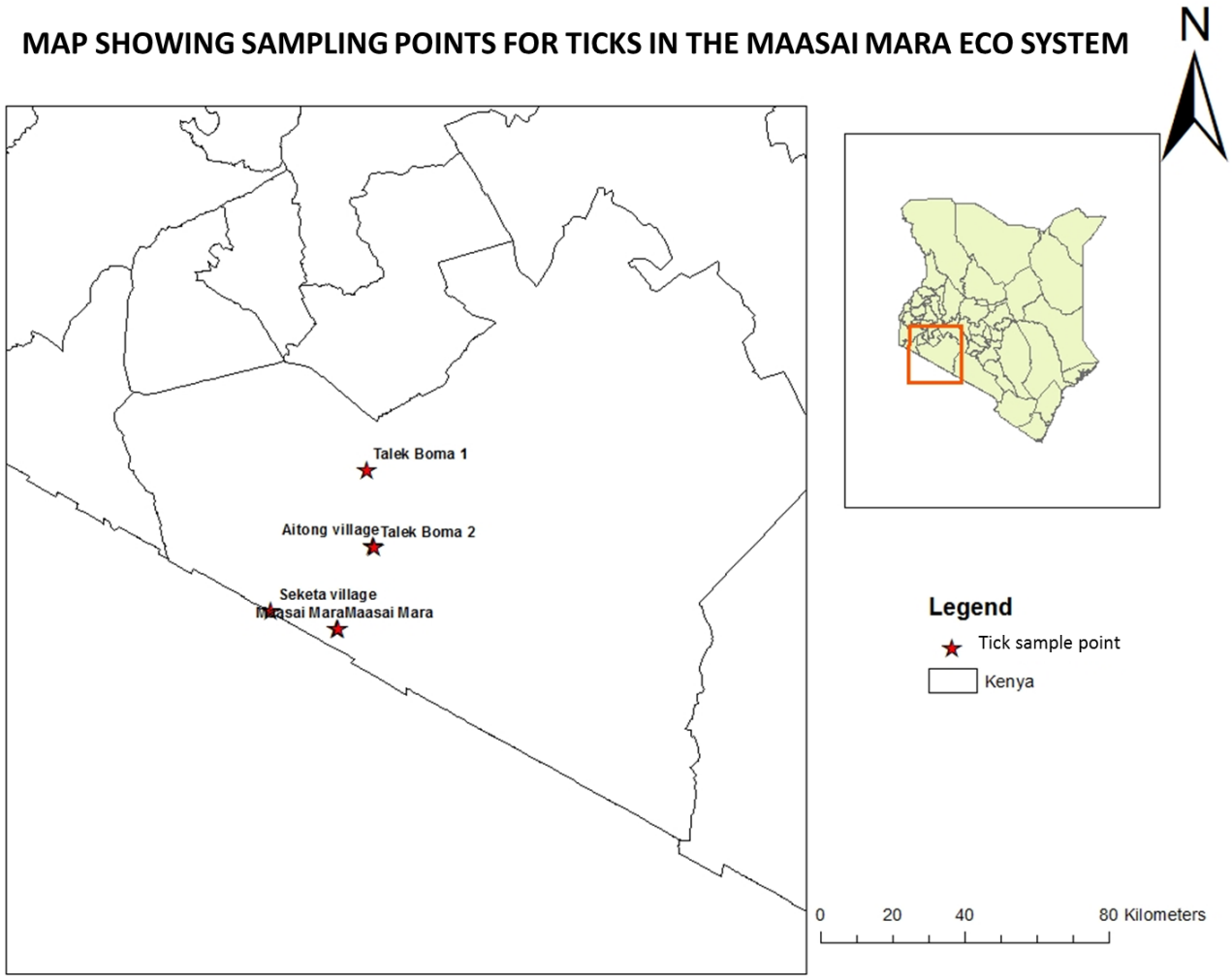

Figure 3. 1: A sampling map of Maasai Mara indicating sites where ticks were collected

3.2 Morphological identification of ticks

Morphological identification of the ticks was done at the acarology laboratory at the Directorate of Veterinary Services, Kenya under the guidance of an acarology technician. The ticks were sorted according to their stages, sex and species. Species identification was done by examining features of the ticks under a dissecting light microscope. The main morphological features studied included the colour of the tick and ornamentation of the scutum, shape of the tick, size and distribution of punctuations, grooves and colour of legs. Identification manuals by Walker *et al*., (2003) and (Hoogstral, 1956) were used as illustration aids towards identification.

3.3 Extraction of whole tick genomic DNA

Briefly, each individual adult tick was placed in a 1.5ml epperndorf tube and ground thoroughly in liquid nitrogen using mini pestles. 180µl (microliters) of cell lysis buffer and 20µl Proteinase K was

added followed by vortexing for 30 seconds. Incubation at 56^{o}C was done overnight and the samples were then vortexed for 10 seconds the next morning. Tissue lysis buffer (AL) was added to each of the vortexed samples, which were vortexed again for 10 seconds. Samples were pipetted into DNeasy Mini spin columns nested in 2ml collection tubes. Centrifugation was done at 10,000rpm in a micro-centrifuge. The flow-through was poured out. The spin columns were replaced in the collection tubes and 500µl of wash buffer (AW1) was added followed by centrifugation for 1 minute at 10,000 rpm. The flow-through was again discarded. The columns were placed in sterile 2ml collection tubes and 500µl of wash buffer (AW2) was added, centrifugation was done for three minutes at 14,000 rpm and this was followed by another centrifugation for 1 minute at 14,000 rpm without any buffer to get rid of any residual ethanol. The spin columns were placed into clean 1.5ml centrifuge tubes and elution of DNA was done twice, first using 70µl of nuclease free water and a second elution using 40µl of nuclease free water. Incubation was done at room temperature for 2 minutes followed by centrifugation for 1minute at 10,000 rpm. Quantities of DNA were determined using both Nano Drop spectrophotometer and agarose gel electrophoresis.

3.4 Determination of nucleic acid concentration and purity

A 1µl aliquot of genomic DNA from each tick sample was pipetted and used for spectrophotometric analysis by measuring the absorbance of ultra violet light at 260 nm and 280 nm in Nano Drop 2000 (Thermo Scientific, USA). The yield and quality of genomic DNA was analysed through agarose gel electrophoresis, 2µl aliquots of genomic DNA from each sample was resolved on 1% agarose gel in 1X tris acetic acid EDTA (TAE) buffer, alongside DNA size markers (100bp, Bioline UK). Prior to electrophoresis and Amplification by PCR, DNA samples were standardized to 50 nanograms per microliter using nuclease free water (Bioline, UK) by following the formula;

$$C_1 V_1 = C_2 V_2$$

Where C1 refers to the initial concentration, C2 the final concentration, V1 is the initial volume while V2 is the final volume.

3.5 Optimization of primers

Primers pairs for use in amplification of tick cytochrome oxidase subunit 1, Internal Transcribed spacer 2 gene, 12S ribosomal DNA and protozoan 18S ribosomal DNA fragments were optimized to determine the optimum conditions for the PCR reactions. Gradient PCR was used to get the best annealing temperatures for each of the primer sets used. Each primer set was tested across a gradient of twelve annealing temperatures to determine the optimal PCR reaction conditions. Temperature ranges were determined by considering 5º C below and above the lower Tm of the primer sets on the primer synthesis report. The PCR reaction was carried out in the Arktik PCR system (Thermo Scientific, USA). Resulting amplicons were analyzed by agarose gel electrophoresis as described in section 3.1.5.

3.6 Amplification of tick molecular markers

3.6.1 Amplification of cytochrome oxidase subunit 1 (CO1) gene sequences of individual R. appendiculatus and R. evertsi

To confirm species identity of collected tick samples, Cytochrome C Oxidase subunit I gene (CO1) was amplified from the total genomic DNA of each individual *R. appendiculatus* and *R. evertsi* ticks. The primers LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ (forward) and HC02198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (reverse) were used (Vrijenhoek, 1994). The 50µl PCR reaction consisted of 10µl MyTaq reaction buffer, (Bioline, UK) which comprised of 5mM DNTPs and 15mM MgCl2, 1unit of MyTaq DNA polymerase, 1µl of 10pmol of each of the respective primers and 5µl of individual DNA template. The final concentrations of components in the 50µl PCR reaction mixture were 1mM of DNTPs, 1.5mM of MgCl2, 0.02 units of MyTaq DNA polymerase and 10 pmol of each primer. The final volume of the reaction mixture was made up to 50µl with Nuclease free water. The PCR conditions consisted of an initial denaturation at 95°C for 1 minute followed by 35 cycles of 95°C for 15 seconds, annealing was done at 40°C for 30 seconds and extension at 72°C for 30 seconds. The final extension was carried out for 5 min at 72°C. Proflex Thermo cycler (Applied Biosystems, USA) with heated lid was used.

3.6.2 Amplification of internal transcribed spacer 2 (ITS2) region of individual R. appendiculatus and R. evertsi

A 700bp fragment of ITS2 region was amplified by PCR amplification of individual *R. appendiculatus* and *R. evertsi* tick genomic DNA samples using the forward primer ITS2IntF1 5'-AAGAGCCTGCAGGGAAAG-3' and reverse primer ITS2IntR1 5'- CACGTTCGTAAACCCATC-3'. The 50µl PCR reaction consisted of 10µl MyTaq reaction buffer, (Bioline, UK) which comprised of 5mM dNTPs and 15mM MgCl2, 1unit of MyTaq DNA polymerase, 1ul of 10pmol of each of the respective primers and 5µl of individual DNA template. The final concentration of these components in the 50ul reaction mixture were 1mM of dNTPs, 3mM of MgCl2, 0.02 units of MyTaq DNA polymerase and 10pmol of each primer. The final volume of the reaction mixture was made up to 50µl with Nuclease free water. The PCR conditions consisted of an initial denaturation at 95°C for 1 minute followed by 35 cycles of 95°C for 15 seconds, annealing was done at 45°C for 30 seconds and extension at 72°C for 30 seconds. The final extension was carried out for 5 minutes at 72°C. Proflex Thermo cycler (Applied Biosystems, USA) with heated lid was used.

3.6.3 Amplification of 12S ribosomal DNA (rDNA) sequences of individual R. appendiculatus and R. evertsi

Approximately 400bp fragments of the 12SrDNA region were obtained by PCR amplification of individual *R. appendiculatus* and *R. evertsi* tick DNA using the forward primer SR-J-1499 5'-TACTATGTTACGACTTAT-3' and reverse primer SR-N-14594 5'-AAACTAGGATTAGATACCC -3' (Simon *et al.*, 1994). The 50µl PCR reaction consisted of 10µl MyTaq reaction buffer, (BIOLINE) which comprised of 5mM dNTPs and 1.5mM MgCl2, 1unit of MyTaq DNA polymerase, 1ul of 10 pmol of each of the respective primers and 5µl of individual DNA template. The final volume of the reaction mixture was made up to 50µl with Nuclease free water. The PCR conditions consisted of an initial denaturation at 95°C for 1 min followed by 35 cycles of 95°C for 15 seconds, annealing was done at 50°C for 30seconds and extension at 72°C for 30 seconds. The final extension was carried out for 5 min at 72°C. Proflex Thermo cycler (Applied Biosystems, USA) with heated lid was used.

