PHYLOGENY AND EVOLUTION OF *THEILERIA* AND *BABESIA* PARASITES IN SELECTED WILD HERBIVORES OF KENYA

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Phylogeny and Evolution of *Theileria* and *Babesia* Parasites in Selected Wild Herbivores of Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This work is dedicated to my beloved parents Mr. Godfrey, Mrs. Naomi and my only beloved sister Caroline for their tireless support throughout my academic journey.

"I can do everything through Christ who gives me strength."

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ABBREVIATIONS AND ACRONYMS

DNA	Deoxyribonucleic Acid
DnaSP	DNA Sequence Polymorphism
EDTA	Ethylenediaminetetraacetic Acid
ECF	East Cost Fever
ExPASy	Expert Protein Analysis
IFAT	Indirect Fluorescent Antibody Test
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KWS	Kenya Wildlife Service
MCF	Malignant Catarrhal Fever
MEGA	Molecular Evolutionary Genetics Analysis
MSA	Multiple Sequence Analysis
MUSCLE	Multiple Sequence Comparison by Log- Expectation
PCR	Polymerase Chain Reaction
18S rRNA	18 Small Subunit ribosomal, RNA
RBC	Red Blood Cell
RNA	Ribonucleic Acid
USA	United States of America

ABSTRACT

Factors that influence transmission dynamics, pathogenicity, and virulence are complex and diverse yet important in understanding the epidemiology of many diseases. *Theileria* and *Babesia* are genera of tick-borne protozoan that are globally spread and infect nearly all warm-blooded animals in which they cause either latent infection or symptomatic and lethal disease. Wild animals are the considered hosts of many species of *Theileria* and *Babesia* and yet their diversity and prevalence is not explicit in the rich wild mammalian host diversity in Kenya. Morever, the close interactions between wildlife and livestock, wide range of grazing, migrations as well as geographical isolation of populations are some of the factors that may cause a parasite to adapt and subsequently evolve. This study identified *Theileria* and *Babesia* species, their prevalence in elephants, wildebeest, impalas and reedbucks and established their phylogenetic relationship. Genomic DNA was extracted from 128 blood samples obtained from elephants, reedbucks, impalas and wildebeest from Tsavo, Narok and Laikipia ecosystem. The genomic DNA was then amplified by an optimized nested amplification method of *Theileria* and *Babesia* 18S rRNA gene. Electrophoresis was then carried out on the secondary PCR products on a 2% agarose gel and the bands viewed under a UV light. The band size for 18S rRNA was found to range from 400bp to 500bp. Amplicons were then sequenced. *Theileria* and *Babesia* were identified in elephants, wildebeest, impalas and reedbucks in Kenya. The prevalence of *Babesia* was high in elephants, while that of *Theileria* was high in reedbucks, impalas and wildebeest. Two haplotypes of *Babesia* were identified in African elephant while nine *Theileria* haplotypes were identified from elephants, wildebeest, impalas and reedbucks of Kenya. The molecular phylogeny revealed presence of potentially novel species of *Theileria* and *Babesia* in elephants, wildebeest, impalas and reedbucks in Kenya. It also revealed the diversity of *Theileria* and *Babesia*

species in the wild herbivores of Kenya. This is the first molecular study in Kenya undertaken to identify *Theileria* and *Babesia* in elephants, wildebeest, impalas and reedbuck.

CHAPTER ONE

INTRODUCTION

1.1 Background

Parasites play important roles in the dynamics of host populations and are increasing conservation concern following their negative impacts on wild and endangered populations (Daszak *et al.,* 2000). Nevertheless, many parasites of economic, medical and veterinary importance have their origin in wild animals, which reiterates the important but often neglected transmission pathway between wild reservoirs and the human-livestock host pool. *Theileria* and *Babesia* parasites are one of the most ubiquitous and widespread blood parasites in the world based on numbers and distribution of species in animals, second to the *trypanosomes*. Their geographic distribution is worldwide, but little is known about the prevalence of *Theileria* and *Babesia* in malaria-endemic countries, where misidentification as *Plasmodium* probably occurs("CDC - DPDx - Babesiosis" 2016).

They infect a wide range of wild and domestic ungulates worldwide, causing diseases that are of great economic importance because of their impact on livestock health and production (Jongejan & Uilenberg, 2004; G. Uilenberg, 1995; Mukhebi, *et al*., 1992).They generally have two classes of hosts, an invertebrate and a vertebrate host (Homer, Aguilar- Delfin, Telford *et al*., 2000).They are transmitted among hosts by a diverse species of Ixodid tick vectors (Bishop *et al*., 2004). Many wildlife species harbor a great diversity of *Theileria* and *Babesia* species and infected individuals are usually asymptomatic carriers equilibrium is disturbed and infections progresses to clinical stage (Githaka *et al*., 2013; Mans *et al*., 2011; Young *et al*., 1978). The high prevalence of and the prolonged carrier state of *Theileria* and *Babesia* infections in many wildlife populations studied so far suggests that

wildlife species are reservoir hosts for *Theileria* and *Babesia* species infecting domestic ungulates (Burridge, 1975; Githaka *et al*., 2013; Mans *et al*., 2011; Otranto *et al*.,2015; Young *et al*., 1978;).

The role of wildlife as reservoir hosts has stimulated research on the molecular characterization, identification and epidemiology of *Theileria* and *Babesia* infecting wildlife. Most of these studies however, have focused on the role of buffalo as a reservoir for *Theileria parva lawrenci* which causes lethal corridor disease in cattle and *Babesia microti* that causes babesiosis disease in humans. Previous studies concentrate on hosts in one location. More recently, studies on wildlife *Theileria* and *Babesia* have expanded to include a number of wildlife host species such as grants gazelle (Hooge *et al*., 2015), waterbuck (Githaka *et al*., 2014), giraffe (Oosthuizen *et al*., 2009) zebras (Bhoora *et al*., 2010; Hawkins *et al*., 2015), sable antelopes (Oosthuizen *et al*., 2008) and rhinos (Otiende *et al*., 2014).However, little is known regarding. *Theileria* and *Babesia* species infecting wildebeest, impalas, reedbucks and elephants which are particularly important as conduits of livestock diseases because of their wide range of free grazing, migrations and interactions with livestock.

Elephants are on the move at least 20 out of every 24 hours and occupy a broad range of habitat types (Poole & Granli, 2009). This can result to hosting different types of ticks and thus diversity in the parasite they carry. Elephants are found mostly in community lands thus outside the national parks and reserves. They end up sharing grazing pastures with livestock thus an opportunity for parasite host switching and exposure to different tick vectors (Kock, 2005). The wildebeest are known to migrate long distances annually for example in the Serengeti-Mara ecosystem in East Africa and in the Kalahari (Holdo 2009; Williamson *et al*., 1988). Long distance migration is suggested to play significant role in the spread of parasites (Cornell *et al*., 2003). Previous study by Morgan 2007 demonstrated how seasonal

movement of Saiga antelope (*Saiga tatarica tatarica*) influenced the spread of helminths within sheep populations along their migratory route (Morgan *et al*., 2007). Historically, the migration of wildebeests in East Africa has been associated with the spread of Malignant Catarrhal Fever (MCF), which is a deadly viral disease in cattle (Cleaveland *et al*., 2001). Impalas and reedbucks share grazing lands and water pools that are sometimes used by livestock that encroach wildlife protected areas. This can result to spilling over of wildlife pathogen to livestock. For instance along the western boundary of the Kruger National Park Impalas were found to transmit foot and mouth disease to cattle (Jori *et al*., 2009). This can result to parasite host switching as well as livestock promoting the transmission of diseases affecting wildlife. These wild herbivores are free wide range grazers the occurrence of disease agents in free-ranging wildlife has been suspected to present a risk to the health of humans and domestic animals (Fischer *et al*., 2003).

Although wildlife harbors a diversity of parasites, usually in latent form, they still exert deleterious effects on their hosts and influence their host populations directly or indirectly (Hudson *et al*., 1998; Albon *et al*., 2002). Like many parasitic diseases of wildlife, *Theileria* and *Babesia* infections are often latent but can progress to fatal disease under conditions of either nutritional or translocation stress (Craig *et al*., 2006; Grobler, 1981; Gulland, 1992; Nijhof *et al*., 2005; Wilson *et al*., 1974). The asymptomatic infection by parasites in wild animals is thought to be due to host-parasite co-evolution. However, with increased modification of environment and subsequent changes in host-parasite ecology, generalist parasites; those that exploit diverse host taxa, are likely to acquire new hosts, exploit wider host range and even switch hosts, an opportunity for adaptation and potential evolution of new species (Chauvin *et al*., 2009). Convergence of these factors raises questions on the genetics, taxonomy and evolution of parasite communities that exploit diverse tick and animal host communities. Several

cases of mortalities caused by *Theileria* and *Babesia* infection have been documented in several species of antelopes such as roan antelope, sable antelope, eland, greater kudu, gray duiker, tsessebe, black rhinoceros and plains zebra (Govender *et al.*, 2011; Lampen *et al.*, 2009; Nijhof *et al.*, 2003). Infections by *Theileria* and *Babesia* have also been implicated as a cause for high calf mortality responsible for the decline in populations of roan and sable antelopes in South Africa (Wilson *et al.*, 1974; Wilson & Hirst, 1977).

Phylogeny and evolution of *Theileria* and *Babesia* has led to identification of various species of *Theileria* and *Babesia* from domestic and wildlife hosts (Hooge *et al.*, 2015). However most of these studies focus on identification and do not go further explaining the genetic divergence of identified *Theileria and Babesia* from known species that they closely related with. These tick-borne parasites tend to be named by the hosts they were isolated from for example *Babesia leo* from lions, *Babesia bicornis* from black rhinos, or by morphological methods and certain general phenotypic characteristics that enable many parasites to be unequivocally assigned to a particular genus. However, with advanced molecular and phylogenetic studies, the identity, taxonomy, host-range and phylogeny of *Theileria* and *Babesia* is subject to review. Molecular techniques such as PCR have been introduced and have found gross inconsistencies in assigning of parasite names because of their high sensitivity and specificity.

1.2 Statement of the Problem

Kenya has a rich diversity of wild mammals but their natural habitat is anthropogenically being altered. For instance, populations of wild animals are isolated in fragmented populations that freely interact with domestic animals thereby creating an opportunity for generalist parasites to switch hosts. Several factors such as host switching, host-parasite migration and use of

antimicrobials have the potential to hasten the evolutionary rate. Although wildlife has been known to be reservoirs of *Theileria* and *Babesia* species, there is paucity of literature on specific *Theileria* and *Babesia* species harbored by wild mammals in Kenya. Blood smear staining has established *Theileria* and *Babesia* infection in samples from varied wildlife in Kenyan national parks. The infecting species and prevalence has not been established. Consequently, no studies on phylogenetic relationship have been carried out to establish the evolutionary trends arising from selection pressures.

1.3 Justification

Through molecular tools, novel species of *Theileria and Babesia* are increasingly being identified especially in wild animals. However, most of the studies on *Theileria* and *Babesia* are based on single hosts confined in one habitat. This study detected *Theileria* and *Babesia* in selected wild herbivores that inhabit different habitats in Kenya, to have a clear understanding of the divergence of *Theileria* and *Babesia* in Kenya**.** Understanding divergence of *Theileria* and *Babesia* will aid in successful vaccine development of these protozoal diseases thus guide in human and livestock vaccine research.

1.4 Hypothesis

There exists diverse species of *Theileria* and *Babesia* infecting wild herbivores in Kenya.

1.5 Objectives

1.5.1 General Objective

To identify *Theileria* and *Babesia* species, determine their prevalence and their Phylogenetic relationships in populations of African elephants, wildebeests, impalas and Reedbuck in Kenya.

1.5.2 Specific Objectives

i. To identify and determine prevalence of *Theileria* and *Babesia* species in populations of African elephants, wildebeest, impalas and reedbucks in Kenya.

ii. To determine the phylogenetic relationship of the *Theileria* and *Babesia* species infecting African elephants, wildebeest, impalas and reedbucks in Kenya.

iii. To determine the presence of Selection Pressures on *Theileria* and *Babesia* species harbored by the African elephant subpopulations, impalas, wildebeests and reedbucks.

1.6 Research Questions

i. Which species of *Theileria* and *Babesia* are present and prevalent in selected elephants, impalas, wildebeests and reedbucks of Kenya?

ii. Is there divergence in *Theileria* and *Babesia* species infecting Kenyan wildlife?

iii. Are there the selection pressures to *Theileria* and *Babesia* species in different subpopulations of selected wild herbivores?

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of *Theileria* and *Babesia*

2.1.1 *Babesia*

Babesia is a tick borne protozoan blood parasite that causes a hemolytic disease referred to as babesiosis. Victor Babes was the first one to identify *Babesia* in 1988. *Babesia* belongs to Kingdom: Chromalveolata, Phylum: Apicomplexa, Class: Aconoidasida, Order: piroplasmida, Family: Babesiidae and Genus *Babesia*. This genus comprises of over 100 species (Chauvin *et al*., 2009).

2.1.2 *Theileria*

Theileria is an obligate protozoan parasite transmitted by tick (Mans *et al*., 2015). *Theileria* belongs to Kingdom: Chromalveolata, Phylum: Apicomplexa, Class: Aconoidasida, Order: piroplasmida, Family: Theileriidae and Genus *Theileria*. *Theileria* comprises of more than 185 different species (Chansiri *et al*., 1999).

2.2 Theileriosis and *Babesiosis*

2.2.1 Babesiosis

Babesiosis is caused by *Babesia.* Babesiosis is an emerging zoonosis with important public health implications, as the incidence of the disease has risen dramatically over the past decade (Teal *et al*., 2012). Other names of this disease include: Prioplasmosis, Red water and Cattle tick fever. The disease varies from subclinical infection to persistent fever, haemolytic anaemia, and lethargy in vertebrate hosts (Ibrahim *et al*., 2013). The symptoms vary depending on the host they include fever, lack of appetite, apathy, haemoglobinuria, bilirubinuria, polichromasia, progressive haemolytic

anaemia, spleeno- and hepato-megally, jaundice, vomiting and death. It mainly affects wide range of mammals leading to high losses in mortality and production. It is of increasing concern as a result of its worldwide distribution. It is considered an emerging zoonosis of humans (Yabsley & Shock 2012). Babesiosis is thus an emerging zoonosis with important public health implications, as the incidence of the disease has risen dramatically over the past decade (Teal *et al*., 2012). A handful of *Babesia* species have been documented as pathogenic in humans (–CDC - DPDx - Babesiosis - Laboratory Diagnosis, n.d.). In the United States, *Babesia microti* is the most common strain associated with humans with other species infecting cattle, livestock and wild animals (Homer *et al*., 2000). People who contract Babesiosis suffer from malaria-like symptoms. As a result malaria is a common misdiagnosis for the disease. Most diagnosed cases of human babesiosis have occurred in the United States of America (USA). Wild animals may demonstrate clinical manifestation of babesiosis similar to that in domestic animals when placed in captivity in non-native areas (Penzhorn, 2006).

2.2.2 Theileriosis

Theileriosis is a complex disease caused by a protozoan parasite of genus *Theileria.* This disease is common to cattle, sheep and goats. East Coast fever (ECF) or Corridor Disease (theileriosis) is a very important livestock disease in East Africa. It is caused by *Theileria parva* and transmitted by three host ticks of the genus *Rhipicephalus*, notably *R. appendiculatus*, the brown ear tick (Norval *et al*., 1992). The African buffalo (*Syncerus caffer*) is the natural host of *T. parva* (Okagawa *et al*., 2012). The first clinical sign of East Cost Fever (ECF) is usually a swelling of the draining lymph nodes, usually the parotid, for the ear is the preferred feeding site of the vector. This is followed by a generalized lymphadenopathy in which superficial lymph nodes such as the parotid, prescapular, and femoral lymph nodes, can easily be seen and

palpated. Fever ensues and continues throughout the course of infection; this rise in temperature is rapid and may reach 106°F (42°C). There is marked petechial and ecchymotic haemorrhage on most mucous membranes of the conjunctiva and the buccal cavity. Anorexia develops, and loss of condition follows. Other clinical signs may include lacrimation, corneal opacity, nasal discharge, terminal dyspnoea, and diarrhea. Before death the animal is usually recumbent, the temperature falls, and there is a severe dyspnoea due to pulmonary oedema that is frequently seen as a frothy nasal discharge (Mans *et al*., 2015)

2.3 Lifecycle and Transmission of *Theileria* and *Babesia*

2.3.1 Lifecycle and Transmission of *Babesia*

Babesia species are transferred with the saliva of the tick to an appropriate mammalian host (Zobba *et al*., 2014). Once inside their mammalian host; sporozoites enter the Red Blood Cells (RBCs) and undergo asexual reproduction through binary fission. Once inside RBC, *Babesia* species are often called piroplasms due to their piriform (pear-shaped) and signet ring appearance. *Babesia* undergoes a complex life cycle that involves both vector and mammalian hosts **(Figure 2.1)**

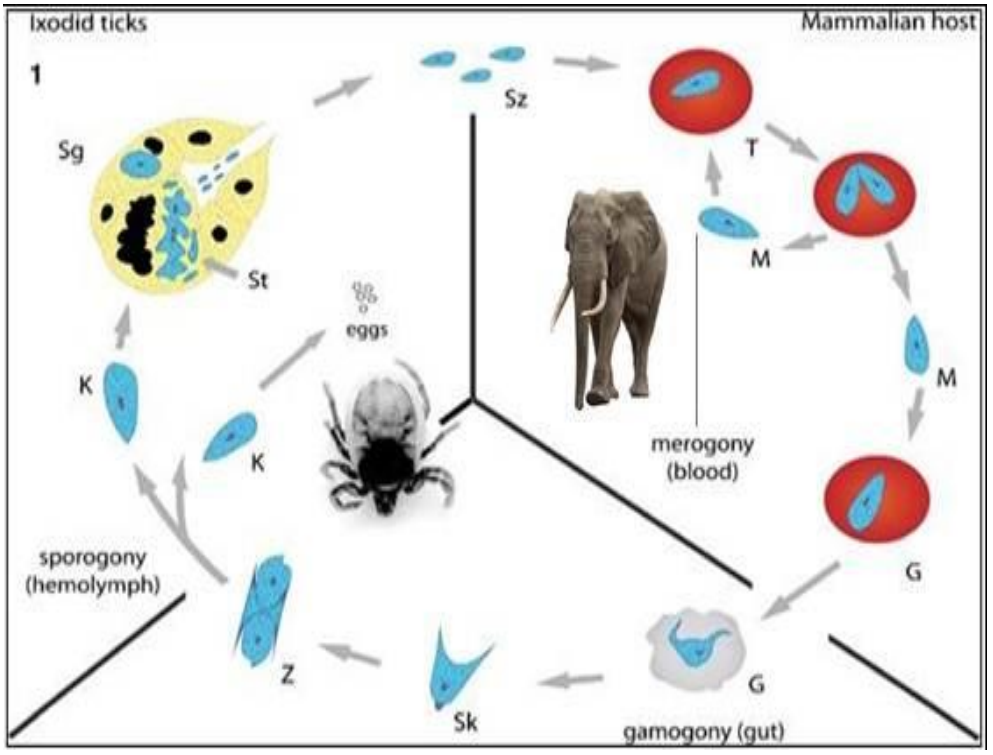

Figure 2.1: Simplified general lifecycle of *Babesia* species modified from (Mehlhorn, 2012)

Babesia life cycle consist of merogony, gamogony, and sporogony. Infection is acquired when sporozoites (Sz) are transferred during tick feeding. Sporozoites then invade erythrocytes and develop into trophozoites (T). Trophozoites divide by binary fission and produce merozoites (M) which continue infection and reinitiate the replicative cycle in the host. Some trophozoites develop into gametocytes (G) which can initiate infection in the tick vector. In the tick gut, gametocytes develop into Strahlenkorper (Sk) which fuse to form a zygote (Z) developing into a kinete (K). Kinetes gain access to the hemolymph of the tick, replicate, and invade various organs. Note that members of the *Babesia* species *sensu stricto* groups can infect the ovaries and be transmitted transovarially via the eggs (E), so that all stages (larvae, nymphs and adults females) are potentially infective, whereas

members of the *Babesia* microti-like groups are only transmitted from one stage to the next (transstadially), so that larvae are rarely if ever infected. Sporogony is initiated when kinetes invade the salivary glands (Sg). Here, the parasite forms a multinucleated sporoblast (St). Newly developed sporozoites (Sz) will then be injected into the host with tick saliva upon the next blood meal. *Babesia* species do not parasitize any vertebrate host cell other than erythrocytes (Cruz-Flores et al. 2010).