3.7 PCR amplification of 18SrDNA of *Babesia* and *Theileria* species

Approximately 437bp and 400bp fragments of 18S rRNA gene sequence of protozoan and Babesia species respectively were amplified from the tick genomic DNA. The amplification was done using the forward primer MWG4/70 5'-AGCTCGTAGTTGAATTTCTGCTGC-3' and the reverse primer; ILO-7782 5'-AACTGACGACCTCCAATCTCTAGTC-3' for protozoans (Jeneby et al, 2011). PCR amplification of Babesia was done using the forward primer PIRO A 5'-AATACCCAATCCTGACACAGGG -3' and the reverse primer; PIRO B 5'-TTAAATACGAATGCCCCCAAC -3'. The 50µl PCR reaction consisted of 10µl of 5x MyTaq reaction buffer (Bioline, UK) which comprised of 5mM dNTPs and 1.5mM MgCl2, 1unit of MyTaq DNA polymerase, 1ul of 10pmol of each of the respective primers and 5µl of individual DNA template. The final concentration of these components in the 50ul reaction mixture were 1mM of dNTPs, 3mM of MgCl2, 0.02 units of MyTaq DNA polymerase and 1µl of 10pmol of each primer. The final volume of the reaction solution was topped to 50µl using nuclease free water. The PCR conditions consisted of an initial step of denaturation at 95°C for 1 minute followed by 35 cycles of 95°C for 15 seconds, annealing was done at 55°C for 30seconds and extension at 72°C for 30 seconds. The final extension was carried out for 5 minutes at 72°C.

3.8 Purification of PCR products, sequencing and data analysis

3.8.1 Purification of PCR products

Purification was done using the Isolate II PCR and Gel Kit (Bioline, UK). The volume of each sample was brought to 50µl using nuclease free water then 100µl of binding buffer was added to each 50µl PCR product. Each sample was then pipette into separate spin columns encased on collection tubes and centrifuged at 11,000 x g for 30 seconds. The flow through was discarded and then 700µl of wash buffer premixed with absolute ethanol was added to each spin column with the bound DNA. Centrifugation was done at 11,000 x g for 30 seconds followed by a second wash with 700µl of the same wash buffer and another centrifugation at 11,000 x g for 30 seconds. The spin columns were

placed back onto the collection tubes and centrifuged at 11,000 x g for 1 minute to dry the silica membrane. The DNA was eluted using 30µl of elution buffer (C).

3.8.2 DNA sequencing

Purified PCR products obtained from amplification of CO1, ITS2, 12SrDNA and 18SrDNA genes were sequenced by the Sanger dideoxy method. This method selectively incorporates chain terminating dideoxynucleotides using DNA polymerase enzyme during in-vitro replication. The sequencing service was outsourced (Macrogen Inc, Netherlands).

3.8.3 Sequence editing and multiple alignments

Resultant sequence chromatograms were inspected and edited using Bioedit (Hall, 1999). The sequences were trimmed to remove low quality 3′ and 5′ ends which showed unclear peaks on the chromatograms (Appendix 1). The 12SrDNA sequences were trimmed to result in 336bp long sequence, the CO1 sequences to 657bp and the ITS2 sequences to 628bp. Consensus sequences were generated from the forward and reverse sequences for each of the COI, 12SrDNA, ITS2 and 18SrDNA fragments. Species identity was confirmed by BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al*., 1990) by comparison and alignment with reference sequences deposited in the Genbank RefSeq database. Multiple sequence alignments were performed in MEGA 6 (Tamura *et al*., 2013) software suite using the MUSCLE algorithm (Edgar, 2004). The *R. appendiculatus* and *R. evertsi* sequences were deposited to Genbank database under accession numbers MF348067 to MF348134 while *R. evertsi* ITS2 sequences were deposited under accession numbers MF348135 to MF348178.

3.8.4 Phylogeny Reconstruction

Phylogeny reconstruction and evolutionary history were inferred using the Neighbour joining method based on the General Time Reversible model (Nei and Kumar, 2000) with 1000 bootstrap replications (Kimura 1980). The trees were rooted using partial ITS2, 12SrDNA and CO1 sequences of

Rhipicephalus turanicus and *Rhipicephalus maculatus* obtained from GenBank database at National Centre for Biotechnology web site (http:www.ncbi.nlm.nih.gov).

3.8.5 Analysis of genetic distance

Pairwise distance analysis between *R. evertsi* ITS2, CO1 and 12SrDNA was conducted on MEGA6. The number of base substitutions per site between sequences are shown in appendices 6,7and 8. Analyses were conducted using the Tajima-Nei model. All positions containing gaps and missing data were eliminated.

CHAPTER FOUR

RESULTS

4.1 Morphological identification of ticks

Morphological identification of a total of 165 adult and nymph ticks was performed. Of these 69 (41.8%) were *Rhipicephalus appendiculatus*, 74 (44.8%) were *Rhipicephalus evertsi* 4 (2.4%) were *Amblyomma variegatum*, 2 (1.2%) were *Rhipicephalus pulchellus*, 8 (4.8%) were *Rhipicephalus boophilus decoloratus* and 8 (4.8%) were nymphs of *R. boophilus decoloratus*. *R. appendiculatus* were collected from wildebeests only while *R. evertsi* were found infesting cattle, wildebeests and sheep. *A. variegatum*, *R. pulchellus* and *R. boophilus decoloratus* species were found only on wildebeests. Table 4.1 summarizes the number of tick species collected from wildebeests, cattle and sheep. Picture images of the five tick species identified are shown in Figure 4.1.

Table 4.1 Ticks identified from wildebeests, cattle and sheep

Tick species	**Hosts collected from**	**Number of ticks Identified**	%
R. appendiculatus	Wildebeests	69	41.8
R. evertsi	Wildebeests, cattle, sheep	74	44.8
A. variegatum	Wildebeests	4	2.4
R. pulchellus	Wildebeests	2	1.2
R. boophilus decoloratus	Wildebeests	8	4.8
R. boophilus decoloratus Nymphs	Wildebeests, cattle	8	4.8

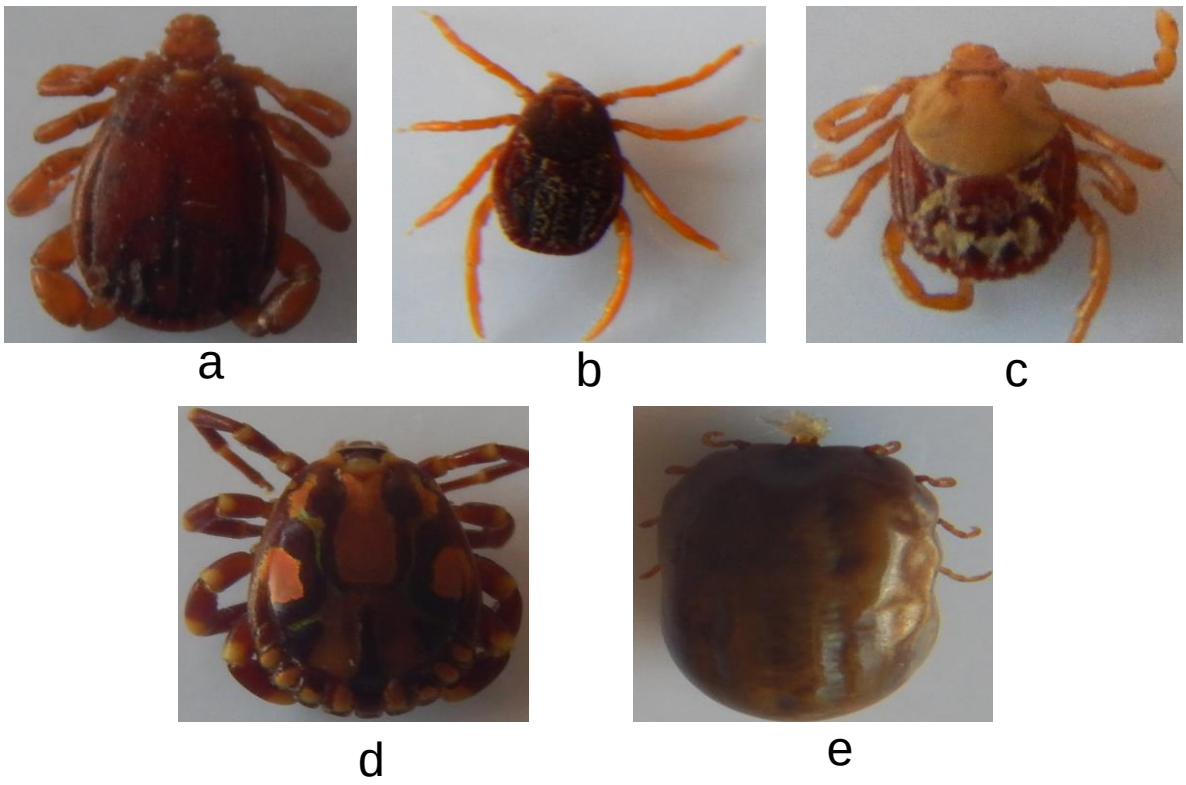

Figure 4. 1: Images of Tick species sampled from cattle, wildebeests and sheep in the Maasai Mara interface a: adult male *R. appendiculatus*, b: adult female *R. evertsi*, c: adult female *R. pulchellus* d: adult male *Amblyomma variegatm*, e: engorged adult female *R. boophilus decoloratus*

Majority of the identified ticks were either *R. appendiculatus* or *R. evertsi* hence these species were investigated further for presence of protozoan parasites. DNA was extracted and pathogen amplification and detection carried out.

4.2 DNA yield and quality

Good quality whole tick genomic DNA was obtained from the ethanol preserved ticks. The average yield was 273.74ng/µl per tick with a range of 17.3ng/µl to 2600.1ng/µl.

4.3 PCR primers optimization

Optimal annealing temperatures for each of the primer pair used for amplification of the three tick genetic markers and protozoan pathogen markers were determined by gradient PCR. Figure 4.2 is an illustration of the amplicons obtained following a gradient PCR for the optimization of the 12S primers.

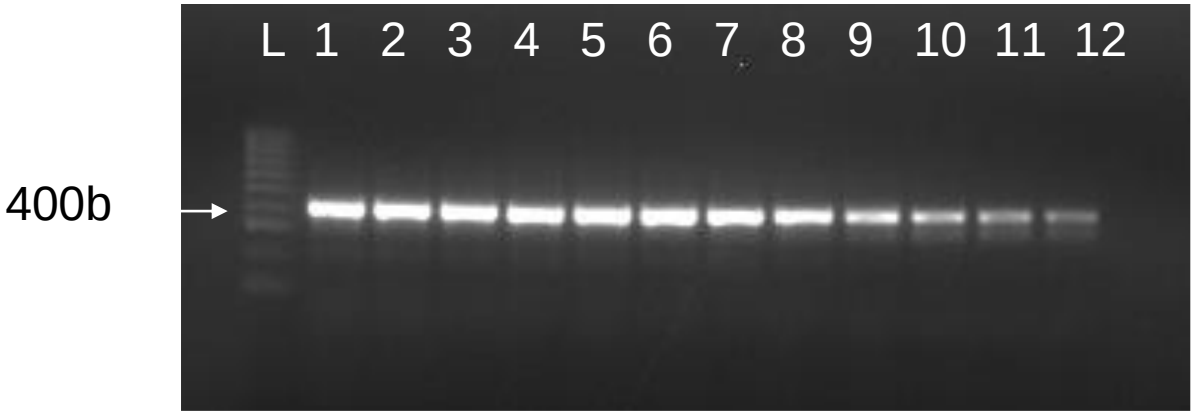

Figure 4. 2: Gel electrophoresis image showing amplicons obtained during optimization of 12S primers. The gradient PCR was carried out at a range of 12 annealing temperatures as follows 1- 42.0, 2-42.3,3-42.9, 4-44, 5-45.4, 6-47.3, 7-49.4, 8-51.3, 9-52.8, 10-54, 11-54.7 and 12-55°C. **Amplicons were analyzed by electrophoresis on a 1.5% gel and the size assessed using 1 kb molecular ladder (Thermofischer Scientific, USA)**

4.4 Molecular identifications of ticks

Of the 165 ticks collected, DNA was obtained from 69 *R. appendiculatus* ticks of which 34 amplified to give an ITS2 amplicon and 29 gave the 12SrDNA product. There was no amplification of the CO1 gene from all the *R. appendiculatus* samples analysed. *R. evertsi* DNA was obtained from 74 ticks of which 37 amplified for the CO1 gene, 49 for the ITS2 and 46 for the 12SrDNA. Figure 4.3 depicts the product quality of the CO1 amplicons.

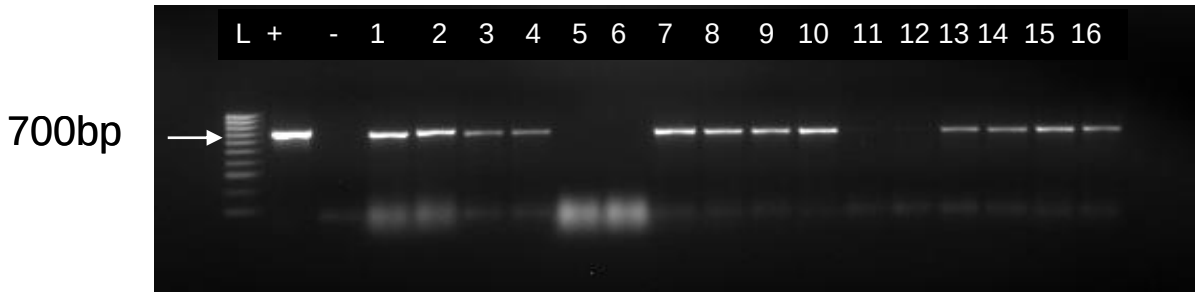

Figure 4.3: Gel electrophoresis image showing CO1 PCR products of different ticks. Lane L represents the ladder followed by a positive (*R. appendiculatus* DNA) and negative control, lanes 1-16 contained CO1 amplicons from 16 ticks. Samples 5, 6, 11, and 12 failed to amplify

From *R. appendiculatus,* 34 ITS2 and 29 12SrDNA amplicons were sequenced while from *R. evertsi*, 37 amplicons of the CO1 gene, 49 for the ITS2 and 46 for the 12SrDNA were sequenced. The sequence trace files were inspected for quality. All the sequenced *R. evertsi* CO1, ITS2 and

12SrDNA sequence chromatograms were of good quality indicated by clear distinct nucleotides peaks on the chromatograms (Appendix 2)

Multiple alignments are shown in appendices 3, 4 and 5.

The percent similarity of *R. evertsi* with reference species in GenBank were 99% for the ITS2 gene 98 to 99% for the CO1 gene and 99% for the 12SrDNA. The percent similarity *R. appendiculatus* to reference sequences in Genbank was 99% for the ITS2 gene and 100% for the 12SrDNA fragment. Table 4.3 summarizes the highest BLAST results for both *R. appendiculatus* and *R. evertsi* sequences.

Table 4.2: Highest BLAST hits and corresponding percent identities of the ITS2, CO1 and 12SrRNA genes from *R. appendiculatus* and *R. evertsi*

Tick species	**ITS2 gene**		**CO1 gene**		**12SrRNA gene**	
	Accession number	% identity	Accession number	% identity	Accession number	% identity
R.e	U97701.1	99	AB934398.1 AF132835.1	99 98	DQ849229.1 DQ901291.1	99 99
R.a	DQ849266.1 DQ849265.1	99 99	-	- -	DQ849209.1 DQ901279.1	100 100

No BLASTN searches were done for CO1 from *R. appendiculatus*, since this marker was not successfully amplified from the tick samples.

The *R. appendiculatus* and *R. evertsi* sequences were deposited to Genbank database under accession numbers MF348067 to MF348134 while *R. evertsi* ITS2 sequences were deposited under accession numbers MF348135 to MF348178.

4.6 Phylogeny of *R. evertsi* found infesting cattle, sheep and wildebeests based on ITS2, 12SrDNA and CO1 genes

Phylogenetic analysis of *R. evertsi* based on the ITS2 gene (Figure 4.5) gave two distinct clades each supported by a bootstrap of 100%. The first clade which further branches into two sub clades showed all the *R. evertsi* ticks obtained from cattle (C), wildebeests (WB) and sheep (Sh). This clade shows close genetic relationship among the *R. evertsi* obtained from the three host species which are equally closely related to *R. evertsi* reference strain MF348170.1. The tree was rooted using reference strains for *R. sanguineus* (AF271283.1) and *R. maculatus* (AF271281.1).

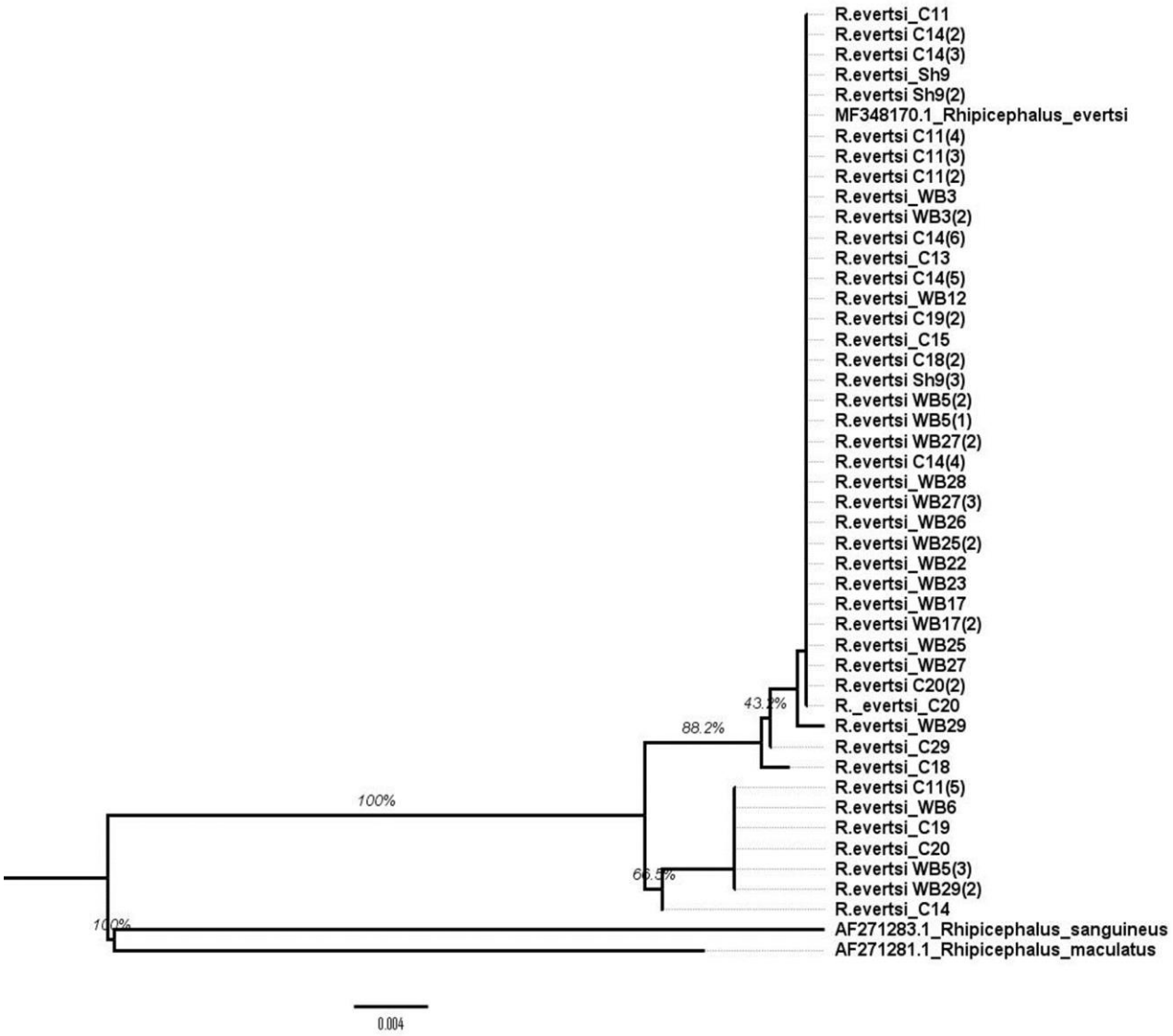

Figure 4.5: Phylogeny of *R. evertsi* based on the partial ITS2 gene. *R. evertsi* from wildebeests are denoted with WB while those from cattle are denoted with C while those from sheep are denoted with Sh. The numbers identify the individual host from which the tick was collected. Bootstrap values greater than 50% are shown as percentages. The nucleotide substitution is 0.005.

Phylogenetic analysis of the CO1 gene (Fig 4.6) gave two distinct clades supported by bootstraps of 100% with the first clade showing relatedness between *R. evertsi* from cattle and wildebeests as well as reference strain AB934398.1. The second clade shows reference strains for *R. sanguineus* (KU214592.1) and *R. turanicus* (KY069271.1) from the NCBI RefSeq database which were used to root the tree.