2.3.2 Life cycle and Transmission of *Theileria*

The life cycles of all *Theileria* species are generally similar (Shaw and Tilney, 1992). It takes place in the tick vector and vertebrate host

(Figure 2.2)

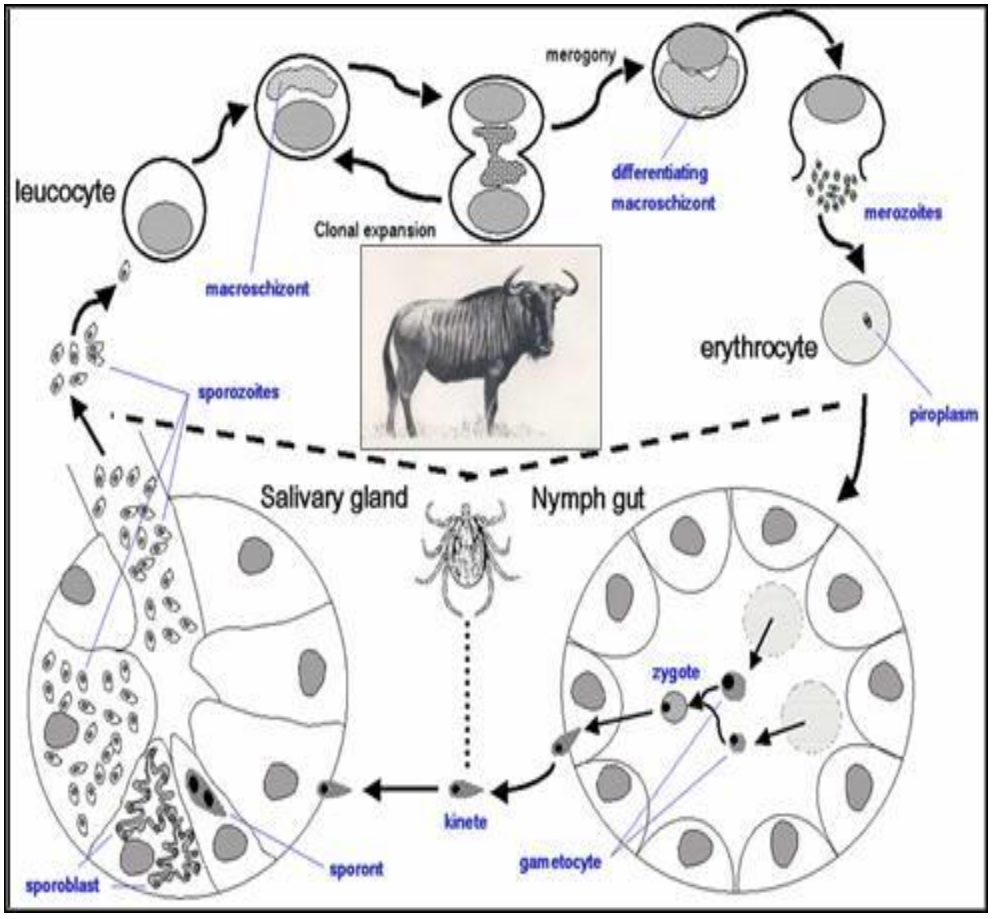

Figure 2.2: A generalised lifecycle of *Theileria* species adopted from (Catalano *et al*., 2015)

Sporozoites are inoculated into a mammalian host when an infected tick takes a blood meal. They are injected into a mammalian host when an infected tick feeds and they enter the lymphoid cells. Inside the lymphoid cells sporozoites develop into schizont or macroschizot. Schizonts differentiate into merozoites and enter the erythrocytes. Ingestion of infected erythrocytes by the tick results to their infection. Gametogenesis and fertilization takes place in the tick gut lumen. Resultant zygote invades occupies epithelial cell gut and remains here during the tick mount cycle. It then develops into a single motile kinete. That egresses the gut cell and invades the salivary glands. The tick enables rapid sporozoite development in

the salivary glands. Sporozites that survive in the gut epithelium and are infective are then transmitted to another mammalian host during feeding of the resulting post-moult nymphs or adult (Shaw & Tilney, 1992).

2.4 Distribution of *Theileria* and *Babesia* species

2.4.1 Distribution of *Theileria* species in Livestock and Wildlife

The two most important species of *Theileria* are found in cattle and buffalo that is *Theileria parva* and *Theileria annaluta* respectively. *T.parva* can infect cattle, African buffalo (*Syncerus caffer*), water buffalo (*Bubalus bubalis*), and waterbucks (*Kobus* spp.). Mildly pathogenic and nonpathogenic species found in cattle include *T. mutans, T. buffeli, T. velifera, T. taurotragi* and *T. sergenti*. *T. annulata* also occurs in cattle, yaks and camels. There are a number of *Theileria* species that can infect both domesticated and wild ruminants they include; *T. mutans, T. buffeli, T. velifera, T. taurotragi* and *T. sergenti.* In sheep and goats the most virulent *Theileria* species is *T. lestoquardi* (formerly *T. hirci*). Other *Theileria* species of sheep, goats and small wild ruminants include; *T. separate, T. lestoquardi,* and the nonpathogenic species *T. ovis. Theileria taurotragi* has also been recognized in eland. *Theileria* species have been found in most wild Bovidae in Africa. They have also been reported in wild animals on other continents. *Theileria equi* is a species of horse‘s, donkeys and giraffes and has been recently isolated in a dog. *T. capreoli and T. cervi* are common to deers. *T. bircornis* is a species of white and black Rhinos in Africa. *T. uilenbergi* is common to sheep, goats, sika and red deer. *Theileria annae* is found in dogs and foxes (Mans *et al*., 2015). *Theileria parva* is widely distributed in sub-Saharan Africa. *T. annulata* occurs from southern Europe and the Mediterranean coast through the Middle East and North Africa, and into parts of Asia. *T. mutans* has been found in African and on some Caribbean islands, and was reported from the U.S. in 1950 and 1975. *T. velifera* and *T.*

taurotragi occur in Africa, while *T. sergenti* has been reported from parts of Asia. *T. buffeli* is widespread, and has been reported from Europe, Asia, Australia, North America and parts of Africa. *T. lestoquardi* has been documented in Asia, the Middle East and parts of Africa and Europe. *T. ovis* and *T. separata* have been documented to occur in Asia (Norval *et al*., 1992).

2.4.2 Distribution of *Babesia* Species in Livestock and Wildlife

There exists diverse *Babesia* species occurring both in domestic and wild animals (Uilenberg, 2006), these species are distributed globally (**Table 2.1**).

Table 2.1: *Babesia* species, host and geographic distribution

Babesia Species	Host	Geographic Distribution
B. beliceri	Cattle	Russia
B. bigemina	Cattle, Buffalo, Gazelle, Antelope	Africa, America, Asia
B. bovis	Cattle, Buffalo	Australia, Europe
B. divergens	Cattle	Africa, America, Asia
B. major	Cattle, American Buffalo	Australia, Europe
B. occultans	Cattle, Antelope	Europe
B. ovate	Cattle	Europe,America,France
B. orientalis	Buffalo, Wild Sheep	Africa
B. crassa	Sheep, Goats	Asia
B. motasi	Sheep, Goats	Asia
B. ovis	Sheep, Goats	Asia
B. caballi	Horse, Donkey, Mule, Zebra	Africa, Asia, Europe
B. perroncitoi	Pig	Africa, Asia, Europe
B. trautmanni	Pig	Africa, America, Asia, Europe
B. canis	Dog, Cat	Europe, Asia (Israel)
B. rossi	Dog	Africa
B. vogeli	Dog	Africa, America, Asia
B. gibsoni	Dog	Europe
B. felis	Cat	Africa, Europe
B. microti	Humans	Asia, America
B. motasi	Sheep, Goat, Ovine	Netherlands, Spain
B. capreoli	Deer	
B. odocoilei	Deer	
B. motasi	Ovine	Netherlands, Spain

2.5 Evolution and Genetic Diversity of *Theileria* and *Babesia*

Theileria and *Babesia* are collectively referred to as piroplasms. There is a controversy in time scale on evolution of *Theileria* and *Babesia* and piroplasms as a whole (Uilenberg, 2006). When they exactly branched from the ancestor is not explicit. In addition whether they evolved in ticks or a vertebrate host first is a query that has driven different responses from researchers. Detailed research has linked evolution of piroplasm to the host animal (Criado- Fornelio *et al*. (2003). Centered on this research hypothesis piroplasmids fall into five groups: Babesids, Theilerids, Archaeopiroplasmids, Prototheilerids, and Ungulibabesids, centered on their sequences of the 18S rRNA. Archaeopiroplasmids which include *Babesia microti*, *Babesia felis*, *Babesia rodhaini*, *Babesia leo,* and *T. annae* are regarded as the piroplasmids ancestors (Criado-Fornelio *et al*., 2003). This view has been supported by genomic research data on piroplasmids.

Theileria and *Babesia* are believed to have evolved in genetic diversity in order to survive hosts immunologically unfavorable environments. In addition to host immunity, tick vectors are considered to be a subject to the evolution of transforming *Theileria* and *Babesia* (Bishop *et al*., 2004). Genetic variation in *Theileria* populations is known to be one of the survival strategies used by these pathogens. They use a number of processes to produce populations that are genetically diverse and the principal process is recombination (Sivakumar, Hayashida *et al*., 2014). *Babesia* use sophisticated mechanisms to achieve genetic and epigenetic diversity for example VESA1 gene family of *B.bovis*. Other mechanisms, such as genetic drift and mutations, can also contribute to genetic variation. Tick species distribution is highly correlated to diversity and geographical distribution of *Babesia* species. *Babesia* expansion can be as a result of tick expansion, as seen for *Babesia canis,* transmitted by *D. reticulatus* in Hungary. Generally both vertebrate host species and tick need to be present in a new environment to allow expansion of *Babesia* species. In

contrast, wide host collection for *Babesia* species and the tick will enhance new host transmission as well as adaptation and potential evolution pointing to novel species (Uilenberg, 2006).

2.6 Identification of *Theileria* and *Babesia*

2.6.1 Microscopic Identification of *Theileria* and *Babesia*

Microscopic examination and accurate identification of *Theileria* and *Babesia* from blood smears, requires trained personnel, experience and parasitological knowledge. It is a challenge to differentiate species of *Theileria* and *Babesia* correctly using microscope method and further, *Babesia* can be confused with the early trophozoite stage (ring forms) of *Plasmodium* parasites. *Theileria* parasite in blood is detected by finding schizonts in Giemsa- stained thin smears from blood or lymph node biopsies. On the other hand in identifying *Babesia* the tetrad morphology, which can be seen with Giemsa staining of a thin blood smear, is unique to *Babesia* and serves as a distinguishing feature from *Plasmodium falciparum*, a protozoan of similar morphology that causes malaria. *Theileria* and *Babesia* occasionally occur in the blood of carrier animals, but in many cases, they cannot be detected by direct examination. *Theileria* and *Babesia* spp. resemble each other, but they can be differentiated with DNA assays. Therefore to allow diagnosis that is more accurate in a format that is accessible to a wider variety of laboratories, the Polymerase Chain Reaction (PCR) method is adopted (Teal *et al*., 2012).

2.6.2 Serological Identification of *Theileria* and *Babesia*

Indirect fluorescent antibody test (IFAT) is common serological method used in detection of *Theileria* and *Babesia*. Schizont or piroplasm antigen, derived from infected animals or cell culture can be used in antigen preparation. IFAT method has a number of limitations in that it has low throughput, has a lot of difficulty in standardization and subjective operator- dependent interpretation

of results. IFAT‘s major problem is the significant cross reactivity that is seen between closely related species. For instance an IFAT assay for *T. lestoquardi* showed significant cross-reactivity with *T. annulata* and *T. parva* anti-sera and vice versa (Leemans *et al*., 1997). Therefore use of IFAT in assigning parasites to particular species is a poor measure (Norval *et al*., 1992) particularly when the species are closely related like in the genotypes found in *T. buffeli*, *T. mutans* and *T. velifera* clades (Mans *et al*., 2015).

2.6.3 Molecular Identification of *Theileria* and *Babesia*

Application of molecular genetics techniques, such as the PCR for gene amplification, and DNA sequencing, have revealed gross inconsistencies in the assignation of some parasite genetic variants, particularly those of the *B. gibsoni* and *B. microti* complexes, to the genus *Babesia* (Allsopp & Allsopp, 2006). Traditional methods have been complemented or even ousted by the molecular ones, of which PCR is the best technique. These techniques are fast, highly sensitive and have high specificity as compared to Giemsa staining (Mosqueda *et al*., 2012). The use of polymerase chain reaction (PCR) and sequencing in the diagnosis and detection of protozoan‘s has thus led to the discovery of unparalleled species diversity in the genus *Theileria* and *Babesia* and also in the revelation of new hosts for *Theileria* and *Babesia* species that were thought to be host- specific (Criado-Fornelio *et al*., 2004). The gene mostly used for molecular identification of *Theileria and Babesia* is 18S small subunit ribosomal RNA gene. The knowledge of the genetic polymorphism in the 18S rRNA of *Theileria* and *Babesia* is essential in designing molecular-genetic probes for the detection of species and strains of *Theileria* and *Babesia* of economic importance to livestock production and wildlife conservation (Oosthuizen *et al*., 2009; Nagore *et al*., 2004).

2.6.4 18S Small Subunit Ribosomal RNA Gene used in Identification of *Theileria* and *Babesia*

The catalytic component of the ribosomes is Ribosomal RNA (rRNA). Four different rRNA molecules: 18S, 5.8S, 28S and 5S rRNA are contained in Eukaryote ribosomes. The 18S rRNA in most eukaryotes is in the small ribosomal subunit, this gene is divided into variable regions (**Figure 2.3**). The V 4 region is mostly used in phylogenetic studies on piroplasms. 18S rRNA has most sequence information available for phylogenetic analysis (Rooney, 2004). The size of this gene is different in different species and contains between 1 720 and 1 770 base pairs. There exists a conserved sequence that is with sequences of nucleotides identical in most or in all *Theileria* and *Babesia* species and in species closely related to them. The conserved region shows limited nucleotide sequence variation. The gene exhibits a steady accumulation of mutations on an evolutionary scale and is therefore valuable in distinguishing different species. Such an organization of the gene enables the designing of primers complementary to the conserved sites in PCR and the detection of the DNA of a large group of related organisms (Skotarczak, 2008).

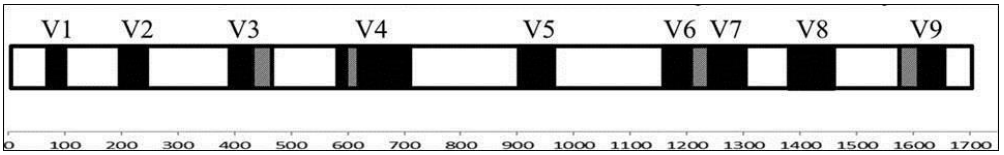

Figure 2.3: A map of the full-length protozoal 18S rRNA gene, including variable (V1 to V9) Modified from (Ishaq & Wright, 2014)

2.7 Economic Importance of *Theileria* and *Babesia*

Theileriosis, heartwater, babesiosis and anaplasmosis are considered the most important tick- borne diseases of livestock in sub-Saharan Africa, resulting in extensive economic losses to livestock farmers in endemic areas (Eygelaar *et*

al., 2015). Theileriosis is considered an important disease because of its severe economic impact on the livestock production. Theileriosis of cattle in Africa has been considered to have had more negative impact on the development of the beef and dairy industries, veterinary infrastructure, legislation, policies and research in Africa than any other livestock disease complex (McCosker, 1979). *Theileriosis* caused by *T. parva* affects cattle in Burundi, Kenya, Malawi, Mozambique, Rwanda, Sudan, Tanzania, Uganda, Zaire, Zambia and Zimbabwe. The total regional loss in 1989 was estimated by Mukhebi *et al*., (1992) at US$168 million, including estimated mortality of 1.1 million cattle (Mukhebi *et al*., 1989). The mortality rate from East Coast fever can be up to 100% in cattle from non- endemic areas, however, in indigenous zebu cattle in endemic areas, mortality is usually low even with a morbidity of approximately 100%. Parasites in the genus *Babesia* are among the most extensively studied protozoan parasites whose economic burden in livestock industry in Africa is impoverishing (Bock *et al*., 2004). *Babesia* is of great economic, medical and veterinary importance because it causes severe morbidity and mortality as well as production losses in livestock industry (Muhanguzi *et al*., 2010).

2.8 *Theileria* and *Babesia* in Wildlife Conservation

In nature, wild mammals harbor diverse species of *Theileria* and *Babesia* in latent state. However, several factors that suppress host immune defense cause latent infections to progress to clinical theileriosis and babesiosis, which is similarly fatal in wildlife as in livestock. Stress, especially stress associated with wildlife capture and translocations have been extensively linked to post-translocation morbidity and mortalities. Captures and translocations have been associated with post-translocation *Babesia*-induced morbidity and mortality (Nijhof *et al*., 2005). For instance, Babesiosis has been associated with morbidity and mortality of black and white rhinos in South Africa and Tanzania (Brocklesby, 1967). A co-infection of

Trypanosomes, Theileria and *Babesia* were linked to the post translocation of the endangered black rhinos in Kenya (Obanda *et al*., 2011). *Babesia bicornis* was identified in four fatal cases of babesiosis in black rhinoceros (*Diceros bicornis*) in South Africa and Tanzania. Three of the four rhinoceros died soon after capture. This was assumed to have happened during periods of nutritional or pregnancy- related stress, or during extreme climatic conditions (Nijhof *et al*., 2003). *Babesia* species was identified in a sable antelope. This antelope died from an unknown illness on a game ranch in the Limpopo province, South Africa (Oosthuizen *et al*., 2008).

Since wildlife translocation is a key conservation strategy for population management especially of the endangered metapopulations, *Theileria* and *Babesia* are potential threats to the conservation of the endangered species. They are especially a threat to the population recovery and conservation of the critically endangered black rhino in Kenya and in the entire African rhino range. It is thus, important to determine their diversity to guide in population health management of wildlife.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Areas

3.1.1 Narok Ecosystem

Narok ecosystem consists of Maasai Mara National Reserve and Siyiapei Narok. Maasai Mara National Reserve (MMNR) is located on the South-Western part of Kenya along the Kenya – Tanzania border between 1°13‘ and 1°45‘ South and 34°45‘and 35°25‘ East. It occupies an area of approximately 1510 square Kilometers and hosts a high diversity of wildlife including large mammals such as African elephants, lions, leopards, African buffaloes, black rhinoceros, wildebeests and several antelope species (Mijele *et al*., 2013).The reserve is contiguous with the Serengeti National Park on the Tanzanian side forming a single ecosystem, the Serengeti-Mara Ecosystem, which is an important habitat for the blue wildebeest migration in East Africa. The MMNR maintains two populations of wildebeest, a small population of resident wildebeests which are present all year round and millions of wildebeests which migrate from the Serengeti to occupy the MMNR for a period of 3-4 months before they retreat (Holdo *et al*., 2009; Serneels & Lambin, 2001). The MMNR is an open conservation area, without fences and wildlife and livestock frequently graze in close proximity.

Siyiapei is 10 km from Narok town is an agro-pastoral zone though in the recent years, commercial crop farming is dominating land use. Altitude is mainly about 1000 m but hilly areas peak at 2300m. Long rains occur in March to May while short rains are received sporadically. From December to May, temperatures of 22º C to 26º C characterize the warm periods that then occur from September to February. This area is increasingly being transformed into human settlement, pushing wild animals out of the area.

Elephants, antelopes, zebra and buffalo are some of the dominant wild herbivores in the area, which persistently cause human-wildlife conflicts.

3.1.2 Laikipia Ecosystem

The Laikipia plateau covers an area of 9,500 km2. It‘s located in the Central Highlands of Kenya. Laikipia, despite its high elevation of 1,800-2,000 m above sea level has a cool dry climate as it lies on the leeward side of Mt. Kenya. The area straddles the equator and as such is endowed with minimal temperature variations all year round but with a marked diurnal temperature range. Mean monthly temperatures are 25° C. Rainfall pattern is erratic at best with highest amounts of up to 1,000 mm and lows of 400 mm. Two rain seasons occur, the long rains are between March and May and the short rains occurring between September and November. The area supports an abundant and diverse wildlife. This includes the "Big five" mammals: lions, elephants, buffalos, black rhino and leopards; as well as plain game like gazelles and Impalas.

3.1.3 Tsavo Ecosystem

Tsavo ecosystem comprises the protected areas of Tsavo East and Tsavo West National parks, private and community sanctuaries and community land. The ecosystem is on the South Eastern part of Kenya about 300 km from Nairobi. This ecosystem carries the largest population of elephants (10 000) in Kenya, according to the 2012 census. However, it also has a rich diversity of wild animals. Temperature ranges from 20º C-30º C and rainfall from200mm–700 mm. The Two rain seasons are long rains experienced between March and April and short rains that are experienced in November and December.

3.2 Study Design

This study was done retrospectively. The study animals included elephants, impalas, reedbucks and wildebeest (**Table3.1**)

Table 3.1: Samples size distribution sampled across locations

Animal	Location	Sample
Elephant *(Loxodonta africana)*	Tsavo	39
Elephant *(Loxodonta africana)*	Narok	40
Impala *(Aepyceros melampus)*	Laikipia	6
Reedbuck *(Redunca redunca)*	Laikipia	11
Wiuldebeest *(Connochaetes taurinus)*	MMNR	32

The total number of animal samples was 128.

3.3 Sample Collection and Sample Clearance

Samples used in this study were obtained from the KWS sample bank. The samples were collected by KWS during population management activities. Specifically, elephant samples in Tsavo ecosystem were obtained during clinical treatments of injured individuals; samples from Narok Ecosystem were obtained during translocation of the population from Narok to Maasai Mara to solve human-elephant conflict. Wildebeest samples were collected during surveillance of Malignant Catarrhal Fever. Impala and Reedbuck were sampled during relocation exercises from wheat farm to conservation area within OlPejeta conservancy, Laikipia. Therefore, this study did not require further ethical clearance, except research permission which was granted by Kenya Wildlife Service.