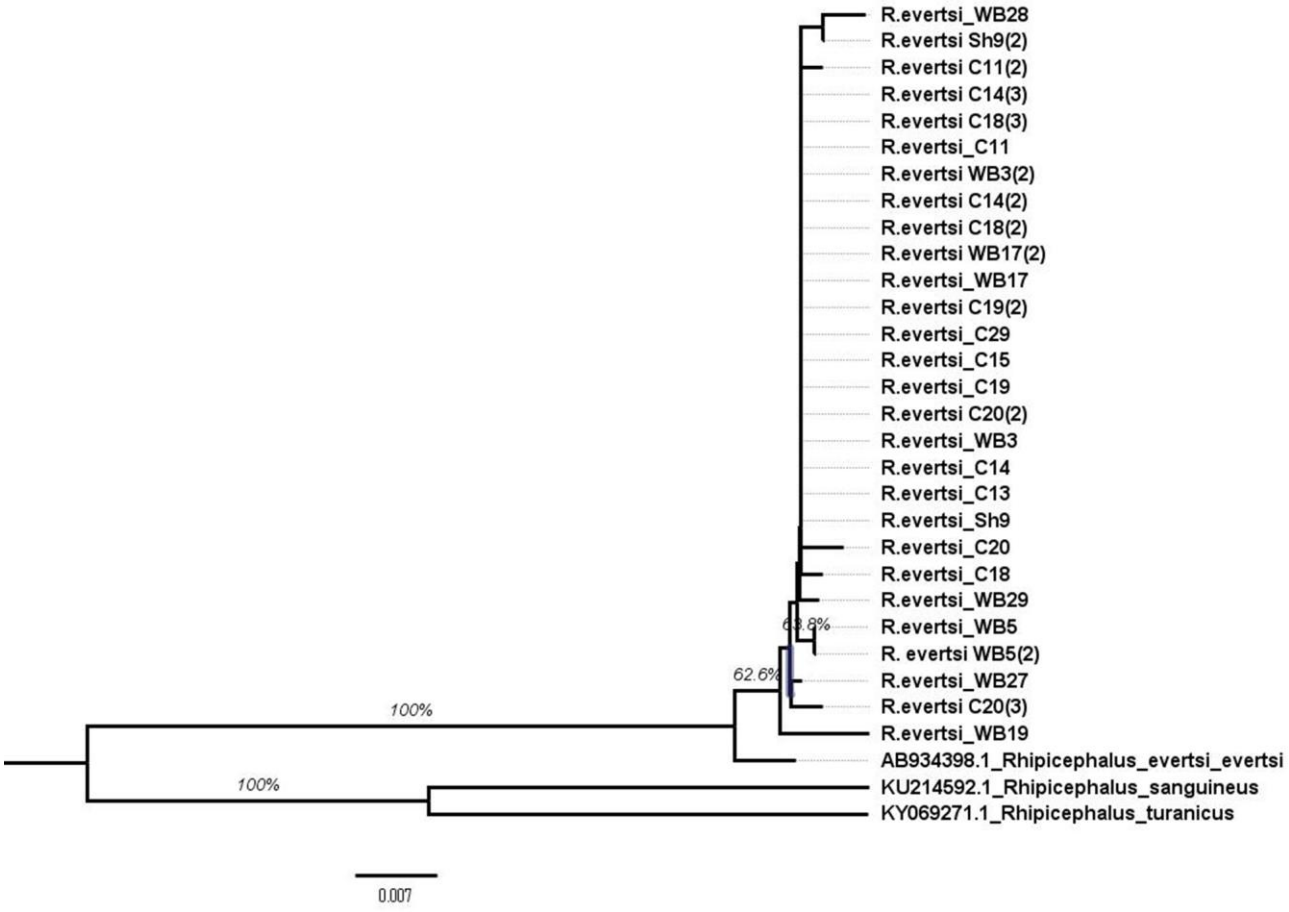

Figure 4.6: Phylogeny of *R. evertsi* based on the 657bp fragment of CO1 gene. *R. evertsi* from wildebeests are denoted with WB while those from cattle are denoted with C. The numbers identify the individual host from which the tick was collected. Bootstrap values greater than 50% are shown as percentages. The nucleotide substitution is 0.009

Phylogenetic analysis of the 336bp partial 12SrRNA gene (Fig 4.7) gave a distinct clade for *R. evertsi* from cattle and wildebeests supported by a bootstrap of 100%. The *R. evertsi* infesting wildebeests and cattle also fell in the same clade as the *R. evertsi* reference strain AF150052.1 deposited in the NCBI RefSeq database as shown in figure 4.8. The tree was rooted using reference strains of *R.*

maculatus (AF150026.1) and *R. sanguineus* (JQ480847.1) which fell on the second clade supported by a bootstrap of 100%.

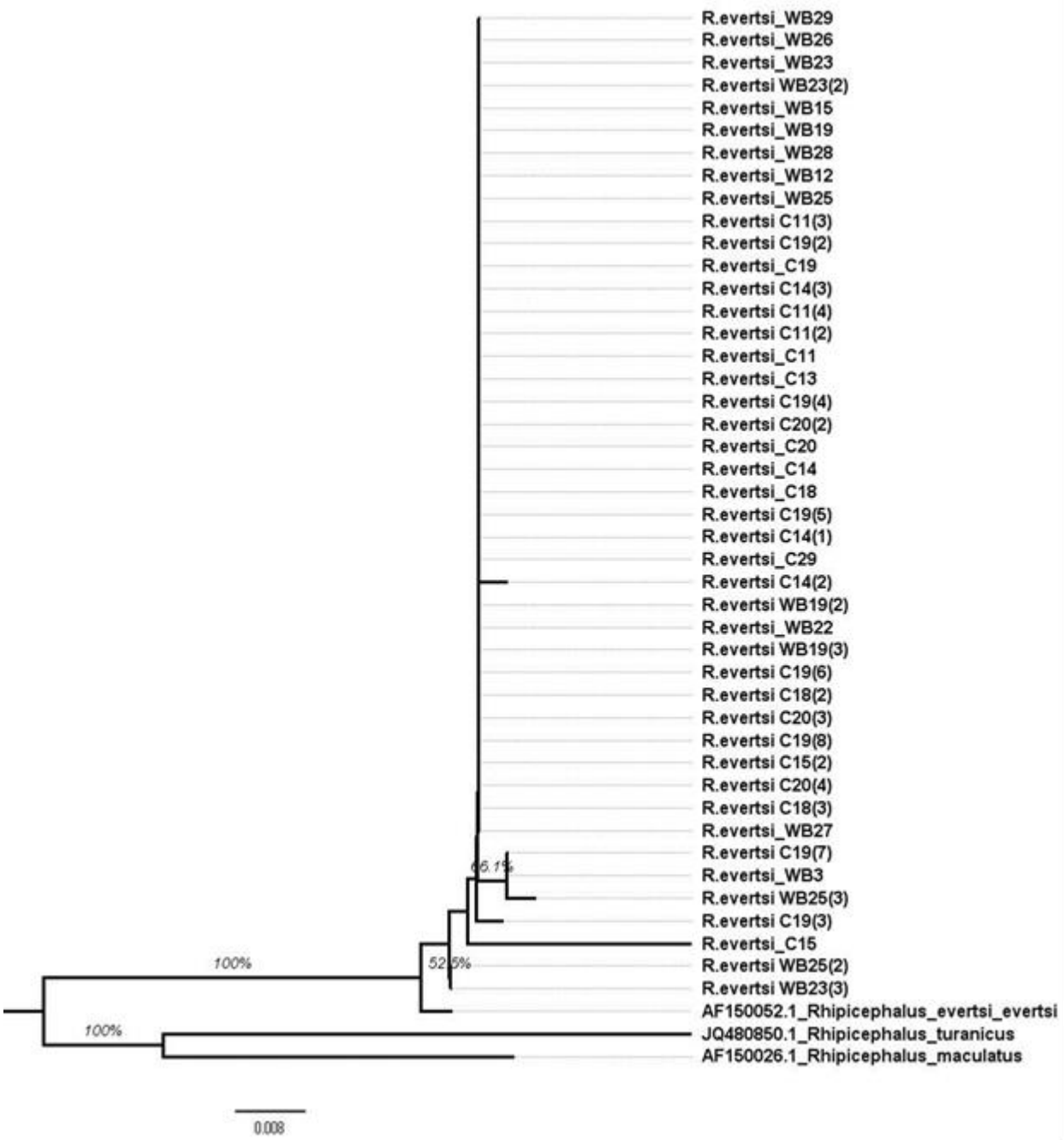

Figure 4.7: Phylogeny of *R. evertsi* based on the 336bp 12srRNA gene. *R. evertsi* from wildebeests are denoted with WB while those from cattle are denoted with C. The numbers identify the individual host from which the tick was collected. Bootstrap values greater than 50% are shown as percentages. The nucleotide substitution is 0.008

4.7 Analysis of genetic distance between *R. evertsi* infesting cattle, wildebeests and sheep

After the pairwise distance analysis, the pairs generally showed low genetic differentiation (Table 4.3). The distance matrices are shown in appendices 6,7 and 8.

Table 4.3: A summary of pairwise genetic distance between R. evertsi from cattle, sheep and wildebeests

***R. evertsi* genetic marker**	**Pairwise distance range**	**Average distance**
CO1	0.000-0.017 (0 - 1.7%)	0.005 (0.5%)
12SrDNA	0.000-0.015 (0 – 1.5%)	0.002 (0.2%)
ITS2	0.000-0.007 (0 – 0.7%)	0.003 (0.3%)

4.8 Tick-borne pathogens detected

Universal 18S primers were used to amplify protozoan pathogens harboured by both *R. appendiculatus* and *R. evertsi*. The *Theileria* species identified from *R. appendiculatus* were *T. parva* and *T. equi* while *T. ovis, T. equi* and two unidentified *Theileria* were detected from *R .evertsi. T. ovis* was amplified from one *R. evertsi* from cattle while *T. equi* was amplified from one *R. evertsi* tick infesting cattle and *R. appendiculatus* from wildebeest. The identity percentages following BLASTN searches for these *Theileria* species ranged from 95 – 100.

Phylogenetic analysis of the *Theileria* species detected from *R. evertsi* and *R. appendiculatus* showed two clading together with both *T. parva* from the NCBI RefSEq database and a strain from South Africa supported by a bootstrap of 100%. A *Theileria* strain isolated from *R. evertsi* infesting cow fell on the same clade with *T. ovis*, while two other *Theileria* isolated from *R. evertsi* infesting cows claded together with *T. equi* from South Africa and Brazil. Figure 4.8 shows the Phylogeny of *Theileria* Species detected from *R. evertsi* and *R. appendiculatus* shown in blue in relation to *Theileria* strains from other regions as well as referenced *Theileria* strains from NCBI RefSeq database.

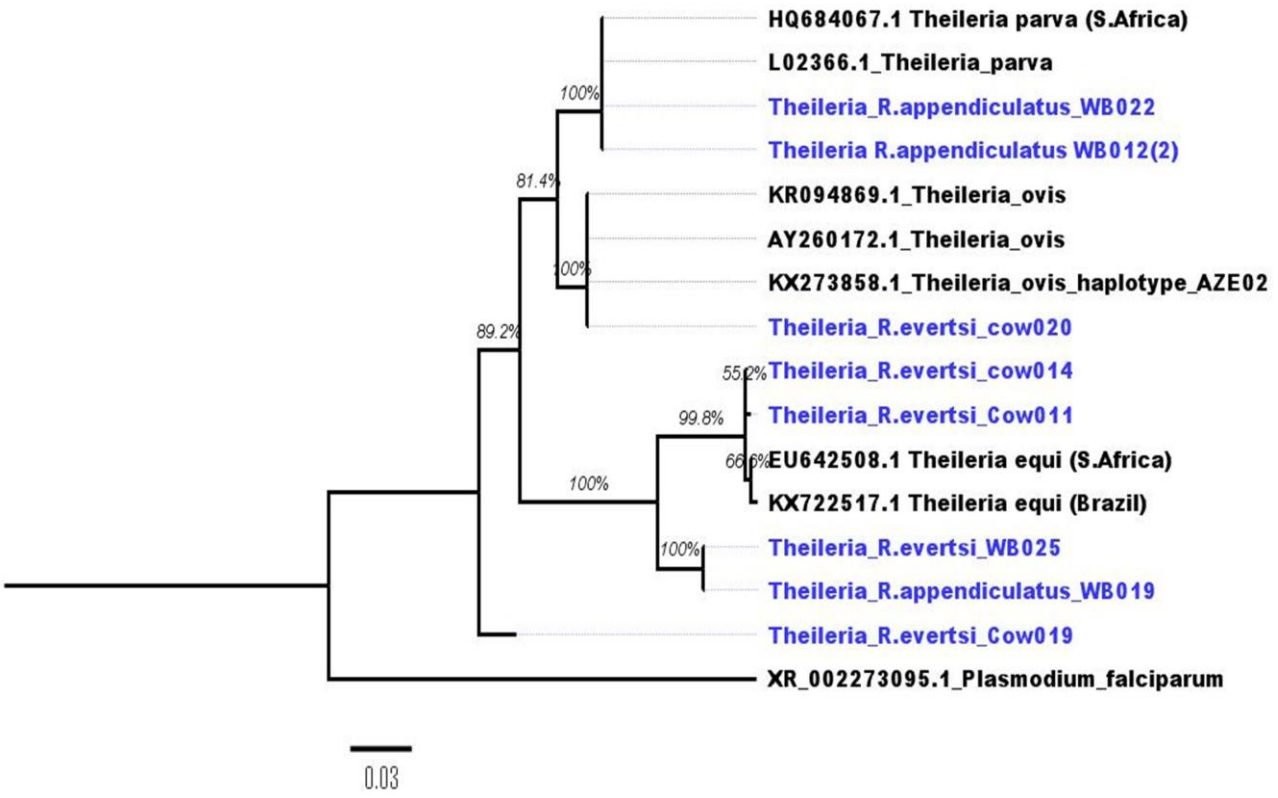

Figure 4.8: Phylogeny of *Theileria* Species detected from *R. evertsi* and *R. appendiculatus.* *Theileria* species shown in blue are from *R. evertsi* and *R. appendiculatus* infesting Cattle (Cow) and wildebeests (WB) with the numbers identifying the specific host from which the ticks were collected.