3.4 DNA Extraction and Amplification

Frozen blood samples in KWS sample bank were thawed prior to DNA Extraction. DNA was extracted from 200µl of blood, using the Qiagen DNeasy blood and tissue extraction kit (QIAGEN, Southern Cross Biotechnologies, South Africa) following the manufacturers protocol. DNA yields were determined with a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, DE, USA), and DNA was stored at 20°C until subsequent analysis. The hypervariable V4 region of the 18S rRNA gene of the genera *Theileria* and *Babesia* was amplified from genomic DNA using a nested polymerase chain reaction. Two sets of primers were used, one set for primary amplification and a second set for secondary amplification. Primary amplification was performed using primers; ILO-9020 (Foward), ILO-9029 (Forward), (5‘CGGTAATTCCAGCTCCAATAGCGT-3‘) and ILO-9030 (Reverse) (5‘- TTTCTCTCAAAGGTGCTGAAGGAGT-3‘). The conditions for the primary amplification consisted of an initial denaturation step of 5 min at 95°C, followed by 30 cycles of denaturation each at 95°C for 30 sec, annealing for 30 sec at 53°C, extension for 1 min at 72°C and terminated by a final extension for 8.5 min at 72°C. Secondary amplification was carried out by ILO-9029 (Forward), (5‘CGGTAATTCCAGCTCCAATAGCGT-3‘) and ILO-7782 (Reverse) (5‘AACTGACGACCTCCAATCTCTAGTC-3‘). The conditions for secondary amplification consisted of an initial denaturation step of 5 min at 95°C, and 30 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 50°C, extension for 1 min at 72°C and terminated by a final extension for 8.5 min at 72°C. Extraction controls and PCR- product negative (water) were included in each PCR reaction as negative controls. Both primary and secondary amplifications were carried out based on a final volume of 10 µl which consisted of 1.5 µl of genomic DNA, 0.1µl of each primer, 5 µl of Thermo Scientific™ DreamTaq™ Green

PCR Master Mix (2X) and 3.3µl of water. The primers were amplifying 400-500 base pairs.

3.5 Gel Electrophoresis and Sequencing of PCR Products

The final PCR product (1 µL) was separated using gel electrophoresis on 2% agarose gel stained with GelRed™ Nucleic acid gel stain. The gel was visualized for positive amplification of the target region on a UV trans-illuminator, and photographed. One kb and100 bp DNA Ladder was used to identify the approximate size of the molecule run on a gel. All PCR products that were positive upon visualization on agarose gel were purified and sequenced at Macrogen in Europe. The products were sequenced in both the forward and reverse directions using the Sanger method with the same primers used to amplify the 18S rRNA during PCR.

3.6 Sequence and Phylogenetic Analysis

The chromatograms were visualized and edited using ChromasLite version 2.1.1. Primers were trimmed out using the software for Molecular Evolutionary Genetics Analysis (MEGA6) (Tamura *et al*., 2013). The consensus nucleotide sequences were aligned using MUSCLE v. 3.8.31 (Edgar, 2004) and visualized using SeaView v.4 (Gouy *et al*., 2010). Unique sequences, herein referred to as haplotypes, were identified from aligned sequences using DNA sequence polymorphism (DnaSP v 5.10), (Librado & Rozas, 2009). Haplotype distributions were done using Microsoft excel. Sequences that were Orthologous to these haplotypes were identified from GenBank (Benson *et al*., 2009) using the Blastn algorithm. Closest sequence match to these haplotypes based on expectation value of greater than 1e-10 were selected. In order to classify the haplotypes into species or clusters of species, at least 3 representative sequences of each known *Theileria* and *Babesia* species from GenBank were obtained for comparison.

Phylogeny was constructed using sequences of V4 region of 18s rRNA gene of *Theileria* and *Babesia*. Prior to phylogenetic analyses, multiple sequence alignment was performed using the program MUSCLE. MEGA 6 was used to determine the model of sequence evolution as well as the rate heterogeneity of aligned sequences (Tamura *et al*., 2013). The phylogeny was inferred using the maximum likelihood method based on a Tamura-Nei (1993) model (Tamura & Nei, 1993) with a gamma parameter to model the nucleotide substitution pattern and rate of evolution. Statistical support for internal branches of the trees or their reliability was evaluated by bootstrapping with 1000 iterations (Felsenstein, 1985). The resultant trees were viewed and edited in Dendroscope v.3 3 (Huson & Scornavacca, 2012). To test the robustness of the phylogenetic analyses, sequence clusters were also detected by the analysis of phylogenetic networks inferred from uncorrected p-distances with the phylogenetic split decomposition network implemented with Split Tree v. 4.13.1 (Huson & Bryant, 2006). Phylogenetic network diagrams produced from these analyses was used to validate the haplotypes. Lastly, DnaSP was used to investigate sequence divergence and polymorphism between the haplotypes and the GenBank references. Nucleotide divergence or the average number of nucleotide substitutions per site between haplotypes and was estimated using the Jukes and Cantor model. The section of the sequences with high polymorphism was detected using a sliding window in DnaSP and visualized using Jalview v 2.8.2 (Waterhouse, Procter *et al*., 2009).

3.7 Analysis of Selection Pressures on *Theileria* and *Babesia*

DnaSP v 5.10 was used in analyses of the genetic differentiation and gene flow among populations, determining Linkage disequilibrium and generating graphical outputs for an easy visualization of results.

CHAPTER FOUR

RESULTS

4.1 Identification and Prevalence of *Theileria* and *Babesia*

Gel electrophoresis confirmed positive amplification of V4 region of the 18s rRNA gene of eitherTheileria or Babesia since the primers used could amplify both species. It also led to the detection of expected fragments of size 400-500 base pairs. 128 samples were positive for either *Theileria* or *Babesia* from gel results. Only 95 samples were sequenced. The sequenced samples included 32 wildebeest from Maasai Mara in Narok ecosystem, 26 elephants from Narok ecosystem, 26 elephants from Tsavo ecosystem, 5 reedbucks and 6 Impalas from Laikipia Ecosystem.

4.1.1 Identification and prevalence of *Theileria* and *Babesia* in African Elephants

Figures 4.1 and 4.2 are 2% agarose gel electrophoresis showing representative results obtained with primers targeting the V4 region of the 18SrRNA of *Theileria* and *Babesia* parasites. Amplicon size was found to be ranging between 400-500 base pairs using100 base pairs molecular weight DNA marker on the edges. Elephants DNA samples showed positive amplification for *Theileria* and/or *Babesia*.

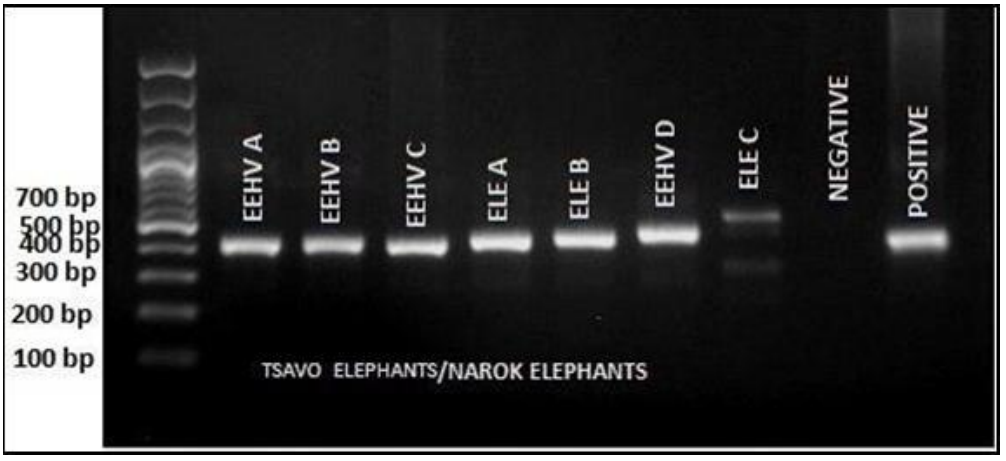

Figure 4.1: PCR amplification gel image of the 18SrRNA gene of *Theileria* / *Babesia* species in elephant DNA samples

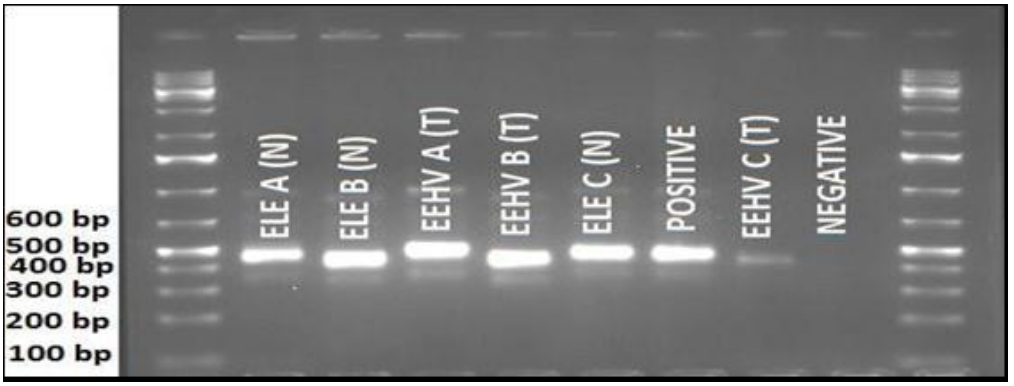

Figure 4.2: PCR amplification gel image of the V4 region of 18SrRNA gene of *Theileria*/*Babesia* species in elephant DNA samples

Key: ELE- Elephant, Narok and EEHV- Elephant, Tsavo.

Out of 26 DNA sequences from elephants in Tsavo ecosystem, 7 sequences were of poor quality and discarded from the analysis. The remaining 19 good quality sequences were analyzed of which 14 sequences were positive for *Babesia* (**Appendix I**) while 5 sequences were positive for *Theileria* (**Appendix II**). For Narok ecosystem out of 26 DNA sequences, 9 sequences were of poor quality and discarded from the analysis. The resulting 17 good quality sequences were analyzed of which 10 sequences were found

positive for *Babesia* (**Appendix I**) and 7 sequences were positive for *Theileria* (**Appendix II**). 26.3% (5/19) elephants from Tsavo were infected with *Theileria* whereas 41.1% (7/17) elephants from Narok were infected with *Theileria*. 73.7% (14/19) of elephant samples in Tsavo were infected with *Babesia* whereas in Narok, 58.8% (10/17) elephant samples had *Babesia*.

All *Theileria* and *Babesia* identified in African elephants were collapsed into haplotypes using DnaSP. Two *Babesia* haplotypes and six *Theileria* haplotypes were identified in African elephants. The two *Babesia* haplotypes were distinct from each other as polymorphisms between the two haplotype sequences were seen at various positions on the sequence chromatograms (**Figure 4.3**) and were also confirmed on the multiple sequence alignment file (**Figure 4.4).** The six *Theileria* haplotypes identified in African elephants of Tsavo and Narok ecosystem were also different as polymorphisms could be seen in various regions in the sequence chromatograms and were confirmed on the multiple sequence alignment file (**Figure 4.5 and Figure 4.6**).

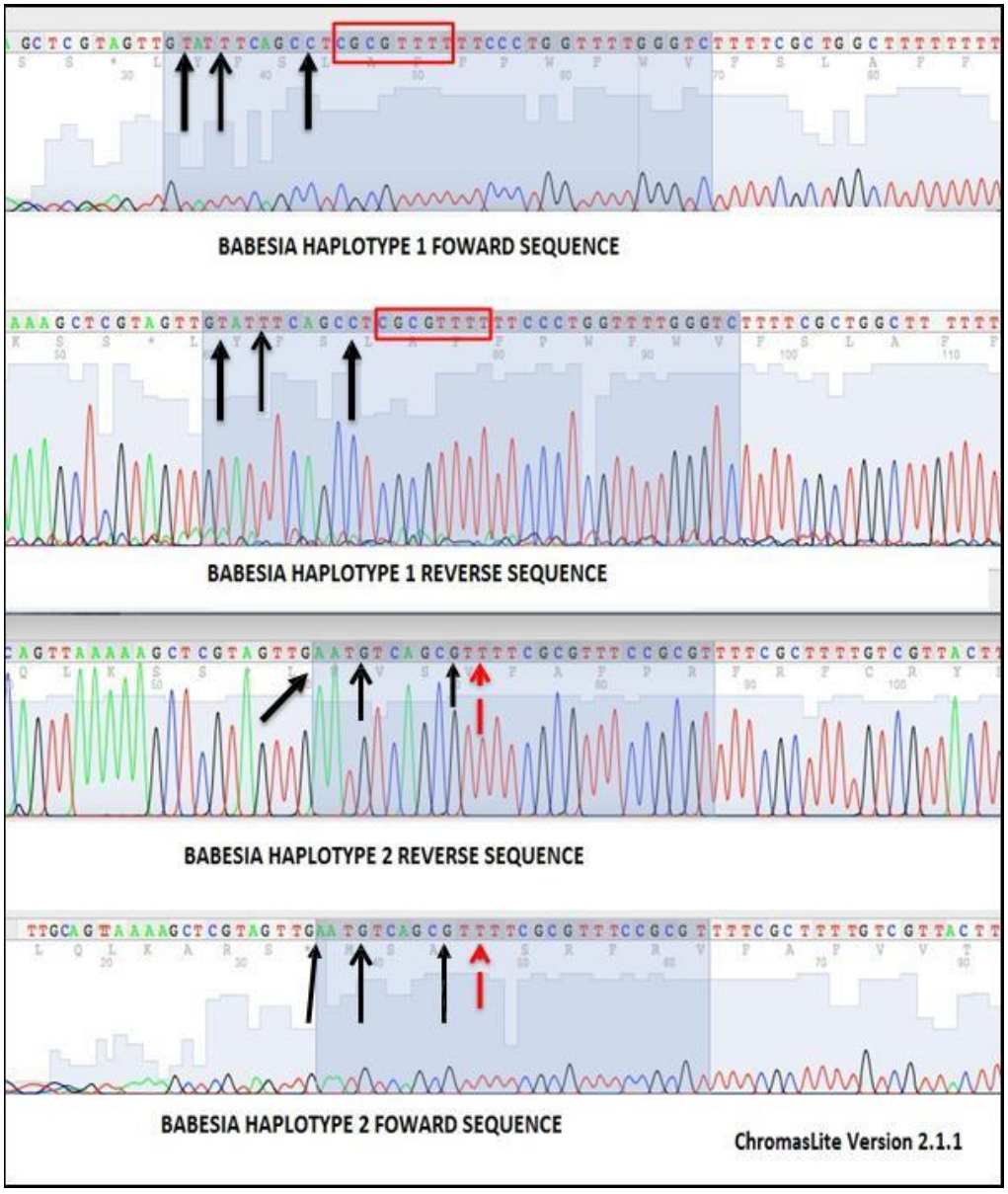

Figure 4.3: Sequence chromatograms showing areas of polymorphism in the 2 *Babesia* haplotypes identified in African elephants of Narok and Tsavo ecosystem

The sequence chromatogram **Figure 4.3** shows the polymorphism result at nucleotide positions 37, 40, 46 and 48 on the 2 *Babesia* haplotypes. The red boxes indicate nucleotide insertions and the red arrows are indicating region of nucleotide deletions. The black arrows on the other hand show the areas

where nucleotide substitutions have occurred. In *Babesia* Haplotype 1 there is nucleotide CGCGTTTT insertion at position 48-55. These insertions are absent in *Babesia* Haplotype 2 at the same position (**Figure 4.3 and Figure 4.4)**. The chromatogram result indicate nucleotide T/A substitution at position 37, nucleotide T/G substitution at position 40 and C/G substitution at position 47 (**Figure 4.3 and Figure 4.4)**. The blue arrows **(Figure 4.4)** show areas where there is polymorphism. Polymorphisms are also observed in other nucleotide positions other than ones listed in the (**Figure 4.3 and Figure 4.4)**.

Various noticeable polymorphism were identified in the V4 region of the 18s rRNA gene of the 6 *Theileria* haplotypes identified in African elephants. The red arrow and black arrow indicate substitutions observed especially at nucleotide position 48 where there was a G/A substitution and at position 118 and 119 where there was nucleotide G/A and T/A substitution respectively **(Figure 4.5 and Figure 4.6.)**

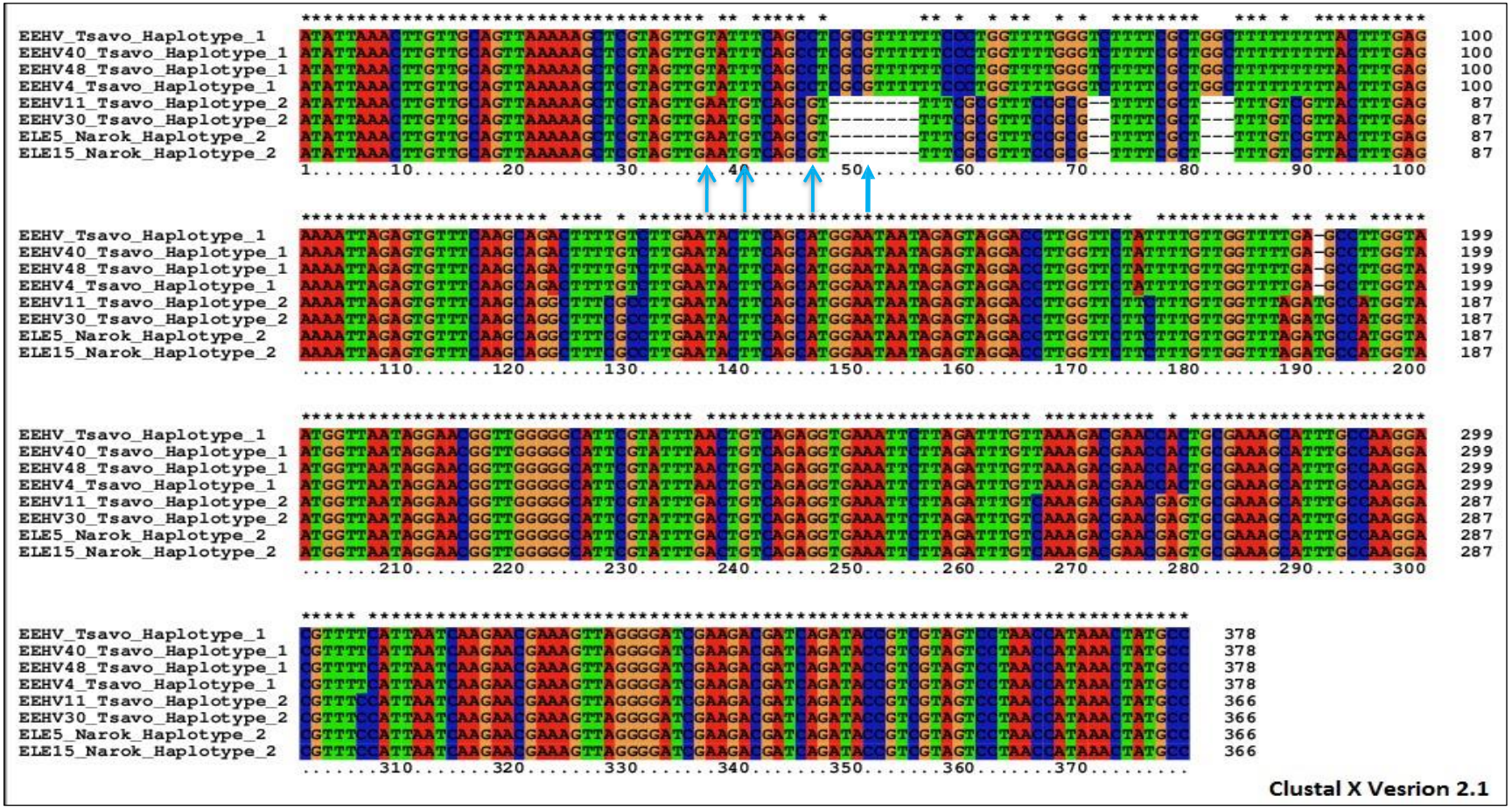

Figure 4.1: Multiple sequence sequence alignment of V4 region of 18s rRNA gene of 2 *Babesia* haplotypes identified in African elephants of Narok and Tsavo ecosystem

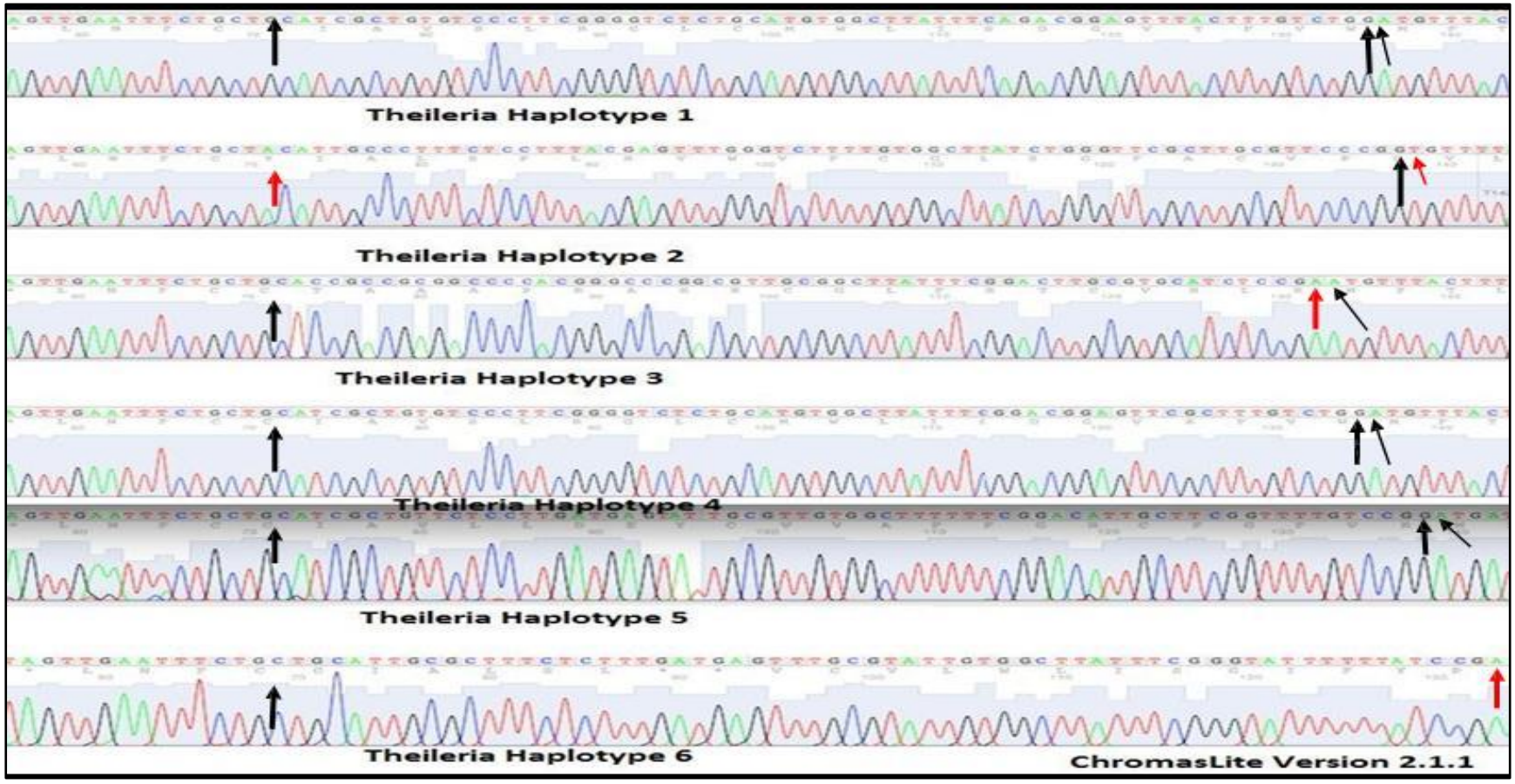

Figure 4.5: Sequence chromatograms showing areas of polymorphism in the 6 *Theileria* Haplotypes identified in African Elephants