The highest BLAST hits obtained for *Babesia* pathogens detected pointed to *Babesia microti* and *Babesia canis.* The identity percentages were however low ranging from 84 to 91 suggesting that the identified species are genetic relations of the above two *Babesia* species but not the exact species. Phylogenetic analysis of *Babesia* strains isolated from *R. evertsi* and *R. appendiculatus* against reference *B. microti, B. canis* and *B. equi* deposited in the NCBI RefSeq database showed genetic relatedness but did not conclusively identify them as either *B. microti* or *B.canis* as in Figure 4.9.

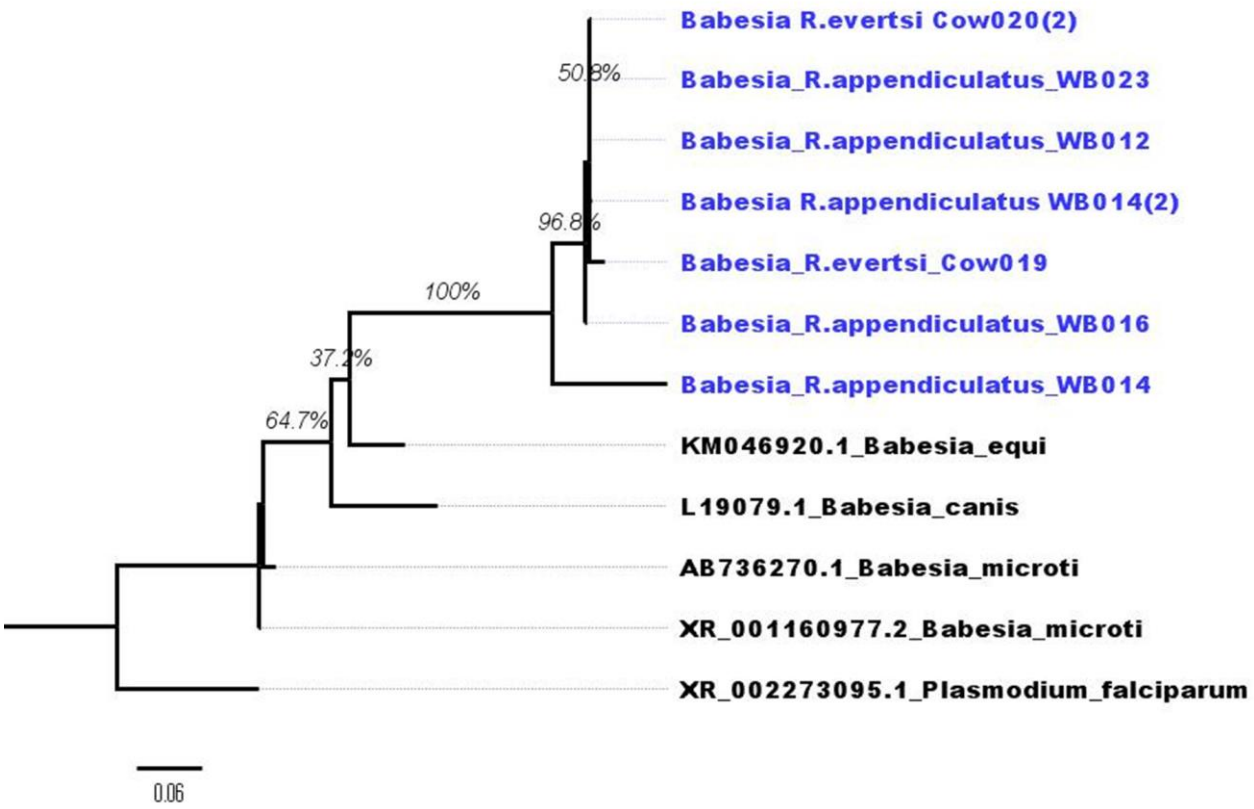

Figure 4.9: Phylogeny of *Babesia* Species detected from *R. evertsi* and *R. appendiculatus. Babesia* species shown in blue are from *R. evertsi* and *R. appendiculatus* infesting Cattle (Cow) and wildebeests (WB) with the numbers identifying the specific host from which the ticks were collected.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

This study assessed the tick species infesting livestock (cattle and sheep) and wildebeests in the Maasai Mara ecosystem. The findings showed Rhipicephaline ticks as the most prevalent ticks infesting livestock (cattle and sheep0 and wildebeests in this ecosystem. There were differences in the distribution of the Rhipicephaline ticks across the host species whereby *R. appendiculatus* which has been documented as the most economically important tick species in Kenya and a principle vector of *T. parva* (Macaluso *et al*., 2003) as well as *T. taurotragi* and *Anaplasma bovis* (Madder *et al*., 2013) was the dominant species infesting wildebeests. While *Rhipicephalus evertsi* a vector of *Theileria* parasite species including *T. ovis, T. equi* as well as *Babesia caballi* and *Anaplasma marginale* (Madder *et al.*, 2013) was found parasitizing cattle, wildebeests and sheep. Other Rhipicephaline ticks were also observed but to a lesser frequency. *R. pulchellus* commonly referred to as zebra tick (Walker *et al.*, 2003) which have been implicated as a probable vectors of Nairobi sheep disease (Asrate and Yalew, 2012) were found only in wildebeest. *Rhipicephalus boophilus decoloratus* was found infesting wildebeests and *Amblyomma variegatum* was the only non-Rhipicephaline tick species that was present only on wildebeests. These findings confirm those of an earlier study carried out in the Maasai Mara National Reserve, which identified among others *Amblyomma variegatum, Rhipicephalus appendiculatus, Rhipicephalus evertsi* and *Rhipicephalus pulchellus* as the common tick species in this ecosystem (Macaluso *et al*, 2003).

In terms of abudance of tick species across the vertebrate hosts i.e. cattle, wildebeest and sheep, findings of this study showed *R. appendiculatus* infesting only wildebeests while *R. evertsi* showed no preference among the three host species infesting cattle, wildebeests as well as sheep showing no host specificity. This observation partly agree with those of a study by Tonetti *et al* (2009) where *R. evertsi* were found in all the sampled wildebeests while *R. appendiculatus* were found only on elands.

Although *R. appendiculatus* has been reported as a known vector of tick-borne diseases in cattle, they were not collected from cattle sampled in this study. The absence of this tick species on cattle could be as a result of acaricide use this conclusion could however not be made due to absence of information on acaricide use or any other tick control method in this study.

This study also aimed to establish the genetic relatedness and phylogenetic relationship of the tick species collected, in a bid to understand whether wildlife and livestock were parasitized by genetically similar or dissimilar ticks on the basis of three molecular markers (ITS2, 12Sr RNA gene and CO1 gene). Phylogenetic analysis of *R. evertsi* based on each of the three markers showed closely related strains infesting cattle, sheep and wildebeests. This study initially used the ITS2 a nuclear marker and CO1 gene a mitochondrial marker for study of tick diversity. It was later noted that CO1 was not amplifying effectively thus 12SrDNA a second mitochondrial marker was also included. Of these three markers, ITS2 had the best amplification success rate followed by 12SrDNA with the CO1 gene giving the lowest amplification for *R. evertsi* and failing completely to amplify from *R. appendiculatus* ticks.

Mitochondrial genes have been shown to have strong advantages as molecular markers since they are easy to amplify due to their high copy numbers per cell and they evolve faster than nuclear genes (Avise, 2012) thus can be used to study variations between closely related individuals within a species. The 650 base pair fragment at the 5'end of the CO1 gene commonly referred to as the 'Folmer region' has been used under the international DNA barcoding initiative because it is flanked by universal primers that have been used for studies on various metazoan taxa (Vrijenhoek, 1994). Although both the ITS2 and CO1 genes have been shown to together provide a powerful tool for study of interspecific variation and phylogenies of closely related species (Cruickshank, 2002), CO1 has been shown to be inadequate in the study of some insect taxa (Francoso and Arias, 2013). The ITS marker has also proved successful in confirming species identities but is limited in environmental barcoding since it is highly conserved across taxa thus can only serve well when used alongside other

markers (Stern *et al*., 2012). Insect data for 12SrDNA on the other hand suggests that although useful, this marker often tends to disappoint in resolving phylogeny due to its rich AT content (Cruickshank, 2002). Seeing that these markers amplify variably and have their associated limitations (Lv *et al*., 2014), there was need to employ a combination of the three in the study. Pairwise distance analysis of *R. evertsi* from cattle, sheep and wildebeests using the three genetic markers showed an overall low genetic variation indicating close genetic relationship among *R. evertsi* infesting the three host species.

BLASTN searches on GenBank with each of the three gene markers ITS2, CO1 and 12S confirmed the morphological identification of the ticks with identity percentages of 100% for the CO1 fragment and 99% and 100% for ITS2 and 12SrRNA genes respectively.

To establish the genetic relatedness and phylogenetic relationship of the tick species collected, phylogenetic analysis based on each of the three markers (CO1, ITS2 and 12SrDNA) was carried out. For each of these markers the analysis showed closely related strains of *R. evertsi* infesting cattle, sheep and wildebeests.