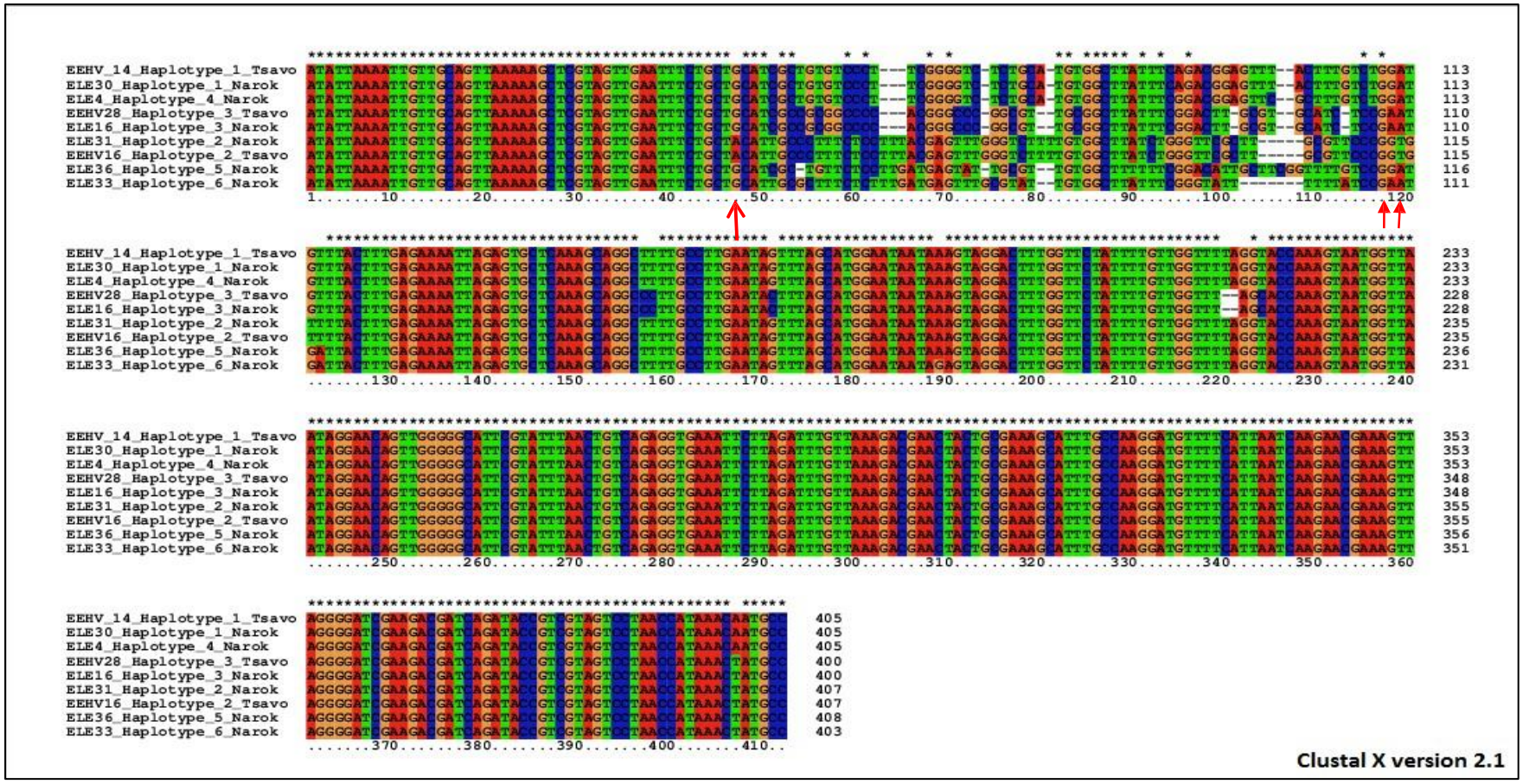

Figure 4.6: Multiple sequence alignment of the V4 region of 18s rRNA gene 6 *Theileria* haplotypes identified in Africanelephants of Narok and Tsavo ecosystem

The 2 *Babesia* haplotypes were distributed in African elephants of Tsavo and Narok ecosystem. Narok ecosystem had only one haplotype of *Babesia* that is haplotype 1.Tsavo ecosystem had two *Babesia* haplotypes; haplotype 1 and haplotype 2 (**Figure 4.7**).

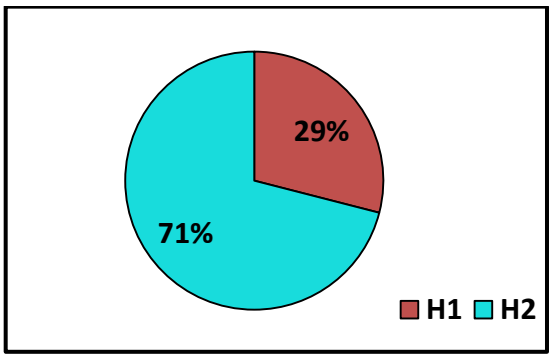

Figure 4.7: Proportions of *Babesia* haplotype distribution in elephant populations of Tsavo ecosystem, Kenya

Key: H- Haplotype.

Babesia haplotype 1 is highly distributed among elephants in Tsavo ecosystem at 71% as compared to *Babesia* haplotype 2 that is at 29%.

Out of the 6 *Theileria* haplotypes identified in elephants of Tsavo and Narok ecosystem; *Theileria* Haplotype 1, 2 and 3 were identified in both Tsavo and Narok Ecosystem while Haplotype 4, 5, and 6 were only identified in Narok ecosystem (**Figure 4.8 and Figure 4.9**).

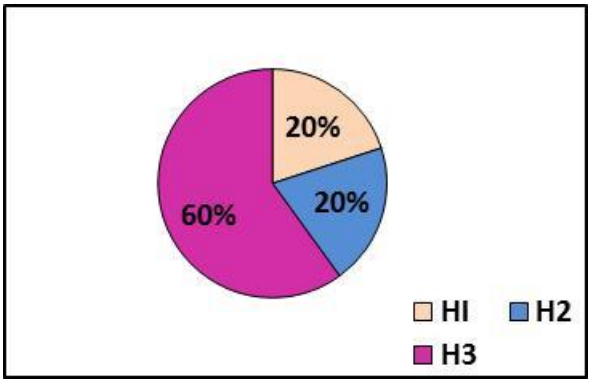

Figure 4.8: Proportions of *Theileria* haplotype distribution in elephant populations of Tsavo ecosystem, Kenya

Key: H- Haplotype.

Theileria haplotype 3 is highly distributed at Tsavo ecosystem with a proportion of 60%while *Theileria* haplotype 2 and 3 are equally distributed having equal proportion of 20%.

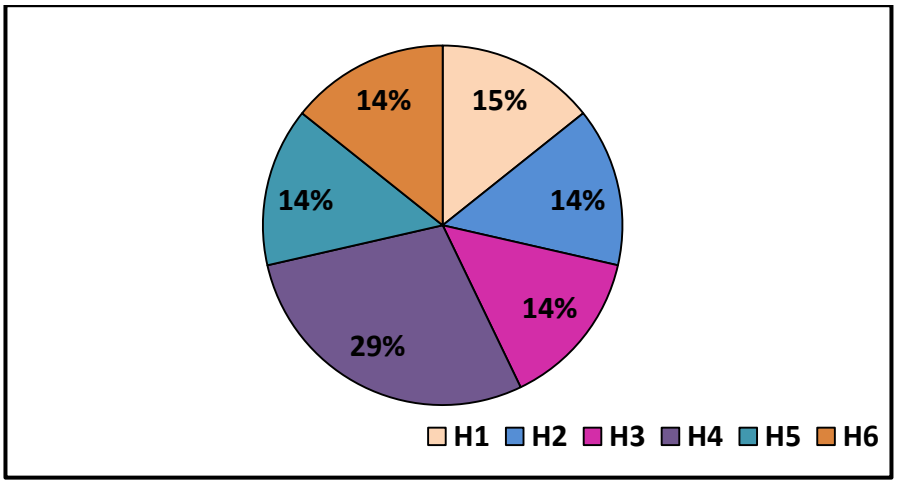

Figure 4.9: Proportions of *Theileria* haplotype distribution in elephant populations of Narok ecosystem, Kenya

Key: H- Haplotype.

Theileria haplotype 4 is highly distributed in elephants of Narok ecosystem at 29%. *Theileria* haplotype 1, 2, 3, 5 and 6 are equally distributed in elephants of Narok ecosystem, all having 14% proportions. Therefore Narok ecosystem has 6 *Theileria* haplotypes while Narok ecosystem has only 3 *Theileria* haplotypes.

4.1.2 Identification and prevalence of *Theileria* and *Babesia* in Wildebeest

The gel image in **Figure 4.10** is a 2% agarose gel electrophoresis showing the representative results obtained with the primers targeting the V4 region of the 18SrRNA of *Theileria* and *Babesia* parasites in wildebeest. The amplicon size was found ranging between 400-500 base pairs clearly indicated using 100 base pairs molecular weight DNA marker on the first and last lanes. All the 32 DNA samples for wildebeest samples showed positive amplification.

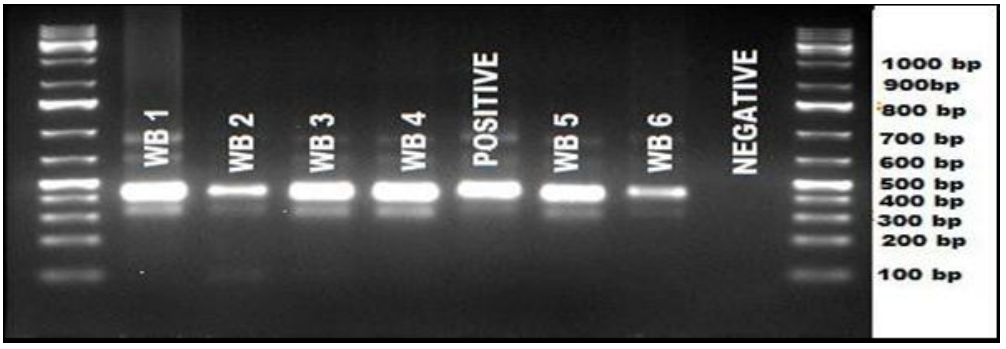

Figure 4.10: PCR amplification gel image of the V4 region of 18SrRNA gene of *Theileria* species in wildebeest DNA samples

Key: W - Wildebeest.

Out of the 32 wildebeest DNA samples sequenced, 3 sequences were of poor quality and discarded from further analysis. 29 good quality DNA sequences were therefore analyzed. Out of the 29 good sequences, 23 were from migratory wildebeest and 6were from resident population of wildebeest.

Babesia was not detected in any of the wildebeest DNA sequences. All the 29 DNA sequences were positive for *Theileria* (**Appendix II**). The occurrence of *Theileria* in Wildebeest was therefore 100%.

All *Theileria* identified in wildebeest were collapsed into haplotypes. Three *Theileria* haplotypes that is haplotype 7, 8 and 9 were identified in wildebeests. Haplotype 7 (GenBank accession number, KT163244) was identified in one resident and one migratory wildebeest; haplotype 8 (GenBank accession number, KT163245) was identified in migratory wildebeest only while haplotype 9 (GenBank accession number, KT163246) was identified in 5 resident wildebeests and 19 migratory wildebeests. The three *Theileria* haplotypes were distinct from each other as polymorphisms were evident in sequence chromatograms (**Figure 4.11)** and confirmed in multiple sequence alignment file (**Figure 4.12**).

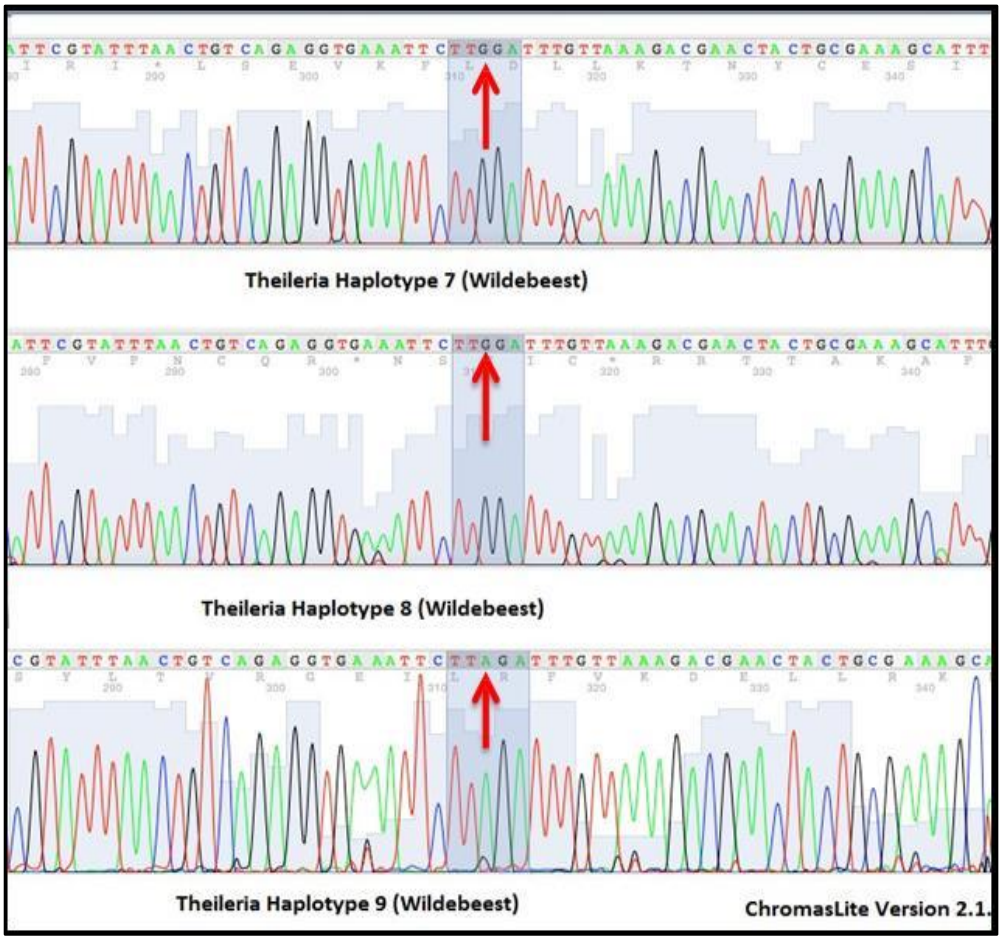

Figure 4.11: Polymorphism in the sequence chromatograms of 3 *Theileria* haplotypes identified in wildebeest of MMNR, Narok ecosystem

Polymorphism within the chromatogram sequence was evident in the 3 wildebeest *Theileria* haplotypes. At nucleotide position 290 shown by the red arrows in the chromatograms above, nucleotide G/A substitution is observed.

The red arrow (**Figure 4.12**) shows G/A nucleotide substitution at position 290. Polymorphisms were also observed in other numerous nucleotide positions other than at the position listed above.

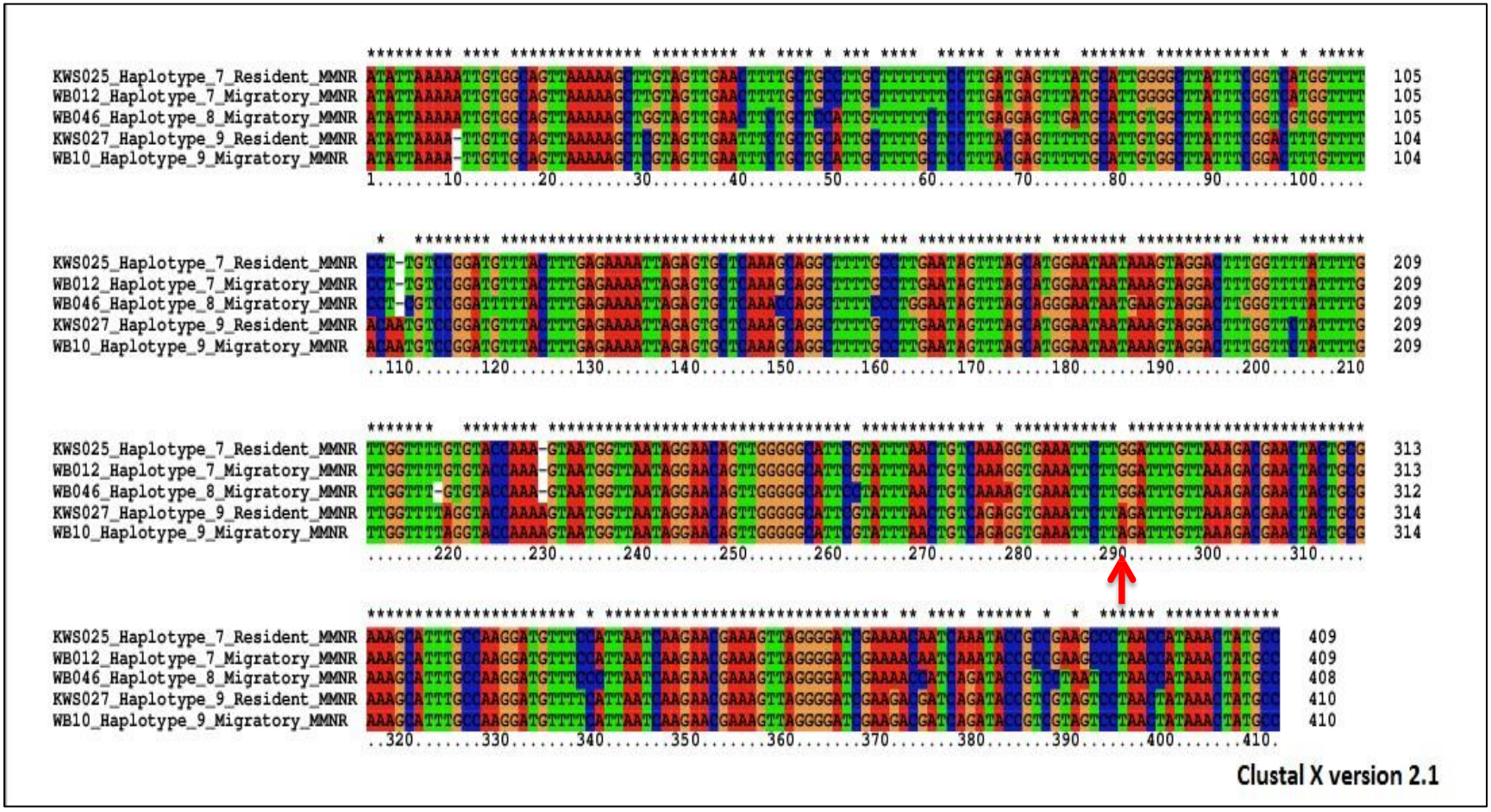

Figure 4.12: Multiple sequence alignment of V4 region of 18s rRNA gene of 3*Theileria* haplotypes from wildebeest of MMNR-Narok

The three *Theileria* haplotypes identified in wildebeest were distributed through the Maasai Mara National Reserve- Narok (**Figure 4.13**).

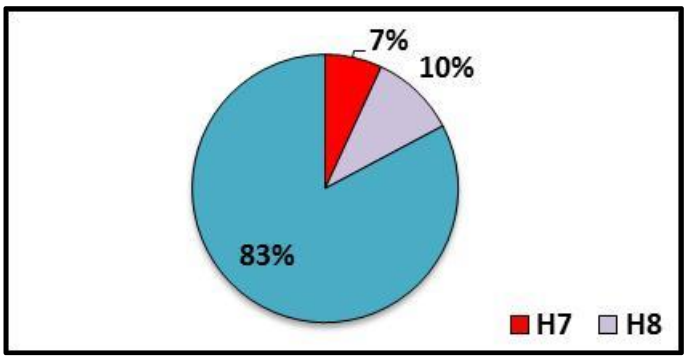

Figure 4.13: Proportions of *Theileria* haplotype distribution in Wildebeest of Maasai Mara National Reserve-Narok ecosystem, Kenya

Key: H- Haplotype.