Finally this study assessed the diversity of protozoan pathogens harboured by ticks infesting wildebeests, cattle and sheep in the Maasai Mara wildlife – livestock interface in order to determine whether ticks infesting wildebeests and livestock harbour similar tick-borne pathogens. The protozoans detected were of the genus *Theileria* and *Babesia. T. ovis* with 100% identity to a sheep isolate from Palestine was detected in *R. evertsi* infesting cattle. *T. equi* was detected from *R. appendiculatus* infesting wildebeests as well as from *R. evertsi* infesting cattle. *T. parva* was detected in *R. appendiculatus* infesting wildebeests while two other *Theileria* that could not be identified to species level were isolated one from *R. evertsi* infesting wildebeests which was closely related to a waterbuck isolate KF597074.1. Tick-borne protozoan pathogens are widespread in wild species in Africa. However, the role of many wild species in the epidemiology of intracellular tick-borne

Babesia and *Theileria* species remain poorly defined (Tonetti *et al*., 2009). In this study, *T. ovis* isolated from *R. evertsi* had 100% sequence similarity to an isolate from an ixodid ticks in Palestine (Azmi *et al*., 2016) while another *Theileria* species with 98% identity to *T. ovis* from sheep in Turkey has previously been isolated from dog blood in Nigeria (Kamani *et al*., 2013). Although *T. ovis* was detected from *R. evertsi*, this finding is not sufficient to conclude on its role as a vector of *T. ovis* within the study area as this would call for transmission trials which were beyond the scope of the current study. However in a previous study *T. ovis* has been isolated from *R. bursa* ticks which were suspected to play a role as natural vectors of this *Theileria* species (Aktas *et al*., 2006) which is the causative agent of ovine theileriosis (Zaeemi *et al*., 2011).

T. equi the causative agent of equine theileriosis was detected from *R. evertsi* with 100% similarity to a horse isolate from the middle east (Ketter-Ratzon *et al*., 2017) while the isolate from *R. appendiculatus* was 100% similar to a donkey isolate from Italy (Veronesi *et al*., 2014). *R. appendiculatus* is a three host tick while *R. evertsi* is a two host tick. Thus detection of *T. equi* from these tick species which were collected from bovine hosts in their adult stage could mean they had previously obtained blood meals from equine hosts before moulting into adults.

The current study also detected *Theileria parva* which is the causative agent of East Coast Fever a disease that has had considerable impact on the development of both the beef and dairy industries in Africa (Norval *et al*., 1992). Presence of *T. parva* harboured by *R. appendiculatus* which is its known natural vector poses a disease risk to cattle at the Maasai Mara wildlife – livestock interface.

This study also detected *Babesia* species which showed close relationship to *B. microti* and *B. canis*. These were detected from both *R. evertsi* and *R. appendiculatus.* Human babesiosis is caused by *B. microti* whose major vector has been documented as *Ixodes scapularis* with wild rodent species serving as the reservoir hosts (Hersh and others, 2012). Babesiosis in dogs on the other hand is caused by *B. canis* with the major Rhipicephaline vector being *R. sanguineus*. Although in the present study *Babesia* species amplified from both *R. appendiculatus* and *R. evertsi* had the highest BLAST

similarity to *B. microti* and *B. canis*, the identity percentages were low ranging from 84% to 91% implying these two can only be concluded to be close relations of the *Babesia* species harboured by these ticks but not the exact species.

The current study had its limitations. First, ticks were collected from only one sheep thus this did not give adequate representation of tick infestation on sheep within the Maasai Mara ecosystem. Secondly, *R. appendiculatus* were collected from wildebeests only despite having been earlier established as common ticks of cattle within this ecosystem. This was speculated to be as result of acaricide use which may have eliminated this tick species from cattle. Information on acaricide use including the chemicals used and frequency of application was not collected during sampling thus this calls for acaricide resistance studies on both *R. evertsi* and *R. appendiculatus* from the study area. Finally the findings showed presence of *Theileria* species responsible for bovine, ovine and equine theileriosis from *R. appendiculatus* and *R. evertsi*. It was however not sufficient to make conclusions on the role of these tick species in transmission of these pathogens since no transmission studies were conducted. Both R. appendiculatus and R. evertsi from which the Babesia and Theileria species were detected are multiple host ticks thus these pathogens could have been picked from previous hosts.

5.2 CONCLUSION

Wildebeest, cattle and sheep in the Maasai Mara ecosystem harbour ticks and tick-borne pathogens of similar species and significant genetic relatedness depicting circulation of ticks and pathogens responsible for bovine, equine and ovine theilerioses. This warrants intensified disease surveillance to mitigate transmission and disease outbreaks in livestock.

R. appendiculatus was found to be common ectoparasites of wildebeests while *R. evertsi* was found to commonly infest cattle, sheep and wildebeests at the Maasai Mara livestock Interface. Very few *A. variegatum* and *R. pulchellus* and *R. boophilus decoloratus* occurred. *R. appendiculatus* ticks from wildebeests were found to be infected with *Theileria parva, T. equi* and *T. ovis*. *Theileria ovis and T. equi* also occurred in *R. evertsi* ticks infesting cattle and wildebeests. They are probably shared across

the two bovine species. There is limited genetic diversity between species of ticks collected from both wildebeests and cattle.

5.3 RECOMMENDATIONS

This study recommends a more detailed sampling of wildebeests in the entire Interface across seasons to identify other tick species whose abundance can be influenced by climatic conditions.

There is a need for collection of questing ticks during sampling in order to show all the genera of ticks occurring in pastures and have potential to infest both livestock and wildlife sharing this ecological habitat.

It would be equally important to investigate further the species identity of *Babesia* and *Theileria* species identified in this study and whether these parasites are also circulating in the Bovine hosts.

There is also need for studies on the acaricides in use within and around the Maasai Mara wildlife-livestock interface followed by investigations on acaricide resistance and acaricide susceptibility especially in *R. evertsi* and *R. appendiculatus*.

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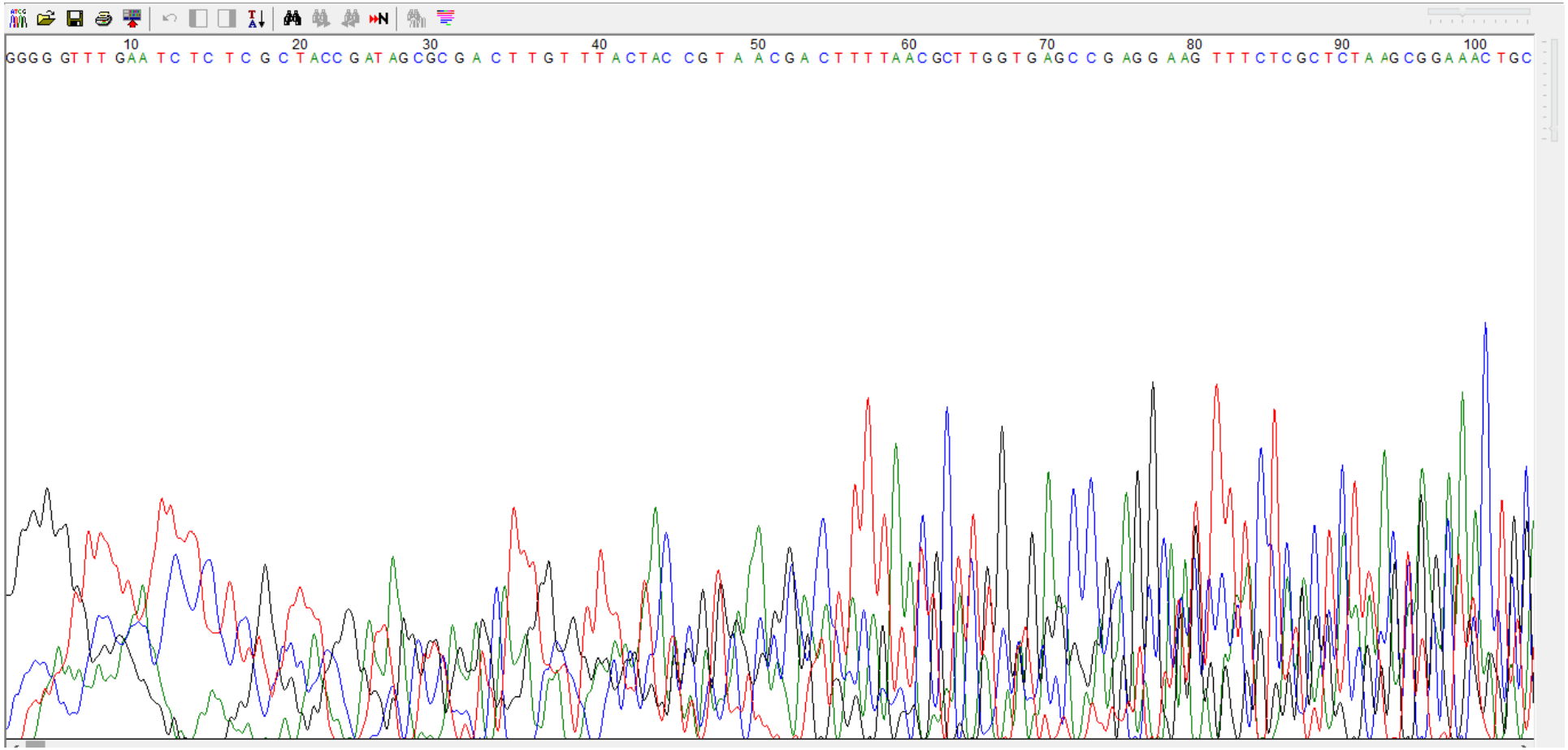
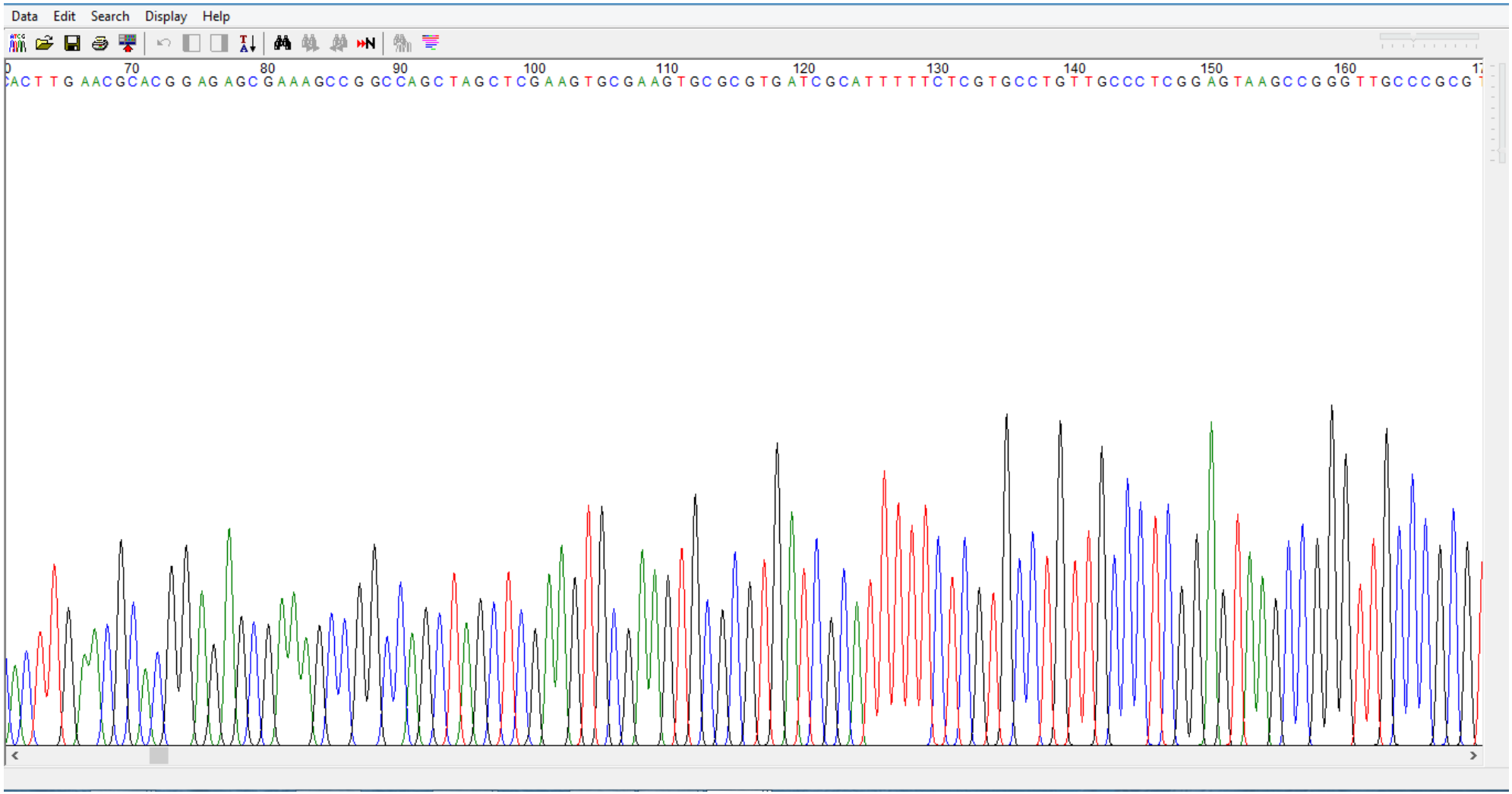
APPENDICES



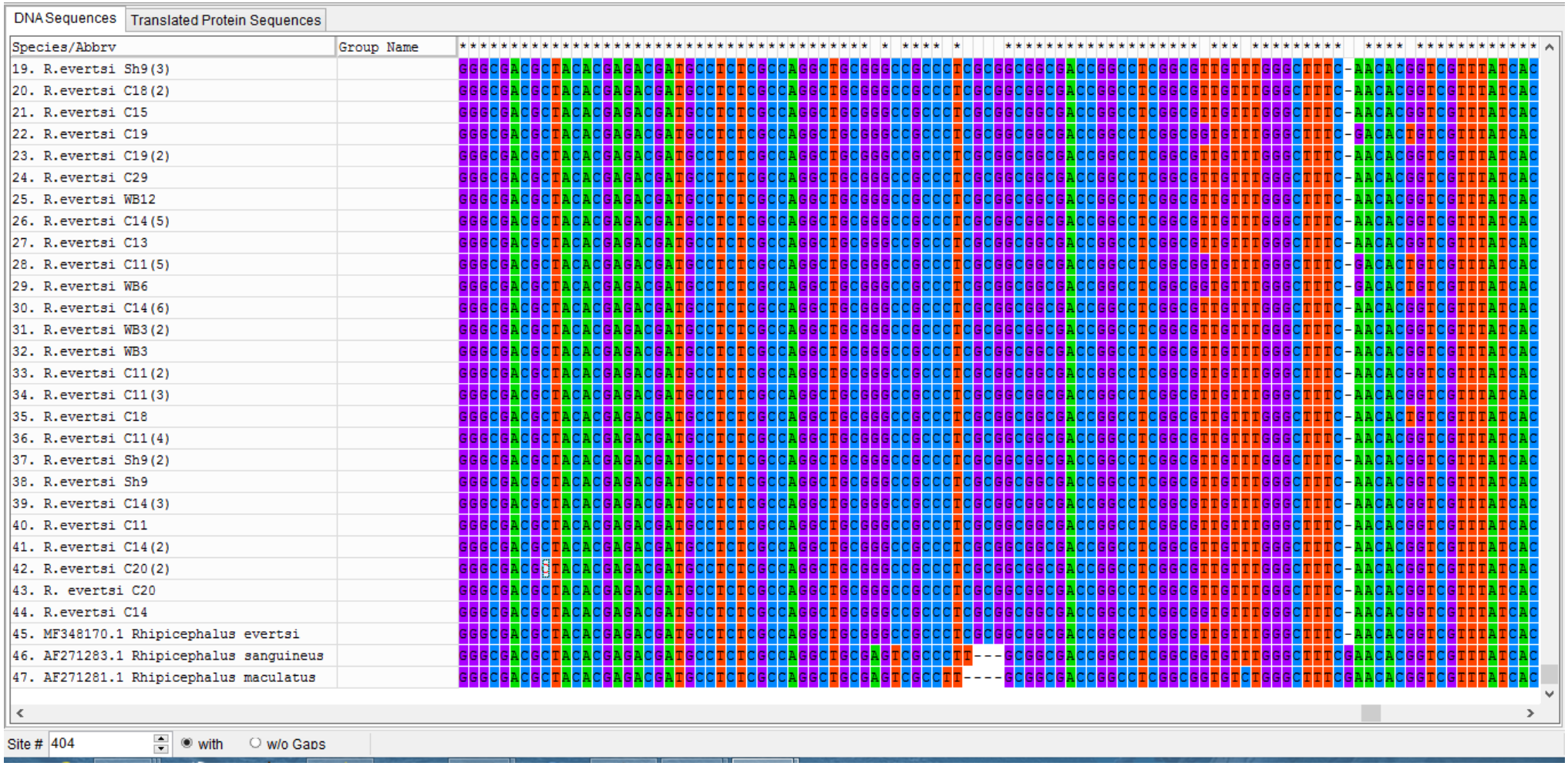
Appendix 1: Low quality 5' end of an ITS2 sequence chromatogram showing unclear nucleotide peaks



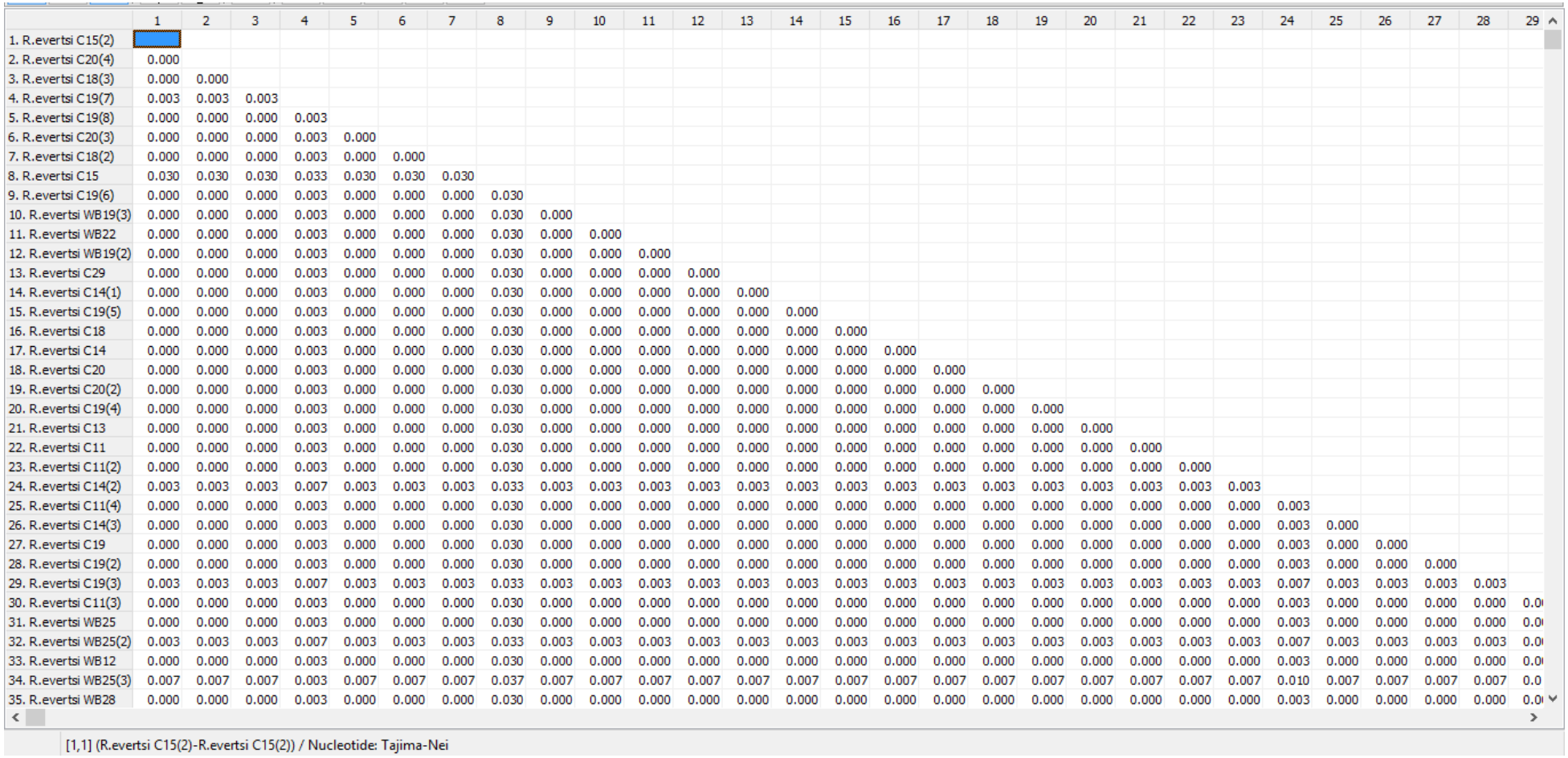
Appendix 2: High quality ITS2 sequence chromatogram showing clear nucleotide peaks