Theileria haplotype 9 was highly distributed in wildebeest of Maasai Mara, Narok at 83%. Haplotype 8 followed with 10% and *Theileria* haplotype 7 at 7%.

4.1.3 Identification and prevalence of *Theileria* and *Babesia* in Reedbucks and Impalas

Figure 4.14 is a 2% agarose gel electrophoresis showing results for samples from impalas and reedbucks after PCR analysis of the V4 region of 18SrRNA in *Theileria* and *Babesia* parasites. Amplicon size was found to be ranging between 400-500 base pairs, using 100 base pairs molecular weight DNA marker. All the 6 impalas and 5 reedbucks DNA samples showed positive amplification.

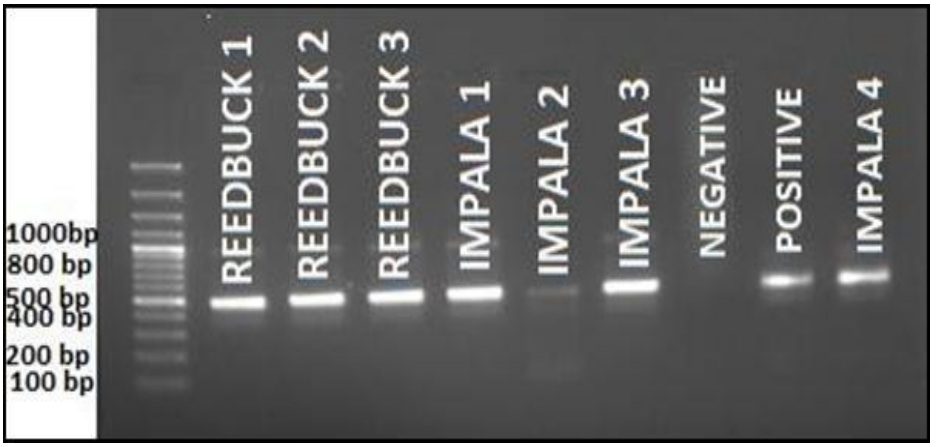

Figure 4.2: PCR amplification gel image of the V4 region of 18SrRNA gene of *Theileria* species in reedbuck and impala DNA samples

All the 5 reedbuck and 6 impala DNA sequences that were sequenced were of good quality and thus analyzed. No *Babesia* was detected in reedbuck or impala. All the 5 reedbucks and 6 impalas were positive for *Theileria* (**Appendix II**).

All the *Theileria* identified in reedbuck and impala was collapsed into haplotypes. Two *Theileria* haplotypes were identified. One *Theileria* haplotype from impala herein referred to as haplotype 10 and one *Theileria* haplotype from reedbuck herein referred to as haplotype 6 since it was identical to *Theileria* haplotype 6 isolated from elephants (**Appendix II**). The two *Theileria* haplotypes from impala and reedbuck were distinct from each other as polymorphisms were evident in sequence chromatograms (**Figure 4.15).** These polymorphisms were confirmed and observed on the multiple sequence alignment file (**Figure 4.16**).

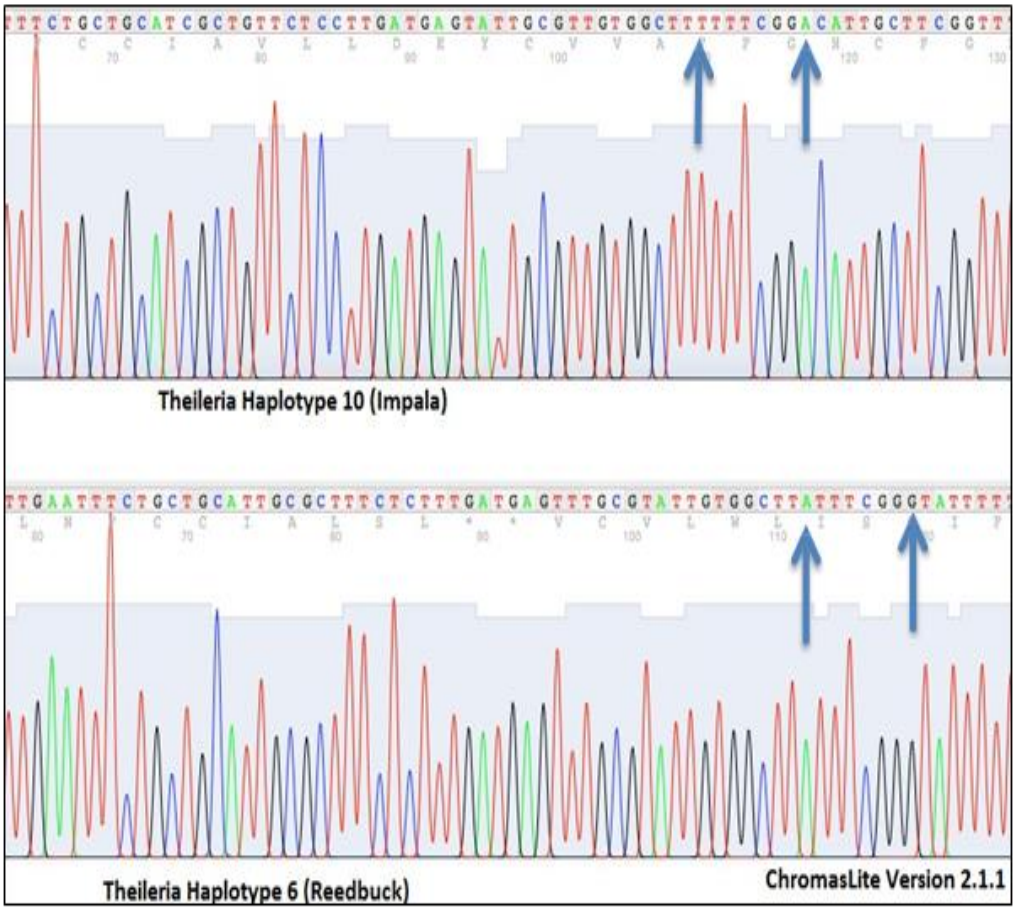

Figure 4.15: Polymorphisms in the sequence chromatograms of 2 *Theileria* haplotypes identified in impalas and reedbucks of Laikipia ecosystem

Polymorphism within the chromatogram sequence was evident in the 2 *Theileria* haplotypes identified in impala and reedbuck. At nucleotide position 89 shown by the blue arrows in the chromatograms above, nucleotide T/A substitution is observed. At nucleotide position 95 there is A/G substitution. Polymorphisms are also observed in other nucleotide positions other than at positions 89 and 95.

The red arrows in (**Figure 4.16**) indicate areas where there are polymorphisms. At position 89 there is nucleotide T/A substitution as well as A/G substitution at nucleotide position 95.

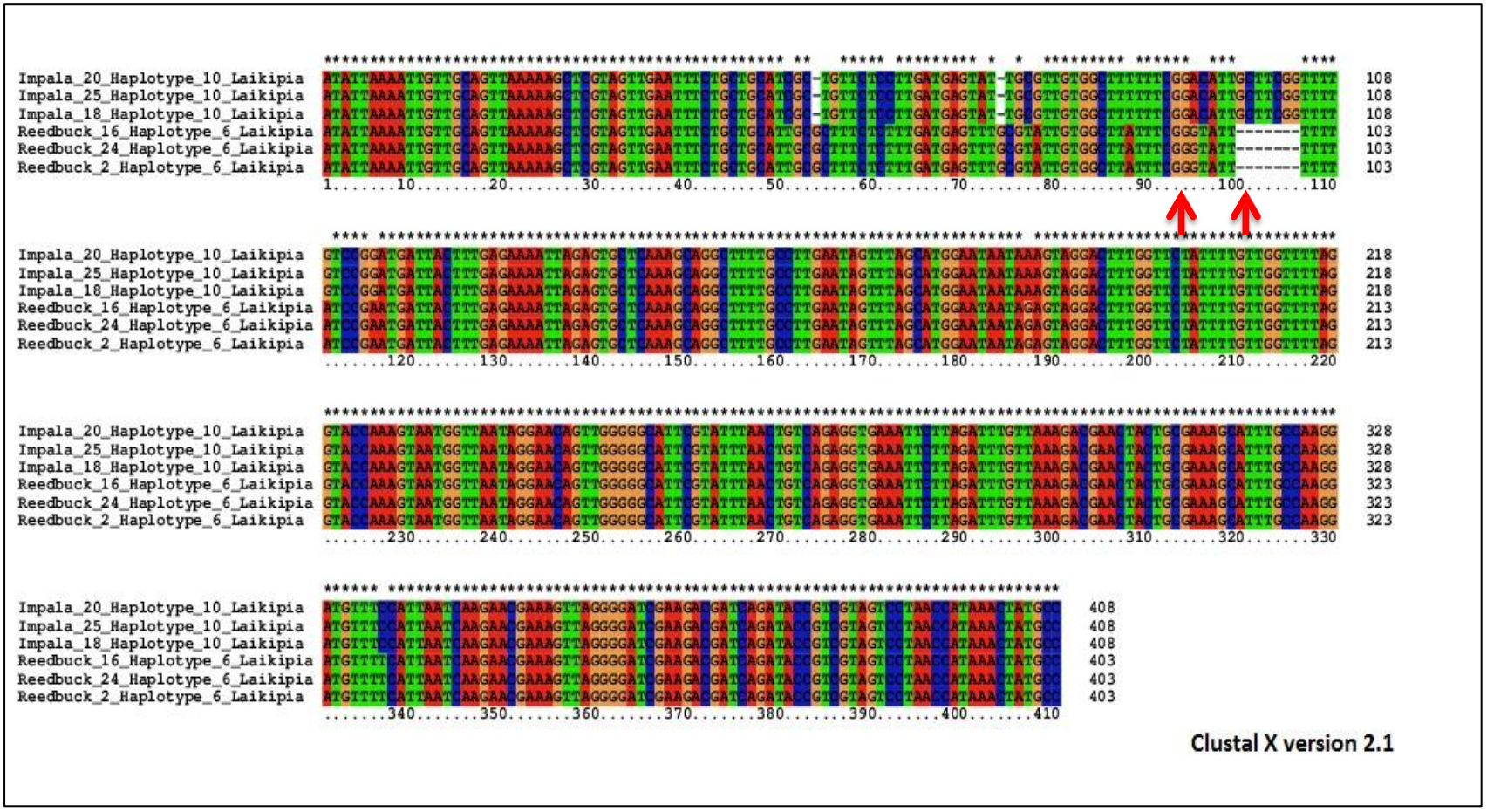

Figure 4.16: Multiple sequence alignment of V4 region of 18s rRNA gene of 2*Theileria* haplotypes from impala and reedbuck 0f Laikipia ecosystem

Laikipia ecosystem therefore had two haplotypes of *Theileria* from reedbuck and impala (**Figure 4.17**).

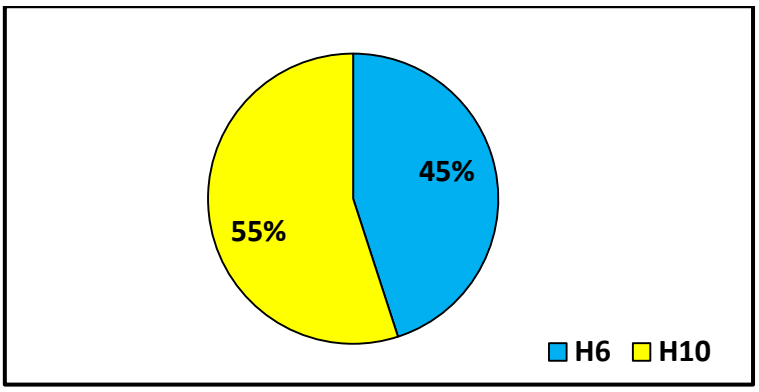

Figure 4.17: Proportions of *Theileria* haplotype distribution in reedbuck and impala in Laikipia Ecosystem, Kenya

Key: H-Haplotype.

Theileria haplotype 10 was more prevalent in Laikipia ecosystem at 55% while that of haplotype 6 was at 45%.

4.2 Phylogenetic relationships, DNA Polymorphism and Divergence of *Theileria* and *Babesia*

4.2.1 Phylogenetic relationships, DNA Polymorphism and Divergence of *Theileria* and *Babesia* in African elephants

Phylogenetic analysis of the 2 *Babesia* haplotypes from elephants with similar sequences based on Blastn (NCBI) search and sequences of known *Babesia* identity revealed that Haplotype 1 clustered with *Babesia bigemina* (**Figure 4.18**). Haplotype 2 formed a distinct clade that share a more recent common ancestry with *Babesia bovis* and *Babesia ovis* clades (**Figure 4.19**). This haplotype may be new and therefore not described earlier.

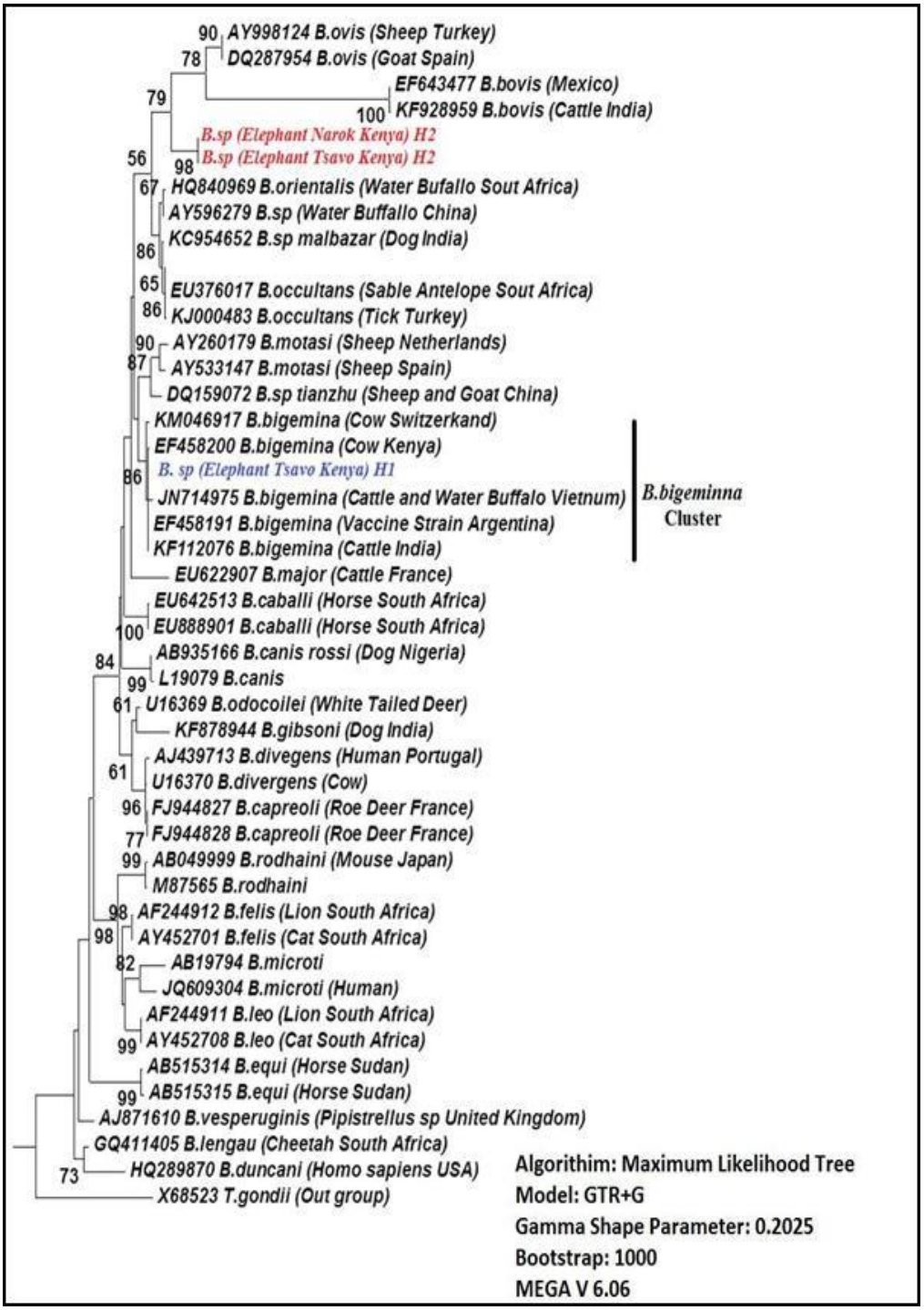

Figure 4.18: Phylogenetic relationship between *Babesia* isolated from African elephants in Tsavo and Narok ecosystem and *Babesia* isolates from the GeneBank

Key: H-Haplotype.

Babesia isolates from elephants of Tsavo and Narok are highlighted in color while the rest accessed from the GeneBank are in Black. The phylogenetic tree was established using maximum likelihood. Numbers above the branches indicate bootstrap values based on 1000 replicates. *Toxoplasma gondii* was used as an out group. The rate of heterogeneity among the species was high with gamma shape parameter of 0.2025.

The Phylogenetic network analysis confirmed that the 2 haplotype clustering was consistent with the topology of the phylogenetic tree (**Figure 4.19)**.

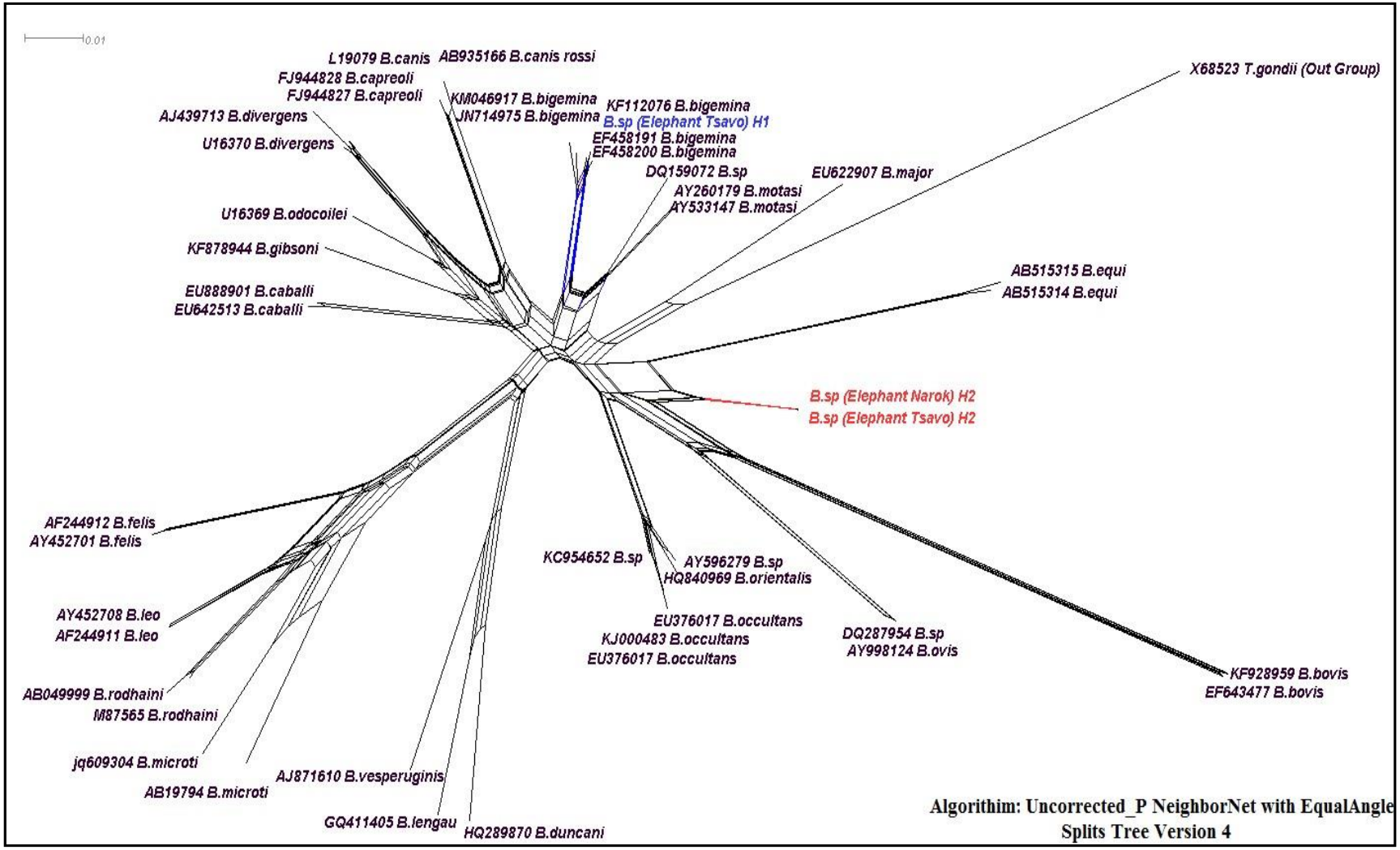

Figure 4.19: Network graph showing the separation and clustering of *Babesia* haplotypes from elephants (in color) and isolates from the Gene B ank (in black). *Toxoplasma gondii* was used as an Out group.

The divergence between *Babesia* haplotypes infecting elephants and two of the most closely associated sequences of known species obtained from GeneBank is shown in (**Table 4.1**).