Appendix 3: A section of the multiple alignment of the 12SrDNA fragment

Appendix 4: A section of the multiple alignment of the CO1 fragment



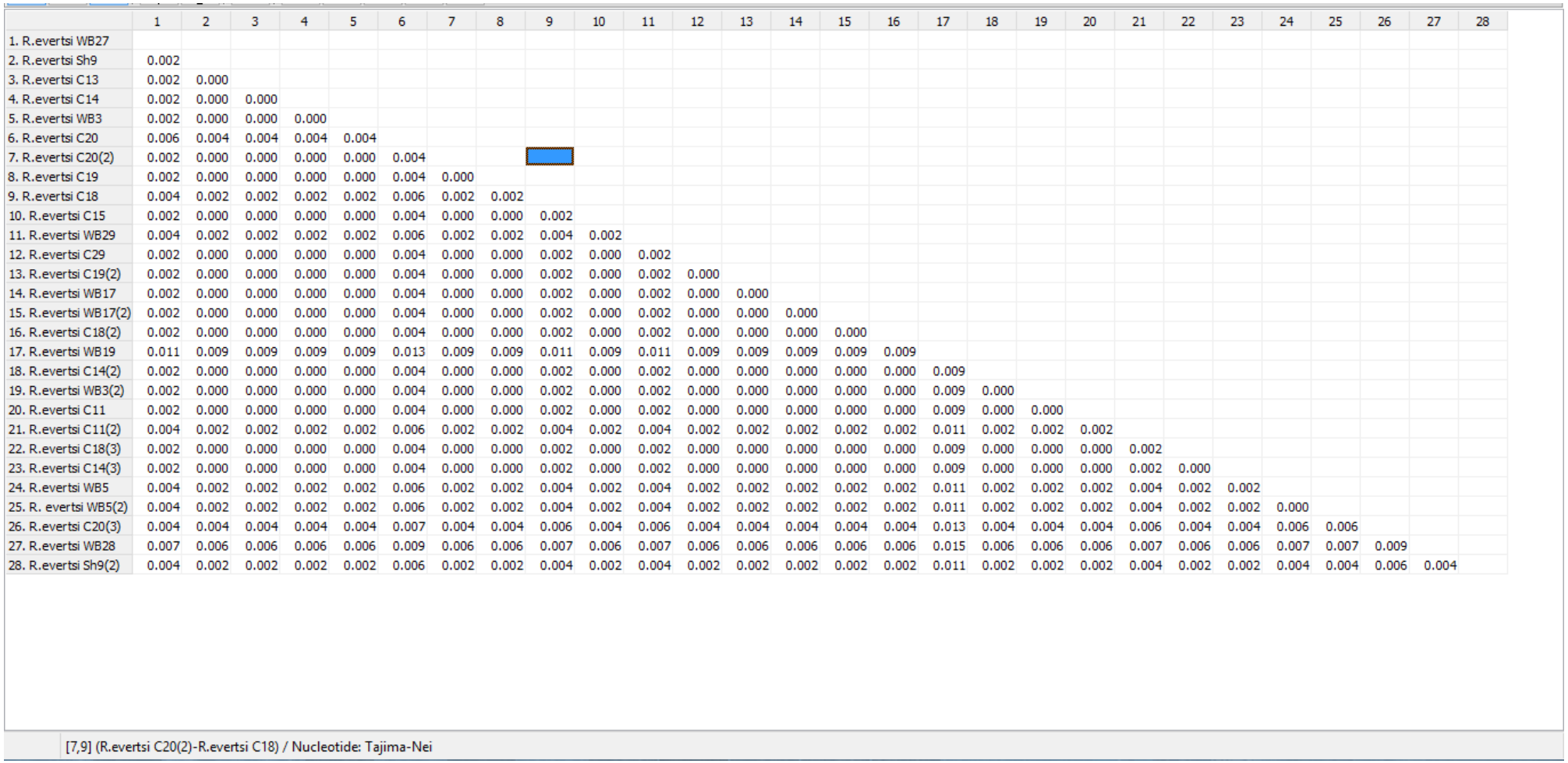
Appendix 5: A section of the multiple alignment of the ITS2 fragment



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1. R.evertsi C15(2)																													
2. R.evertsi C20(4)	0.000																												
3. R.evertsi C18(3)	0.000	0.000																											
4. R.evertsi C19(7)	0.003	0.003	0.003																										
5. R.evertsi C19(8)	0.000	0.000	0.000	0.003																									
6. R.evertsi C20(3)	0.000	0.000	0.000	0.003	0.000																								
7. R.evertsi C18(2)	0.000	0.000	0.000	0.003	0.000	0.000																							
8. R.evertsi C15	0.030	0.030	0.030	0.033	0.030	0.030	0.030																						
9. R.evertsi C19(6)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030																					
10. R.evertsi WB19(3)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000																				
11. R.evertsi WB22	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000																			
12. R.evertsi WB19(2)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000																		
13. R.evertsi C29	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000																	
14. R.evertsi C14(1)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000																
15. R.evertsi C19(5)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000															
16. R.evertsi C18	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000														
17. R.evertsi C14	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000													
18. R.evertsi C20	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000												
19. R.evertsi C20(2)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000											
20. R.evertsi C19(4)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
21. R.evertsi C13	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000									
22. R.evertsi C11	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000								
23. R.evertsi C11(2)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000							
24. R.evertsi C14(2)	0.003	0.003	0.003	0.007	0.003	0.003	0.003	0.033	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003						
25. R.evertsi C11(4)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003					
26. R.evertsi C14(3)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000				
27. R.evertsi C19	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000			
28. R.evertsi C19(2)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000		
29. R.evertsi C19(3)	0.003	0.003	0.003	0.007	0.003	0.003	0.003	0.033	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.007	0.003	0.003	0.003	0.003	
30. R.evertsi C11(3)	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.0
31. R.evertsi WB25	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.0
32. R.evertsi WB25(2)	0.003	0.003	0.003	0.007	0.003	0.003	0.003	0.033	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.007	0.003	0.003	0.003	0.003	0.0
33. R.evertsi WB12	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.0
34. R.evertsi WB25(3)	0.007	0.007	0.007	0.003	0.007	0.007	0.007	0.037	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.010	0.007	0.007	0.007	0.007	0.0
35. R.evertsi WB28	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.0

[1,1] (R.evertsi C15(2)-R.evertsi C15(2)) / Nucleotide: Tajima-Nei

Appendix 6 Estimates of Evolutionary Divergence between 12SrDNA Sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Tajima-Nei model involving 44 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 309 positions in the final dataset. Evolutionary analyses were conducted in MEGA6



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1. R.evertsi WB27																												
2. R.evertsi Sh9	0.002																											
3. R.evertsi C13	0.002	0.000																										
4. R.evertsi C14	0.002	0.000	0.000																									
5. R.evertsi WB3	0.002	0.000	0.000	0.000																								
6. R.evertsi C20	0.006	0.004	0.004	0.004	0.004																							
7. R.evertsi C20(2)	0.002	0.000	0.000	0.000	0.000	0.004																						
8. R.evertsi C19	0.002	0.000	0.000	0.000	0.000	0.004	0.000																					
9. R.evertsi C18	0.004	0.002	0.002	0.002	0.002	0.006	0.002	0.002																				
10. R.evertsi C15	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002																			
11. R.evertsi WB29	0.004	0.002	0.002	0.002	0.002	0.006	0.002	0.002	0.004	0.002																		
12. R.evertsi C29	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002																	
13. R.evertsi C19(2)	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002	0.000																
14. R.evertsi WB17	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002	0.000	0.000															
15. R.evertsi WB17(2)	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000														
16. R.evertsi C18(2)	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000													
17. R.evertsi WB19	0.011	0.009	0.009	0.009	0.009	0.013	0.009	0.009	0.011	0.009	0.011	0.009	0.009	0.009	0.009	0.009												
18. R.evertsi C14(2)	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.009											
19. R.evertsi WB3(2)	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.009	0.000										
20. R.evertsi C11	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000									
21. R.evertsi C11(2)	0.004	0.002	0.002	0.002	0.002	0.006	0.002	0.002	0.004	0.002	0.004	0.002	0.002	0.002	0.002	0.002	0.011	0.002	0.002	0.002								
22. R.evertsi C18(3)	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.002							
23. R.evertsi C14(3)	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.002	0.000						
24. R.evertsi WB5	0.004	0.002	0.002	0.002	0.002	0.006	0.002	0.002	0.004	0.002	0.004	0.002	0.002	0.002	0.002	0.002	0.011	0.002	0.002	0.002	0.004	0.002	0.002					
25. R. evertsi WB5(2)	0.004	0.002	0.002	0.002	0.002	0.006	0.002	0.002	0.004	0.002	0.004	0.002	0.002	0.002	0.002	0.002	0.011	0.002	0.002	0.002	0.004	0.002	0.002	0.000				
26. R.evertsi C20(3)	0.004	0.004	0.004	0.004	0.004	0.007	0.004	0.004	0.006	0.004	0.006	0.004	0.004	0.004	0.004	0.004	0.013	0.004	0.004	0.004	0.006	0.004	0.004	0.006	0.006			
27. R.evertsi WB28	0.007	0.006	0.006	0.006	0.006	0.009	0.006	0.006	0.007	0.006	0.007	0.006	0.006	0.006	0.006	0.006	0.015	0.006	0.006	0.006	0.007	0.006	0.006	0.007	0.007	0.009		
28. R.evertsi Sh9(2)	0.004	0.002	0.002	0.002	0.002	0.006	0.002	0.002	0.004	0.002	0.004	0.002	0.002	0.002	0.002	0.002	0.011	0.002	0.002	0.002	0.004	0.002	0.002	0.004	0.004	0.006	0.004	

[7,9] (R.evertsi C20(2)-R.evertsi C18) / Nucleotide: Tajima-Nei

Appendix 7: Estimates of Evolutionary Divergence between CO1 Sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Tajima-Nei model. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 542 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1. R.evertsi WB17																													
2. R.evertsi WB17(2)	0.000																												
3. R.evertsi WB25	0.000	0.000																											
4. R.evertsi WB27	0.000	0.000	0.000																										
5. R.evertsi WB29	0.002	0.002	0.002	0.002																									
6. R.evertsi WB29(2)	0.017	0.017	0.017	0.017	0.015																								
7. R.evertsi WB23	0.000	0.000	0.000	0.000	0.002	0.017																							
8. R.evertsi WB22	0.000	0.000	0.000	0.000	0.002	0.017	0.000																						
9. R.evertsi WB25(2)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000																					
10. R.evertsi WB26	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000																				
11. R.evertsi WB27(3)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000																			
12. R.evertsi WB28	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000																		
13. R.evertsi C14(4)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000																	
14. R.evertsi WB27(2)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000																
15. R.evertsi WB5(1)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000															
16. R.evertsi WB5(2)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000														
17. R.evertsi WB5(3)	0.017	0.017	0.017	0.017	0.015	0.000	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017													
18. R.evertsi C20	0.017	0.017	0.017	0.017	0.015	0.000	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.000												
19. R.evertsi Sh9(3)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017											
20. R.evertsi C18(2)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000										
21. R.evertsi C15	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000									
22. R.evertsi C19	0.017	0.017	0.017	0.017	0.015	0.000	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.000	0.000	0.017	0.017	0.017								
23. R.evertsi C19(2)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000	0.000	0.017							
24. R.evertsi C29	0.002	0.002	0.002	0.002	0.004	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.015	0.015	0.002	0.002	0.002	0.015	0.002						
25. R.evertsi WB12	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000	0.000	0.017	0.000	0.002					
26. R.evertsi C14(5)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000	0.000	0.017	0.000	0.002	0.000				
27. R.evertsi C13	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000	0.000	0.017	0.000	0.002	0.000	0.000			
28. R.evertsi C11(5)	0.017	0.017	0.017	0.017	0.015	0.000	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.000	0.000	0.017	0.017	0.017	0.000	0.017	0.015	0.017	0.017	0.017		
29. R.evertsi WB6	0.017	0.017	0.017	0.017	0.015	0.000	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.000	0.000	0.017	0.017	0.017	0.000	0.017	0.015	0.017	0.017	0.017	0.000	
30. R.evertsi C14(6)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000	0.000	0.017	0.000	0.002	0.000	0.000	0.000	0.017	0.0
31. R.evertsi WB3(2)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000	0.000	0.017	0.000	0.002	0.000	0.000	0.000	0.017	0.0
32. R.evertsi WB3	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000	0.000	0.017	0.000	0.002	0.000	0.000	0.000	0.017	0.0
33. R.evertsi C11(2)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000	0.000	0.017	0.000	0.002	0.000	0.000	0.000	0.017	0.0
34. R.evertsi C11(3)	0.000	0.000	0.000	0.000	0.002	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.000	0.000	0.017	0.000	0.002	0.000	0.000	0.000	0.017	0.0
35. R.evertsi C18	0.004	0.004	0.004	0.004	0.006	0.013	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.013	0.013	0.004	0.004	0.004	0.013	0.004	0.002	0.004	0.004	0.004	0.013	0.0

[1,1] (R.evertsi WB17-R.evertsi WB17) / Nucleotide: Tajima-Nei

Appendix 8: Estimates of Evolutionary Divergence between ITS2 sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Tajima-Nei model. The analysis involved 44 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 535 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.