Table 4.1*:* Sequence divergence between *Babesia* haplotypes from the African elephants in Kenya and both *Babesia bigemina* and *Babesia bovis*

	Haplotype 1	***Haplotype 2***	***B. bigemina***	***B. bovis***
Haplotype 1		0.0630	0.0000	0.1348
Haplotype 2	0.0630		0.06582	0.1114

Babesia haplotype 1 from elephants and *Babesia bigemina* showed no divergence while there was great divergence and heterogeneity between *Babesia* haplotype 2 from elephants and *Babesia bovis.*

Polymorphism in the two haplotypes was elevated in a small portion of the V4 region of 18s rRNA about 60 bp in length, a property shared by other species (**Figure 4.20**)

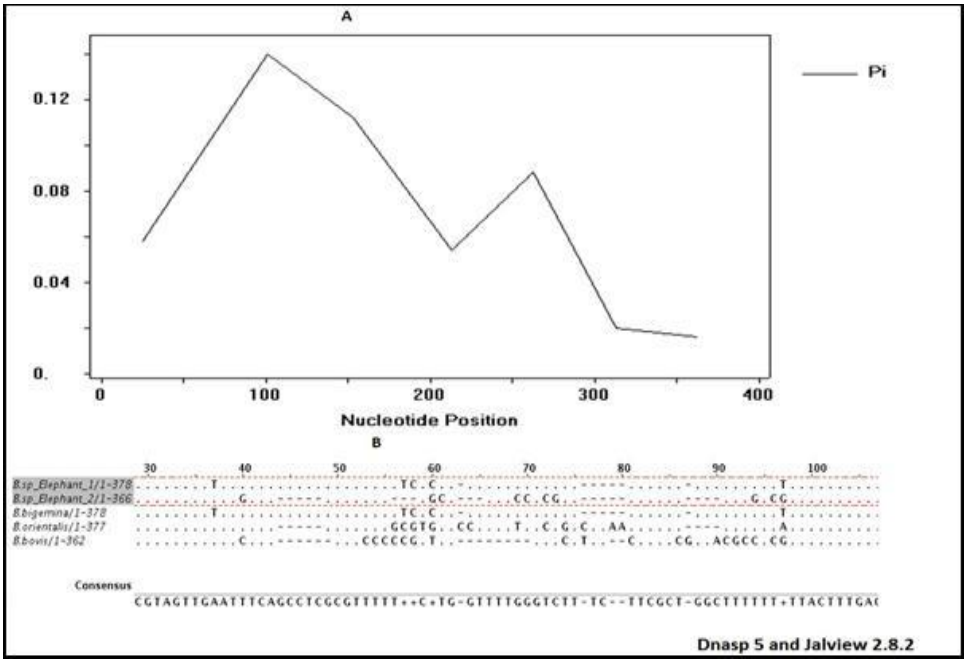

Figure 4.20: Polymorphism in the V4 region of 18s rRNA gene of *Babesia* species

In Figure 4.20, A is showing polymorphism at a region 40-100 bp from the ILO primers. B is a sequence alignment showing variability in the 40-100 base pairs within the two elephant *Babesia* haplotypes, *B.bigemina*. *B.orientalis* and *Babesia bovis* cluster. Phylogenetic analysis of the 6 elephant *Theileria* haplotypes with similar sequences on Blastn (NCBI) and with sequences of known identity showed a clustering of based the haplotypes with other *Theileria* species (**Table 4.2**).

Table 4.2: Table showing known *Theileria* species that clustered with the elephant *Theileria* haplotypes

Haplotypes	Cluster
Theileria Haplotype 1	*Theileria parva*
Theileria Haplotype 2	*Theileria cf. velifera*
Theileria Haplotype 3	*Theeileria cf. mutans*
Theileria Haplotype 4	*Theileria parva*
Theileria Haplotype 5	*Alone*
Theileria Haplotype 6	*Theileria lewenshuni*

Theileria haplotype 1 and 4 were found to be clustering with *T.parva* that has been widely isolated in African buffalo of South Africa. Importantly they also clustered with *T.parva* that has been isolated in cattle in Kenya (**Figure 4.21**). Haplotype 2 clustered with *Theileria cf.velifera* that is commonly isolated in African buffalo. Haplotype 3 clustered with *Theileria cf. mutans* a strain common to the African buffalo (**Figure 4.21**). *Theileria* haplotype 5 formed its own distinct clade. This haplotype may be a new species or novel haplotype not yet described (**Figure 4.21**). *Theileria* haplotype 6 was found clustering with *Theileria lewenshuni*. This haplotype shared a clade with *Theileria* sp that has ever been isolated in waterbuck in Kenya (**Figure 4.21**).

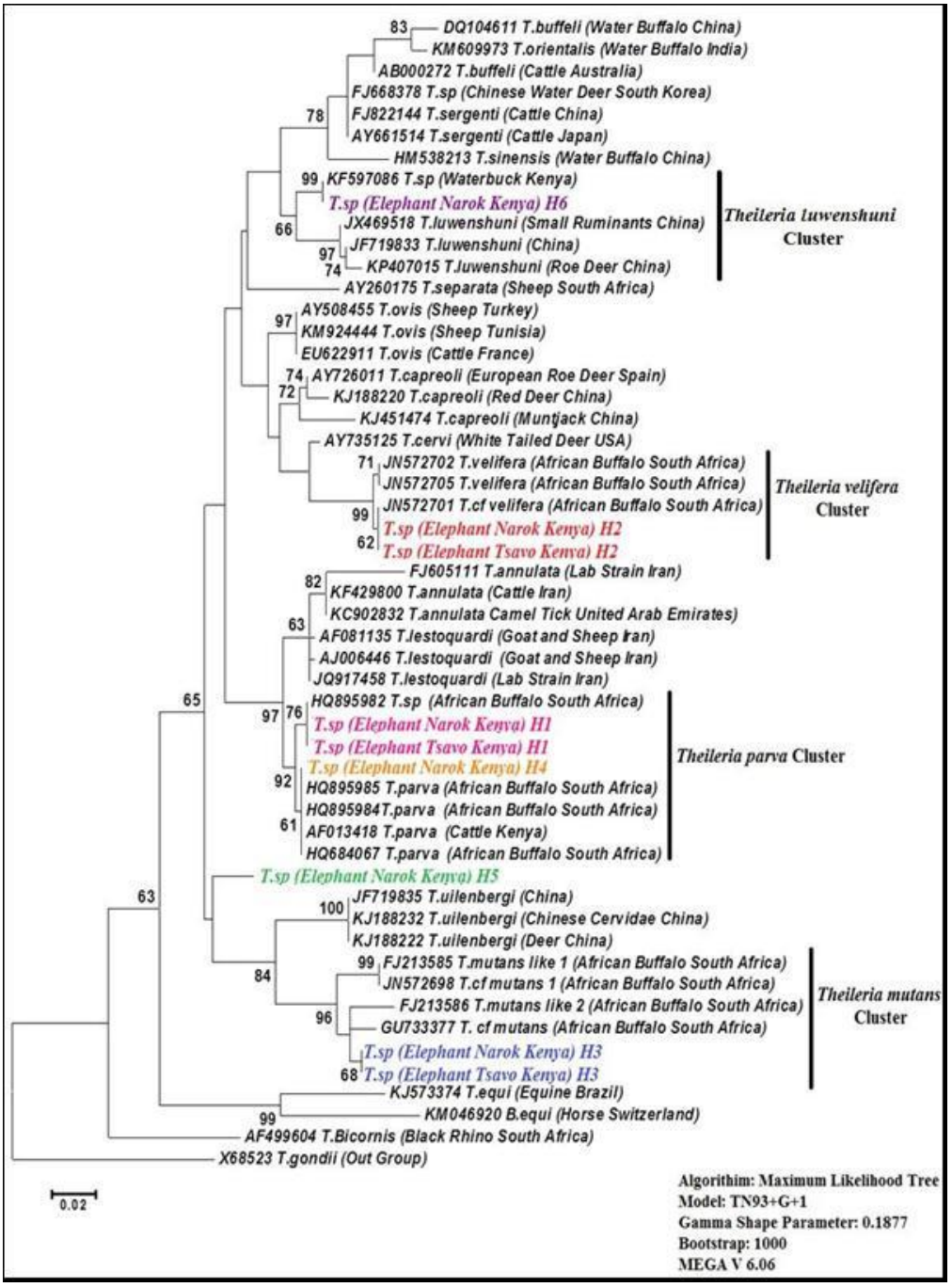

Figure 4.21: Phylogenetic relationship between *Theileria* isolated from African elephants inTsavo and Narok ecosystem and *Theileria* isolates from the GeneBank

Theileria isolates from elephants of Tsavo and Narok are highlighted in color while the rest accessed from the Gene ank are in black. The phylogenetic tree was established using maximum likelihood. Numbers above the branches indicate bootstrap values based on 1000 replicates. *Toxoplasma gondii* was used as an Out group. The phylogeny above represents a high rate of heterogeneity with a gamma shape parameter value of 0.1877. Haplotype 5 and 6 showed no clustering with known *Theileria* species.

The Phylogenetic network analysis confirmed that the 6 haplotype clustering was consistent with the topology of the phylogenetic tree (**Figure 4.22**)

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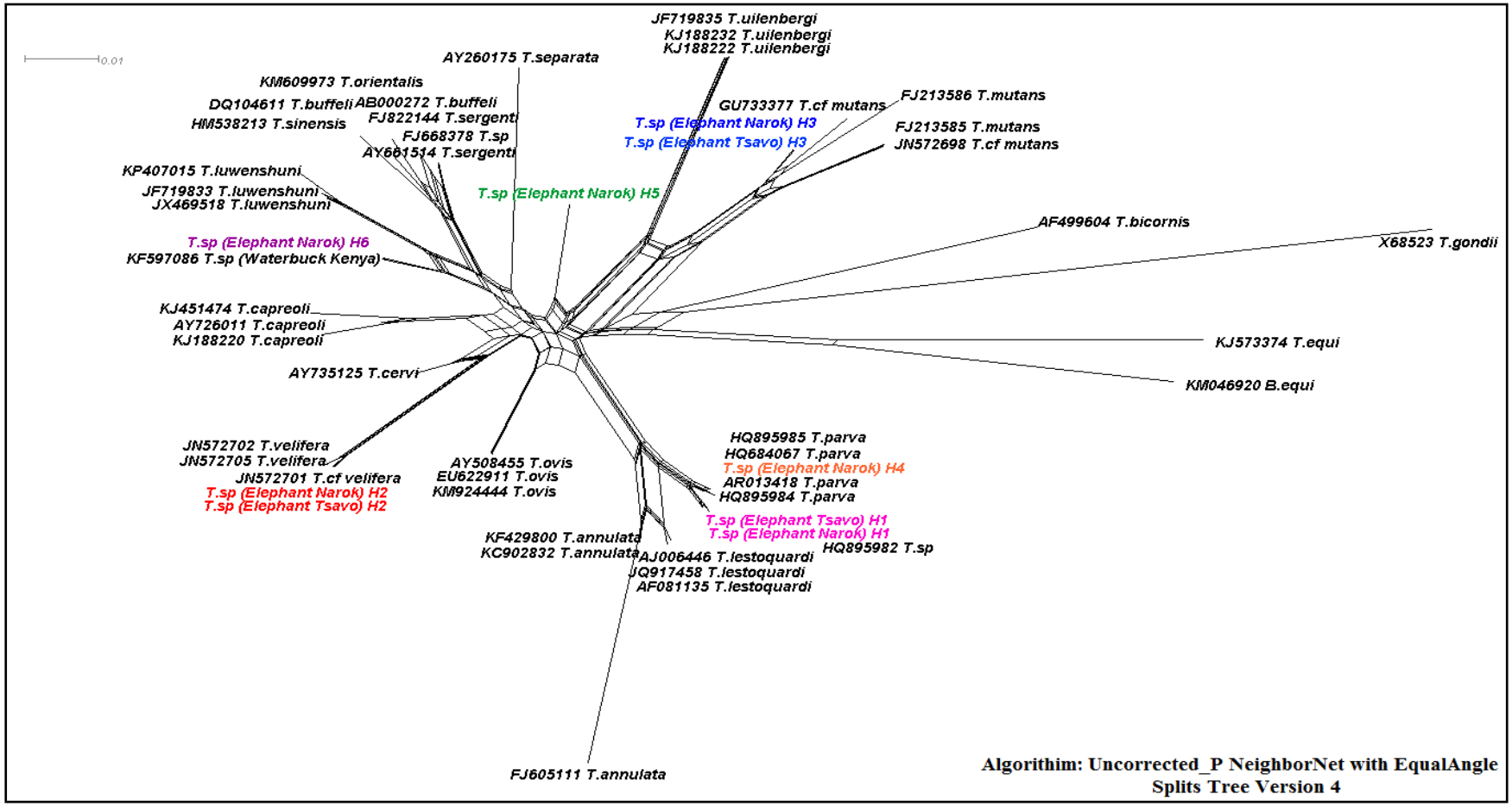

Figure 4.22: Network graph showing the separation and clustering of elephant *Theileria* haplotypes (in color) and isolates fromthe GeneBank (in black)

Genetic divergence between *Theileria* haplotypes isolated in African elephants and *Theileria* species they clustered with was variable (**Table 4.3**).

Table 4.3: Sequence divergence between *Theileria* haplotypes infecting African elephants and known *Theileria* species obtained from GeneBank

	T. parva	*T. cf velifera*	*T. uilenbergi*	*T. mutans*	*T. lewunshuni*
Haplotype 1	0.0074				
Haplotype 2		0.000			
Haplotype 3				0.0739	
Haplotype 4	0.000				
Haplotype 5	0.0668	0.0498	0.0593	0.0759	0.05926
Haplotype 6					0.1597

There was very limited divergence and heterogeneity between *Theileria parva* and haplotype1. There was no divergence between *Theileria* haplotype 2 and *T. cf velifera* but there was considerable divergence between *Theileria* haplotype 3 and *T.mutans.* Haplotype 4 showed no divergence with *T.parva*. There was a greater divergence and heterogeneity between haplotype 5 and *Theileria parva, Theileria cf.velifera, Theileria cf. mutans* and *Theileria lewenshuni.* There was great divergence and heterogeneity between *Theileria* haplotype 6 and *Theileria lewenshuni.*

Polymorphism in the 6 haplotypes was elevated in a small portion of the V4 region of 18s rRNA about 100 bp in length, a property shared by other species (**Figure 4.23**).

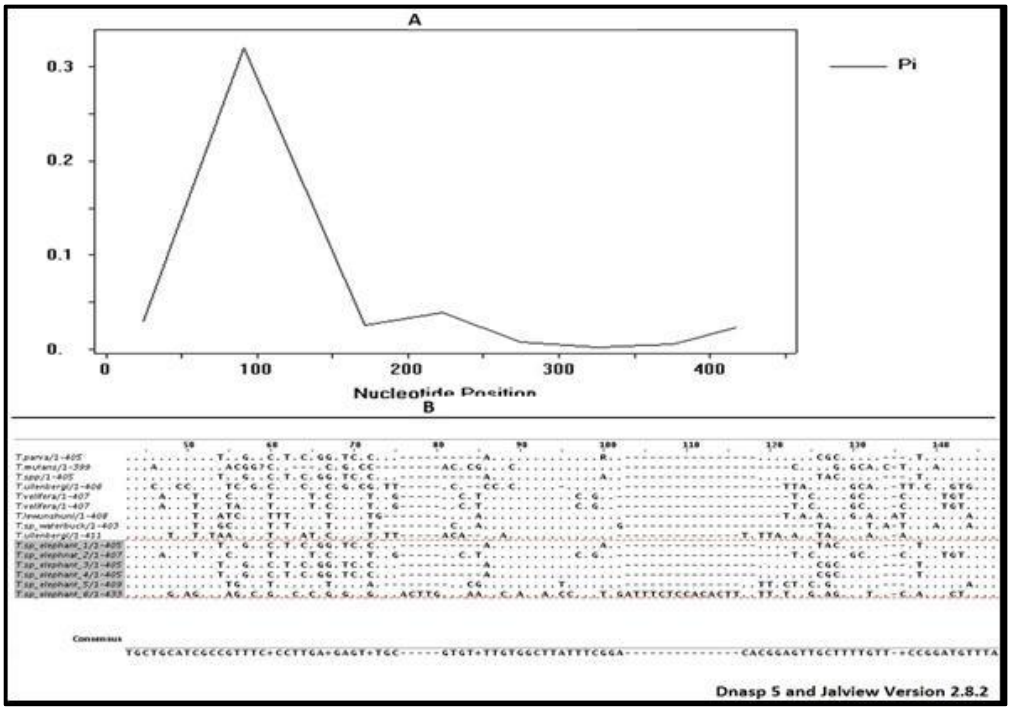

Figure 4.23: Polymorphism in the V4 region of 18s rRNA gene of *Theileria* species

In **Figure 4.23** above A shows that the 18r RNA is localized to a region 1-180 bp from the ILO primers and B is a sequence alignment showing variability in region 1-180 bp within the six elephant *Theileria* haplotypes, *T.parva, T.mutans, T.ulienbergi, T.velifera* and *T.Lewunshuni.*

4.2.2 Phylogenetic relationships, DNA Polymorphism and Divergence of *Theileria* in Wildebeest

Phylogenetic analysis of 3 *Theileria* haplotypes isolated from wildebeest with similar sequences based on Blastn (NCBI) and with sequences of known identity showed a clustering of Haplotype 7 and Haplotype 8 with *Theileria separata* while Haplotype 9 clustered with *Theileria ovis* (**Figure 4.24**).

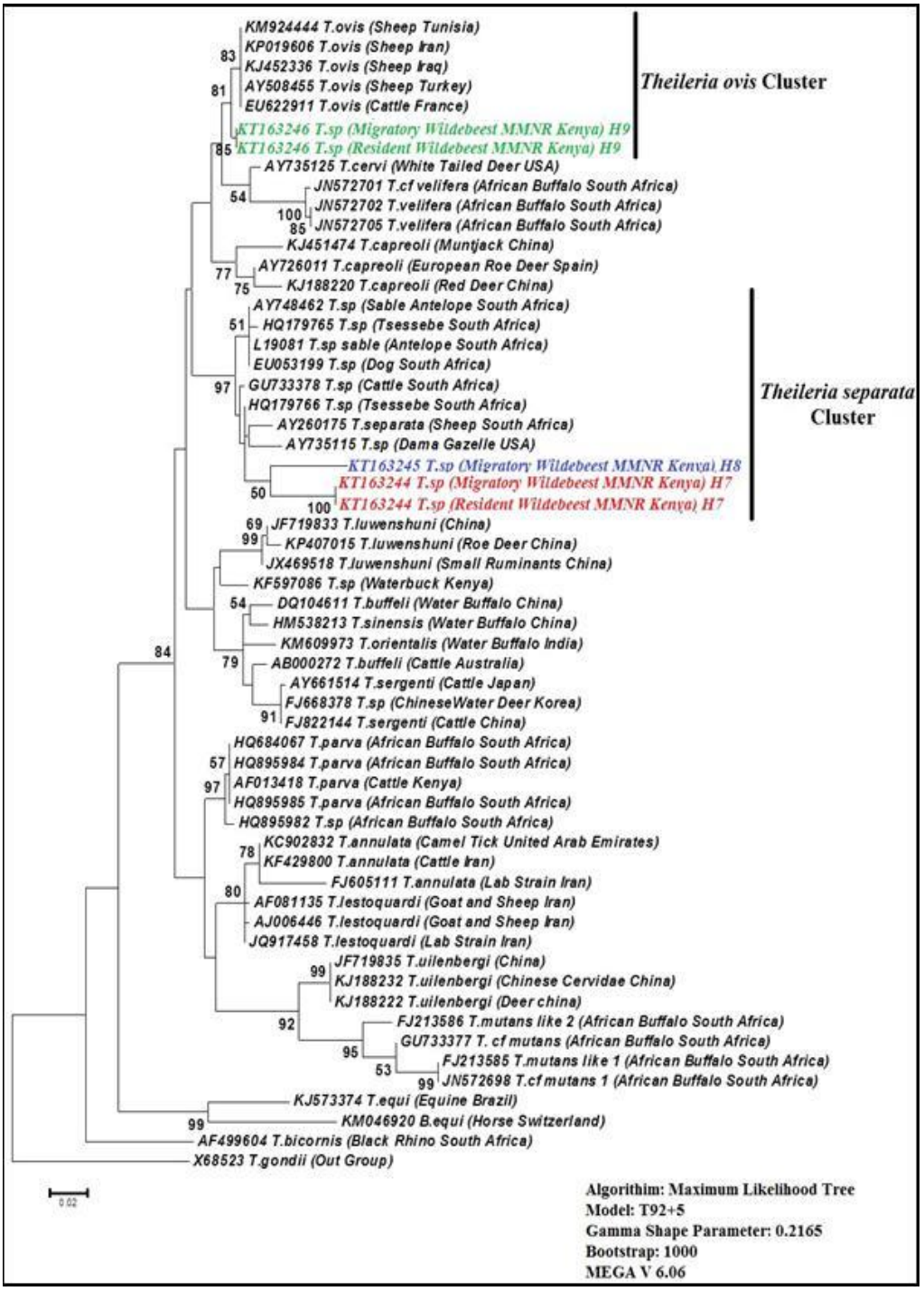

Figure 4.24: Phylogenetic relationship between *Theileria* isolated from wildebeest and *Theileria* isolates from the GeneBank

H-Haplotype

Theileria isolates from wildebeest are highlighted in color while the rest were accessed from the GeneBank. The phylogenetic tree was established using maximum likelihood. The numbers above branches indicate bootstrap values based on 1000 replicates. *Toxoplasma gondii* was used as an Out group. According to the gamma shape parameter value of 0.2165 shows high rate of heterogeneity among the species.

The phylogenetic network analysis confirmed that the haplotype clustering was consistent with the topology of the phylogenetic tree (**Figure 4.25**).

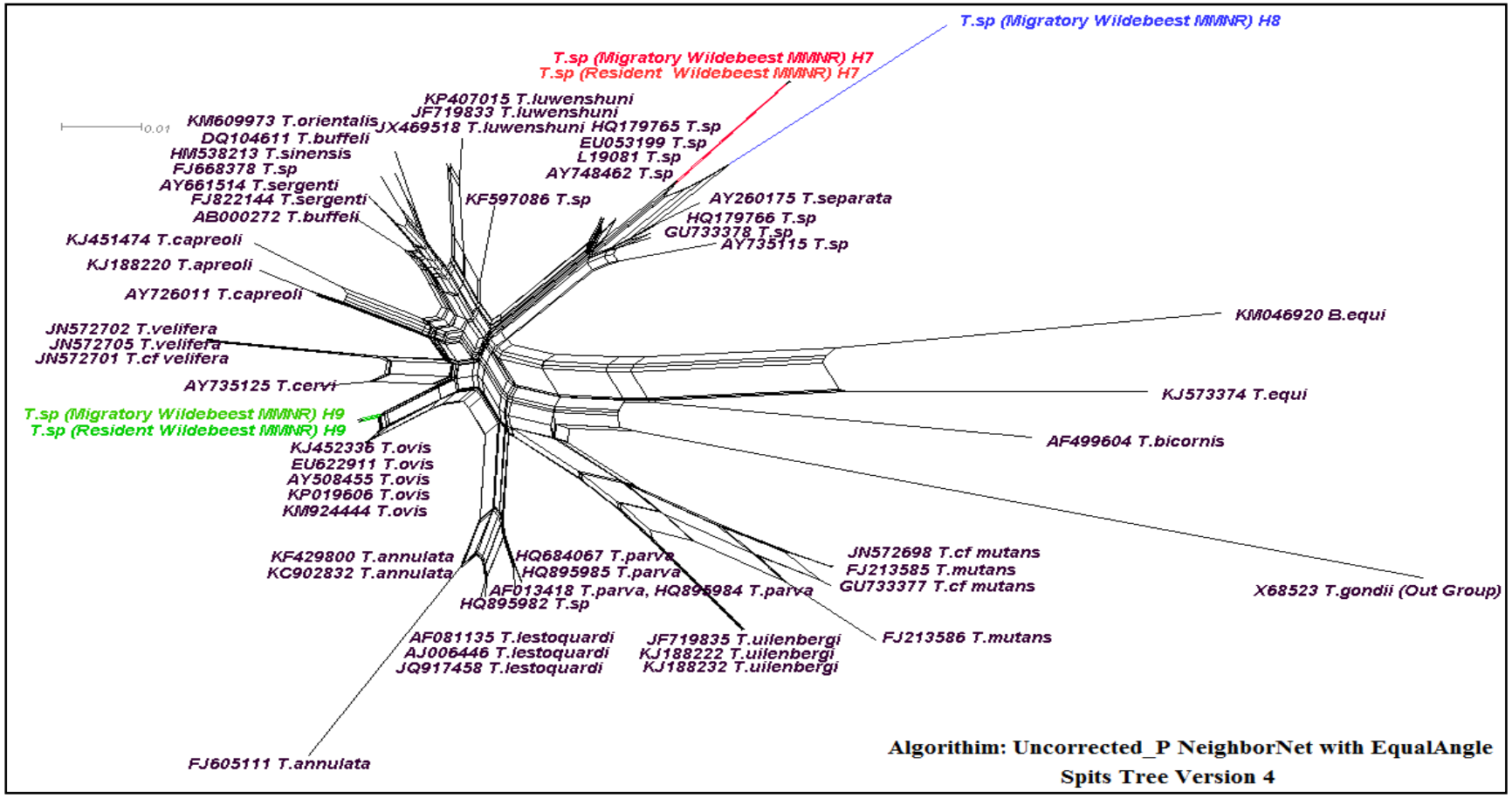

Figure 4.25: Network graph showing the separation of wildebeest *Theileria* haplotypes (in color) and isolates from the GeneBank (in black)

Genetic divergence between *Theileria* haplotypes detected in wildebeest that is KT163246 (Haplotype 7), KTI63245 (Haplotype 8), and KT163244 (Haplotype 9), and *Theileria* species they clustered with was variable (**Table 4.4**).

Table 4.4: Sequence divergence between *Theileria* species infecting wildebeest and closely associated sequences of known species obtained from GeneBank

	KT163244	KT163245	KT163246	*Theileria ovis*	*Thaileria seperata*
KT163244		0.075	0.075	0.077	0.059
KT163245	0.075		0.097	0.100	0.059
KT163246	0.075	0.097		0.007	0.053
Theileria Ovis	0.077	0.100	0.007		0.056
Thaileria seperata	0.059	0.050	0.053	0.056	

There was limited divergence between *Theileria ovis* and KT163246. Greater divergence and heterogeneity was observed between *Theileria separata* and haplotypes KTI63245 and KT163244.

Polymorphism in the three species was elevated in a small region about 100 base pairs in length, a property shared by other species (**Figure 4.26**).

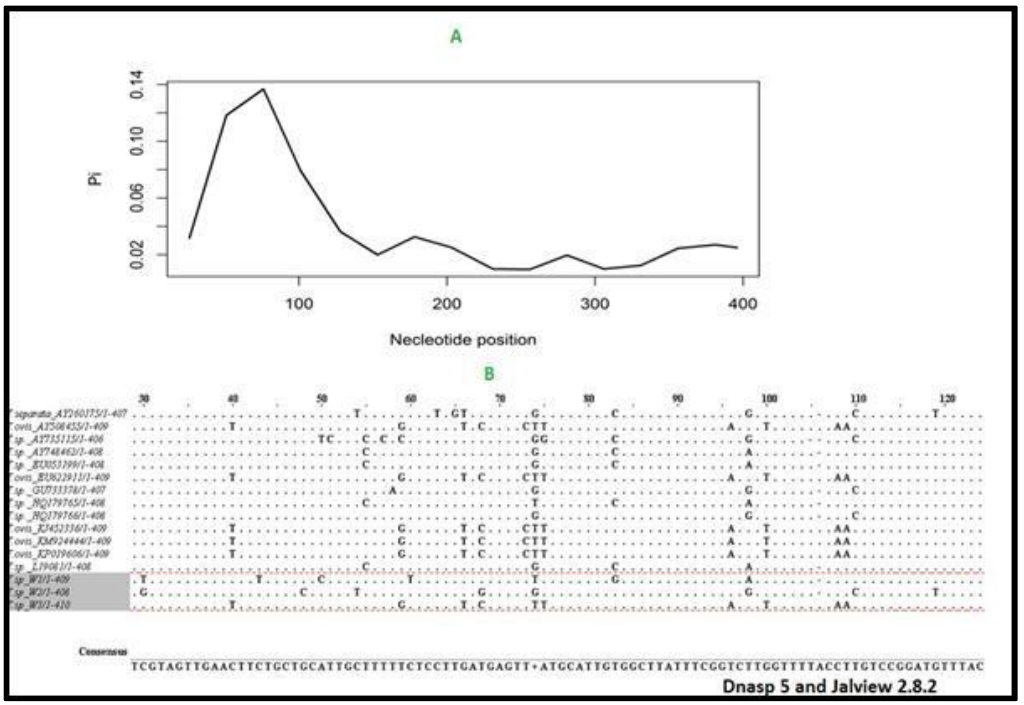

Figure 4.26: Polymorphism in the V4 region of 18s rRNA gene of *Theileria* species

In **Figure 4.26** above, A shows polymorphism localized to a region 1-150 bp from the ILO primers and B is a sequence alignment showing variability in that 1-150 within the three wildebeest haplotypes, *T.ovis* and *T.Seperata* cluster.

4.2.3 Phylogenetic relationships, DNA Polymorphism and Divergence of *Theileria* in Reedbuck and Impala

Phylogenetic analysis of haplotypes with similar sequences to those of known identity showed a clustering of impala haplotype with *Theileria separata* while reedbuck haplotype clustered with *Theileria lewenshuni* (**Figure 4.27**).

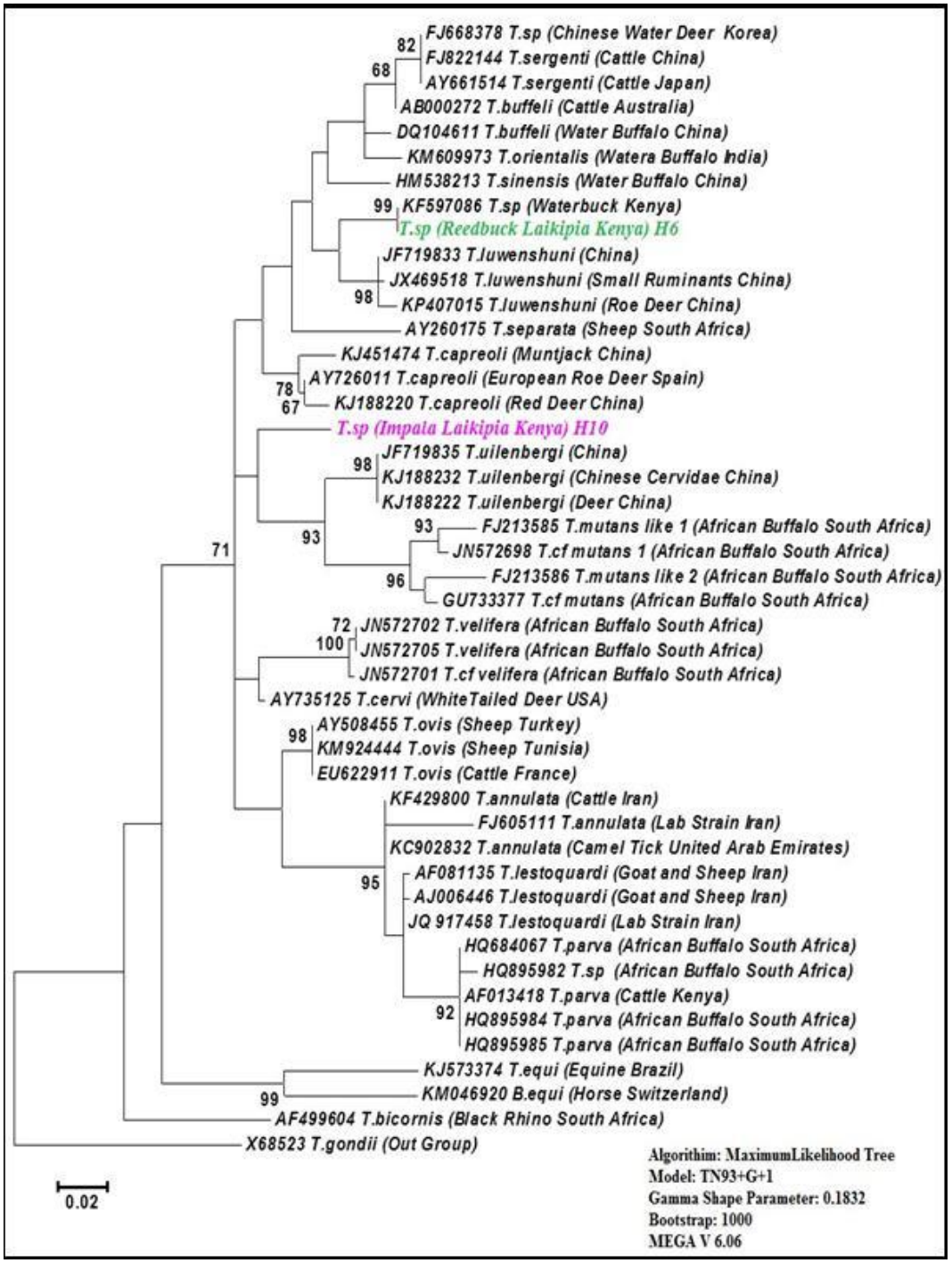

Figure 4.27: Phylogenetic relationship between *Theileria* isolated from impala, reedbuck and *Theileria* isolates from the GeneBank

H: H-Haplotype.

Theileria isolates from impalas and reedbucks are highlighted in color while the rest were accessed from the GeneBank. The phylogenetic tree was established using maximum likelihood. Numbers above the branches indicate bootstrap values based on 1000 replicates. *Toxoplasma gondii* was used as an out group. The rate of heterogeneity among the species is high as the gamma shape parameter is 0.1873.

The phylogenetic network analysis confirmed that the haplotype clustering was consistent with the topology of the phylogenetic tree (**Figure 4.28**).

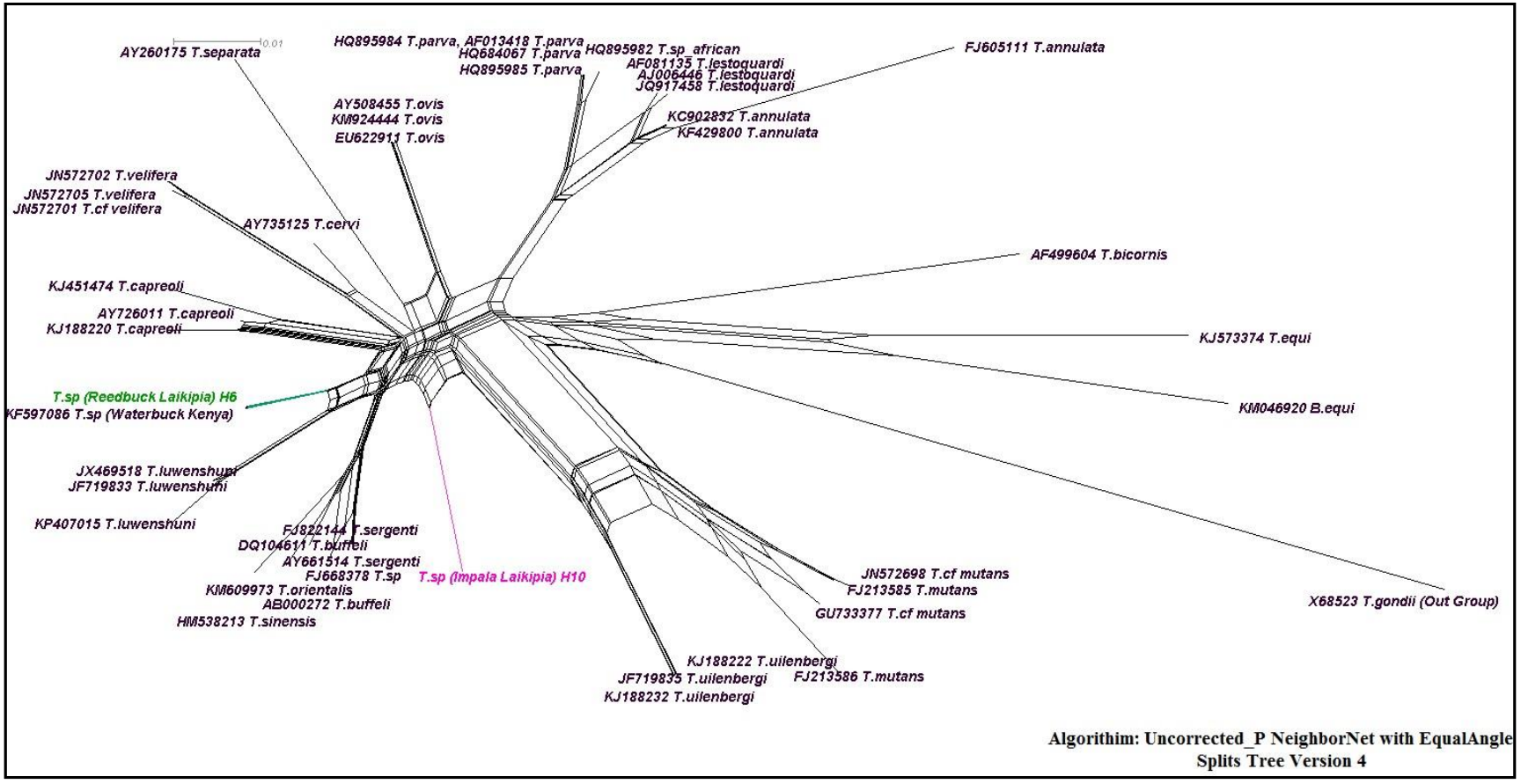

Figure 4.28: Network graph showing the separation of reedbuck and impala *Theileria* haplotypes (in color) and isolates from the GeneBank (in black)

Genetic divergence between *Theileria* haplotypes infecting impala and reedbuck and *Theileria* species they clustered with was variable (**Table 4.5**).

Table 4.4: Sequence divergence between *Theileria* haplotypes infecting reedbuck and impala together with two of the most closely associated sequences of known *Theileria* species obtained from GeneBank

	Haplotype 10	Haplotype 6	*Theileria* separata	*Theileria* lewenshuni
Haplotype 10		0.041	0.064	0.064
Haplotype 6	0.041		0.0676	0.041

There was greater divergence and heterogeneity between *Theileria separata* and *Theileria* species isolated from impala. This divergence was also observed between *Theileria lewenshuni* and *Theileria species* isolated from reedbuck.

Polymorphism in the two haplotypes was elevated in a small region about 100 bp in length in the V4 18s rRNA gene, a property shared by other species (**Figure 4.29**)

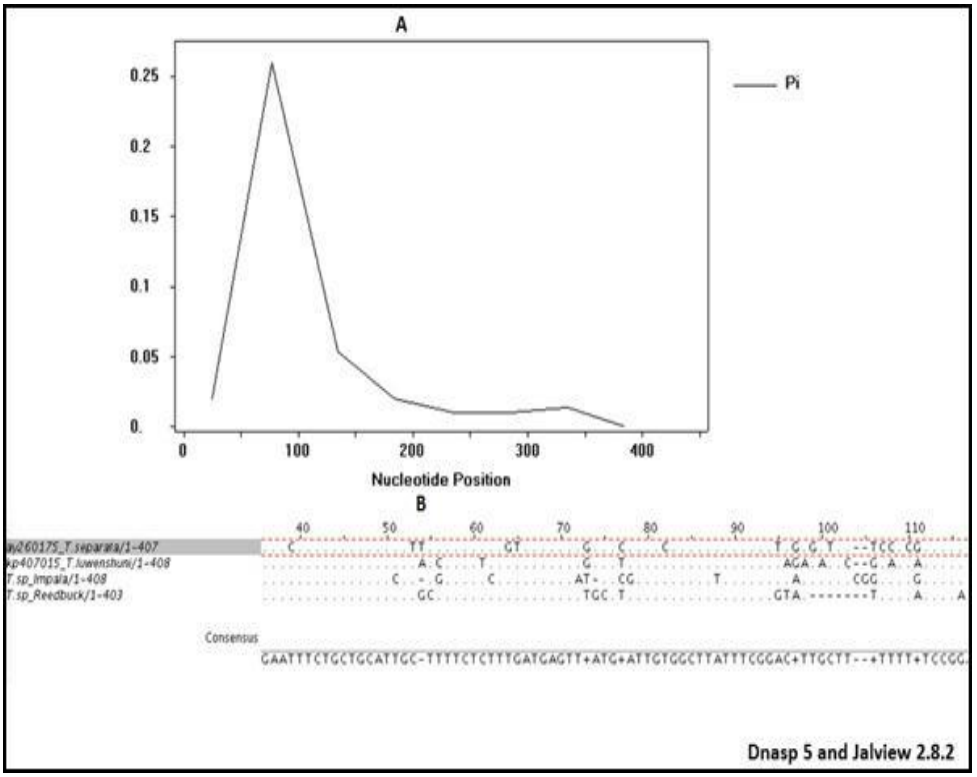

Figure 4.29: Polymorphism in the V4 region of 18s rRNA gene of *Theileria*

A shows that polymorphism is localized to a region 1-150 bp from the ILO primers. B is a sequence alignment showing variability in the region 1-150 base pairs within the two *Theileria* haplotypes from reedbuck, impala, *T.lewenshuni* and *T.seperata* clusters.

4.2.4 Phylogenetic relationships, DNA Polymorphism and Divergence of *Theileria* in Elephants, Wildebeest, Impalas and Reedbucks

Phylogenetic analysis of elephant, impala, reedbuck and wildebeest haplotypes with similar sequences based on BLASTn (NCBI) and with sequences of known identity showed a clustering of impala and reedbuck *Theileria* haplotypes with elephant *Theileria* Haplotypes (**Figure 4.30**). These results revealed several *Theileria* haplotypes unique to each host species but also some common genotypes that are shared between host species. For example *Theileria* haplotype from impala and elephants formed a distinct

clade that had a shared common ancestry with *Theileria luwenshuni*. In addition another distinct clade of *Theileria* potentially representing a new species was shared between reedbuck and elephants. There were no *Theileria* haplotypes shared between elephants and wildebeest (**Figure 4.30**).

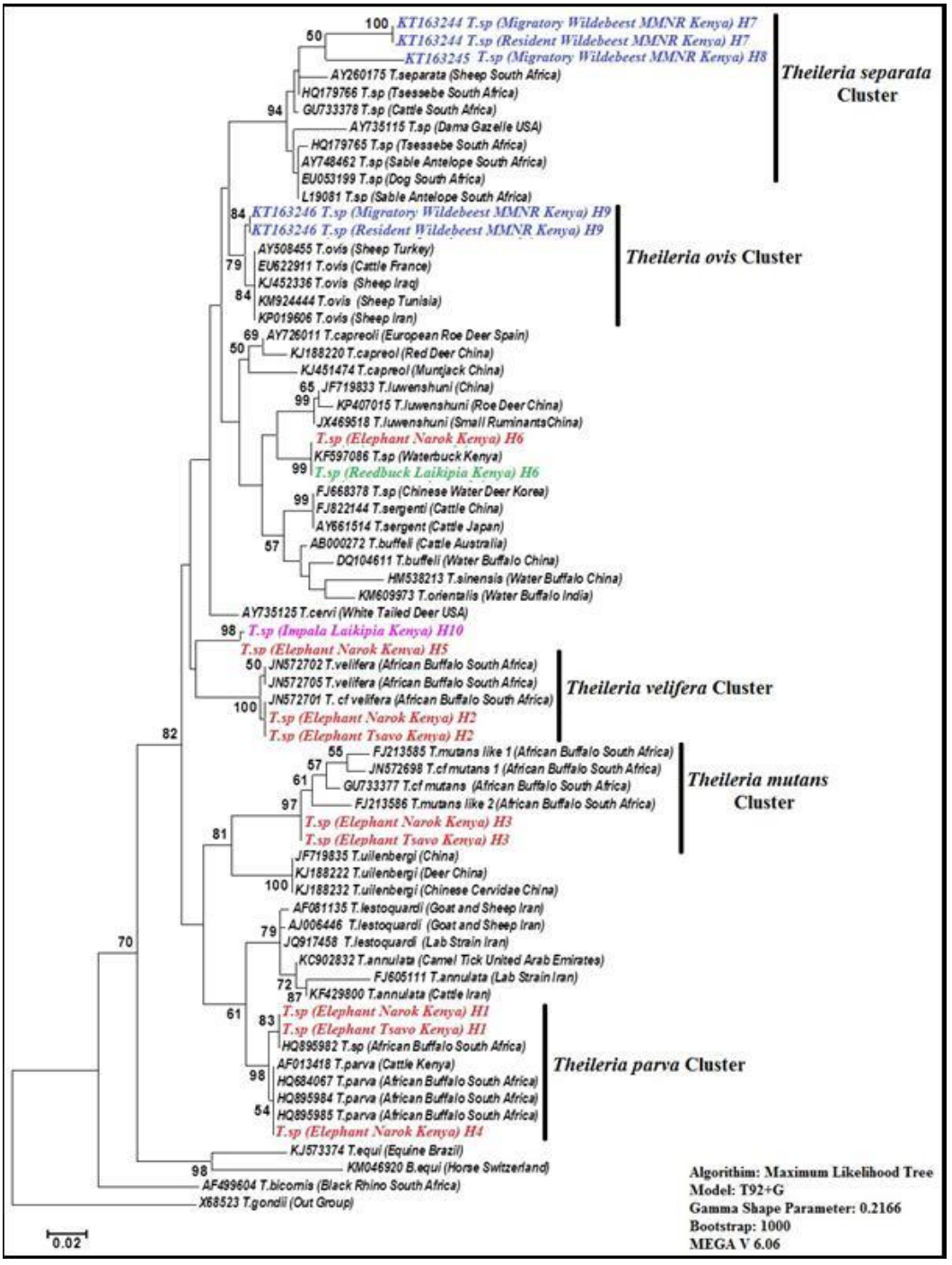

Figure 4.30: Phylogenetic relationship between *Theileria* isolated from wildebeest, elephant, impala and reedbuck and *Theileria* isolates from the GeneBank

The phylogeny was established using maximum likelihood. Numbers above the branches indicate bootstrap values based on 1000 replicates. Wildebeest, elephant, impala and reedbuck *Theileria* isolates are in color while the rest were accessed from the GeneBank. The rate of heterogeneity among the species was high with a gamma shape parameter of 0.2166.

The phylogenetic network analysis confirmed that the haplotype clustering was consistent with the topology of the phylogenetic tree (**Figure 4.31**).

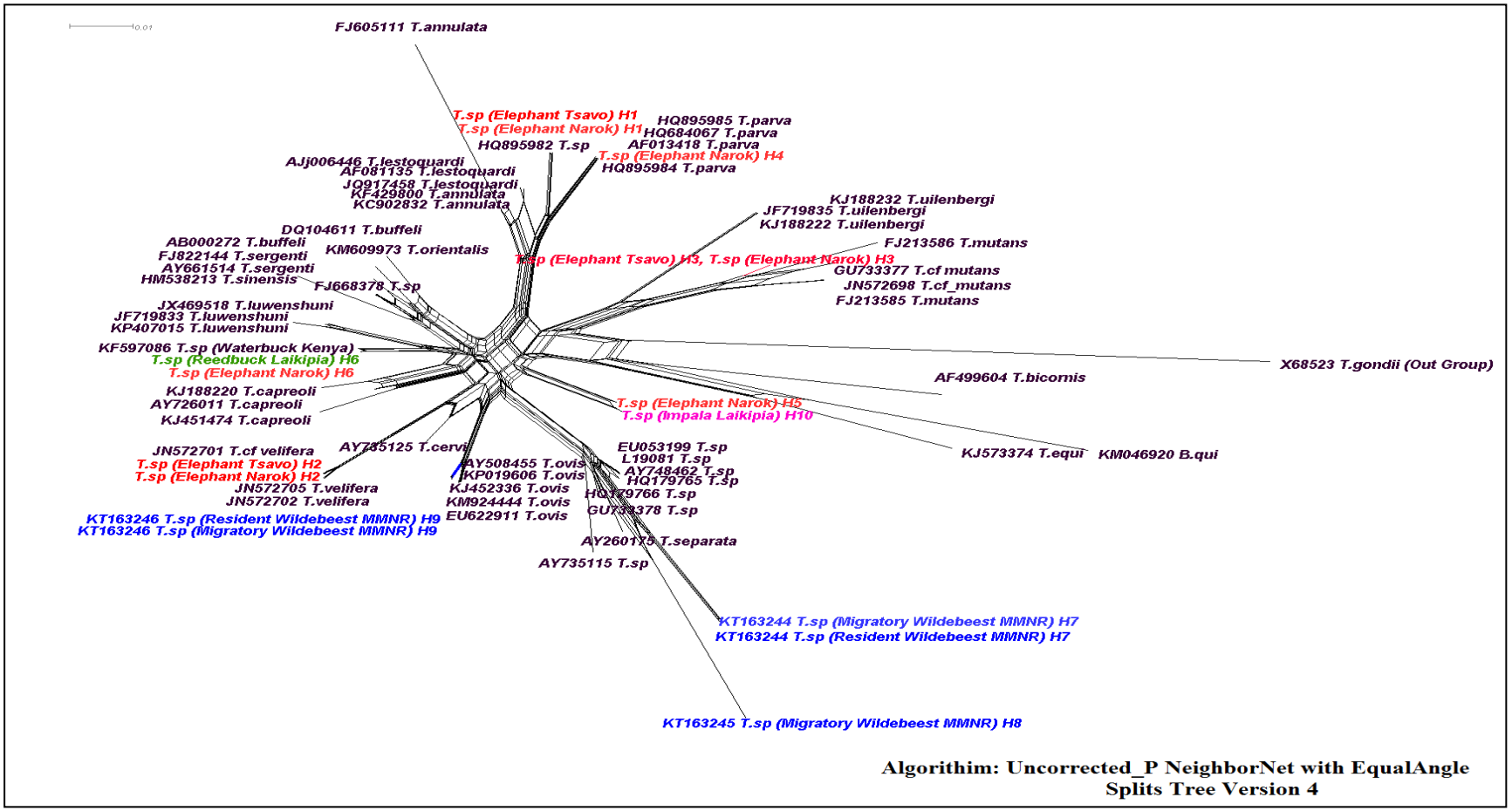

Figure 4.31: Network graph showing the separation of *Theileria* haplotypes from wildebeest, elephant, impala and reedbuck (in color) and isolates from the GeneBank (in black)

4.3. Selection Pressures

4.3.1 Linkage Disequilibrium in the partial 18s rRNA gene of *Theileria* and *Babesia* species

There was linkage disequilibrium in the partial sequence of 18s rRNA gene of *Theileria* species identified in elephants, impalas, reedbucks and wildebeest from Laikipia, Narok and Tsavo ecosystem, Kenya (**Figure 4.32**).

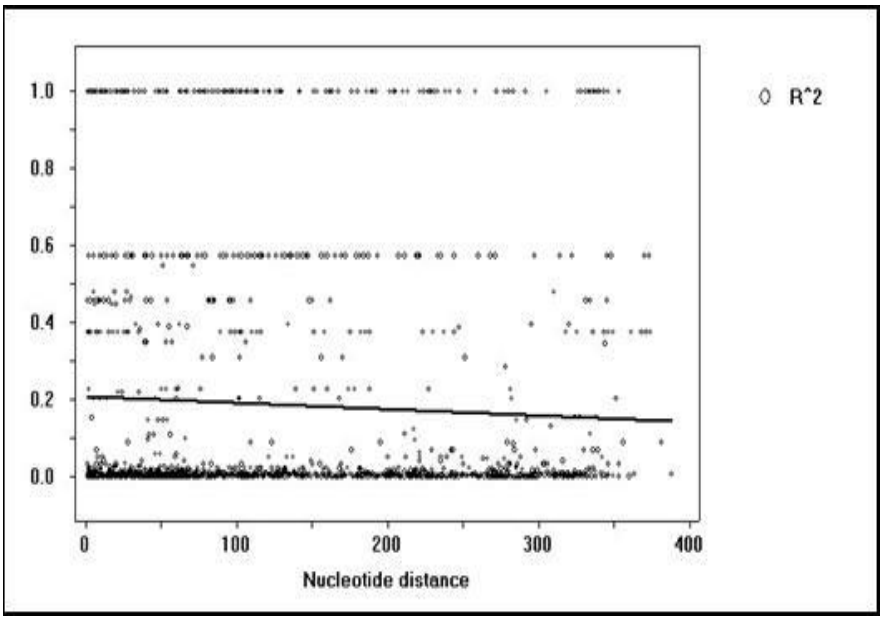

Figure 4.3: Linkage disequilibrium graph of the partial 18s rRNA gene of *Theileria* species

The linkage disequilibrium graph is based on R2 parameter, the y axis represent r2 values and x axis indicate nucleotide distance. The graph show that there is linkage disequilibrium since the R2 value on the graph is greater than zero. Linkage disequilibrium is high between the first1-150 base pairs and declines a little towards end. This linkage disequilibrium

was confirmed by the onferroni procedure and statistical significance by Fishers exact test and Chi-square test where *** P<0.001.

There was Linkage disequilibrium in the partial sequence of 18s rRNA gene of *Babesia* species identified in elephants from Narok and Tsavo ecosystem, Kenya **(Figure 4.33).**

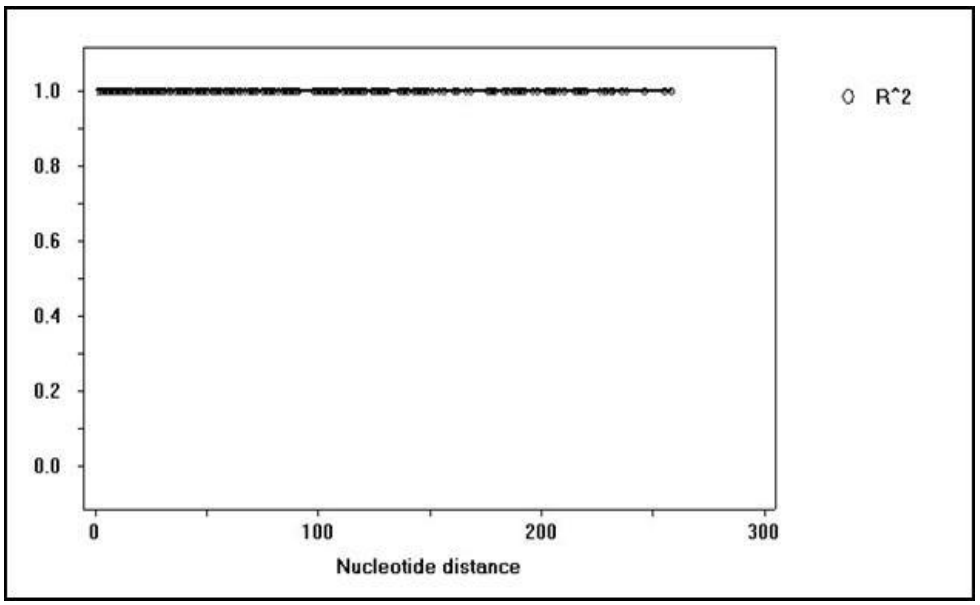

Figure4.33: Linkage disequilibrium graph of the partial 18s rRNA gene of *Babesia* species

The linkage disequilibrium graph is based on R2 parameter, the y axis represent r2 values and x axis indicate nucleotide distance. The graph shows complete linkage disequilibrium since the R2 value on the graph is equal to 1(r2=1). This linkage disequilibrium was confirmed by the Bonferroni procedure and statistical significance by Fishers exact test and Chi-square test where *** P<0.001.

4.3.2 *Theileria* and *Babesia* Haplotype Diversity between and among Populations

Ten haplotypes of *Theileria* were identified in populations of African elephants, wildebeest, impalas and reedbucks from Tsavo, Narok and Laikipia ecosystems. The haplotype diversity of *Theileria* species between the populations was 0.75943. The haplotype diversity among the populations was > 0.5 (**Table 4.6)**.

Table 4.6: *Theileria* haplotype diversity among Tsavo, Narok and Laikipia populations

Population	Haplotype Number	Haplotype Diversity
Tsavo	3	0.
Narok	9	0.553
Laikipia	2	0.545

The table above show high haplotype diversity among *Theileria* species identified in Tsavo ecosystem followed by Narok ecosystem. Laikipia ecosystem was shown to have the lowest *Theileria* species haplotype diversity. Two *Babesia* haplotypes were identified in populations of African elephants from Tsavo and Narok ecosystem. *Babesia* species haplotype diversity between the populations was 0.28986. The haplotype diversity of *Babesia* species among the populations of Narok and Tsavo was P<0.5 (**Table 4.7**).

Table 4.5: *Babesia* Species haplotype diversity among populations

Population	Haplotype Number	Haplotype Diversity
Tsavo	2	0.43956
Narok	1	0

Table 4.7 shows no haplotype diversity in the *Babesia* species from Narok ecosystem. However there was haplotype diversity of 0.43956 in *Babesia* species from Tsavo ecosystem.

4.3.3 Gene flow and Genetic differentiation of *Theileria* and *Babesia* species

Based on haplotype frequencies, there was a statistically significant differentiation among *Theileria* strains isolated from elephants, wildebeest, impalas and reedbucks in , Laikipia and the Tsavo ecosystems in which FST=0.376. Genetic differentiation of *Theileria* species among Laikipia, Tsavo and Narok populations are shown in **Table 4.8**. Genetic differentiation estimate was obtained by the permutation test with 10000 replicates (*,0.01<P<0.05; **, 0.001<P<0.01; ***, P<0.001).

Table 4. 6: Genetic differentiation of *Theileria* species among populations

Population 1	Population 2	Hs	Fst
Tsavo	Narok	0.566	0.338
Tsavo	Laikipia	0.584	0.407
Narok	Laikipia	0.552	0.384

(Fst: F-fixation index, s-subpopulation, t-total population)

Fst is the difference between the average expected heterozygosity of subpopulations and the expected heterozygosity of the total population. As shown in table 10 Fst among Tsavo and Laikipia was considerably high than that of Tsavo and Narok and Narok and Laikipia. These results indicate that there is somewhat heterozygosity on average for the subpopulations that were compared. Hs is the average expected heterozygosity of subpopulations

assuming random mating with each subpopulation. Results show considerably low heterozygosis among the subpopulations as HS >0.5.

There was a statistically significant differentiation among *Babesia* strains isolated from elephants in the Narok and the Tsavo ecosystems where FST=0.23077. Genetic differentiation of *Babesia* species among Tsavo and Narok populations is shown in **Table 4.9**. The Genetic differentiation estimates of *Babesia* species in Narok and Tsavo populations was obtained by the permutation test with 10000 replicates (*, 0.01<P<0.05; **, 0.001<P<0.01; ***,P<0.01).

Table 4.9: Genetic differentiation of *Babesia* species among populations

Population 1	Population 2	Hs	Fst
Tsavo	Narok	0.26	0.23

(Fst: F-fixation index, s-subpopulation, t-total population)

As shown in **Table 4.9** the Fst among Tsavo and Narok was 0.2 indicating less heterozygosity for the subpopulations compared. HS was <0.5 indicating there is little heterozygosity among the subpopulations.

Gene flow estimates of *Theileria* species in Narok, Tsavo and Laikipia populations was significant according to Hudson, Slatkin and Maddison 1992 where Fst was 0.37620 and Nm was 0.83. NM is the effective migration rate. Nm for *Babesia* in Tsavo and Narok subpopulations is low indicating that there is genetic differentiation and gene flow among the subpopulations.

Gene flow estimates in *Babesia* species in Narok and Tsavo populations was significant according to Hudson, Slatkin and Maddison 1992, where Fst was 0.23077 and Nm was1.67. Nm for *Babesia* in Tsavo and Narok

subpopulations is low indicating that there is genetic differentiation and gene flow among the subpopulations.

CHAPTER FIVE

DISCUSSION

5.1 *Theileria* and *Babesia* in African Elephants

Wild animals have been demonstrated to harbor a great diversity of *Theileria* and *Babesia* (Eygelaar *et al*., 2015, Otiende *et al*., 2015). However, since the first microscopic demonstration of piroplasm in Kenyan elephant blood (Brocklesby and Campbell 1963), there is no study that has confirmed which species *Theileria* and *Babesia* infects these charismatic conservation flagship species. This study provides the first molecular confirmation of *Theileria and Babesia* in African elephants. The results from this study have indicated presence of diverse species of *Theileria* and *Babesia* in African elephants in Kenya.

The prevalence of *Babesia* in elephants was higher than that for *Theileria* suggesting a higher prevalence or parasitemia for *Babesia sp* than for *Theileria sp* in elephants. This finding contrast many studies of piroplasms in large mammals where *Theileria* is usually the dominant strain either in terms of prevalence or levels of parasitemia. These results suggest that elephants may be important reservoir of *Babesia* species.

This study confirmed the infection of African elephants by *B. bigemina* (Haplotype 1) and a potentially new species of *Babesia* (Haplotype 2*)*. Phylogeny of *Babesia* inferred using *Babesia* species isolated from African elephant and *Babesia* species sequences retrieved from GenBank revealed presence of *B.bigemina* in African elephant. The *Babesia bigemmina* that clustered with haplotype 1 has been isolated elsewhere in cattle. This Phylogeny showed that the *Babesia* species haplotype 2 isolated from elephants of Tsavo and Narok ecosystem were putatively new genotypes,

which could be new species. Several new genotypes of *Babesia* have been identified in several wildlife species such as rhinos (Nijhof *et al*., 2003), grey kangaroos (Dawood *et al*., 2013), meerkats (Leclaire *et al*., 2015), buffalos (Ibrahim *et al*., 2013), wild dogs, giraffe (Githaka *et al*., 2013), sable antelope (Oosthuizen *et al*., 2008), hyenas and lions (Williams *et al*., 2014). It was interesting that the *Babesia* haplotypes 2 isolated from elephant occurred in two separated elephant populations, which suggests that first, there is no geographical sub- structuring and second, the haplotype is a common infection in the African elephant.

The presence of *B .bigemina* is not only the first in the African elephant, but *B.bigemina* is a species of great economic interest especially because it is associated with severe livestock disease, bovine babesiosis. The losses and control of babesiosis and anaplasmosis in Kenya, cost 5.1 million US dollars annually (Bock *et al*., 2004). Further, presence of *B. bigemina* in African elephants, suggests that African elephant is now an important reservoir, which should not be ignored in the epidemiology and planning control programmes of bovine babesiosis. Previously, African buffalo is thought to be the only significant reservoir of *B. bigemina* and other hemoparasites (Oura *et al*., 2011). This study has confirmed that the African elephant is a host for *B. bigemina.* Specifically the *Babesia bigemmina* it clustered with in the phylogenetic tree has been isolated in cow from Kenya **(Figure 4.18)**. This can possibly indicate that sharing of grazing lands between elephants and livestock contributes to sharing in vectors they host thus the species of *Babesia* they host. Secondly as habitats become more fragmented, animal densities increase in small protected areas due to enhanced protection, anthropogenic mediated stress will increase as wildlife will attempt to colonize their previous ranges now dominated by human activities such as agriculture this can potentially result to parasites switching hosts.

Theileria has not been identified in Elephants yet and thus this is the first study to identify *Theileria* in African elephants. Specifically this study identified 6 *Theileria* haplotypes thus 4 putatively new *Theileria* species, *T.parva* and *T.cf velifera* in African elephants.

This study has indicated a high *Theileria* haplotype diversity in African elephants. The high number of *Theileria* haplotype diversity is consistent with the prediction that in ungulates and other mammals large bodies host species tend to have high parasite diversity (Bordes *et al* 2008).

Phylogenetic relationship showed that *Theileria* haplotype 4 from elephant of Tsavo and Narok clustered with *T.parva* and was completely identical to *T.parva* that has been previously isolated in African buffalo in South Africa and cattle in Kenya. This result shows that elephant interactions with buffalos especially over grazing land can result in hosting similar strain of *Theileria* harbored by buffalos. This could be possibly true as buffalos in Tsavo and Narok ecosystem are found to share grazing lands with elephants. On the other hand over the past few years cattle have been found grazing in National reserves and parks, specifically in the Tsavo ecosystem cattle are widely found in the parks this could results to tick sharing thus hosting similar strains *Theileria*. Elephants are mostly found in the edges of the national parks and reserves and mostly in community lands they end up sharing grazing lands with livestock thus an opportunity for generalist parasites to switch hosts. *Theileria parva* is known to cause East Coast fever in cattle, a disease that has big economic impact on livestock production. Mortality from East Coast Fever caused by *Theileria parva* is a major source of calf mortality in rural areas when tick control is not given much attention.

In Kenya for example, *T. parva* infection is of significant threat to the

Livestock industry in different ways: via economic impact of East Coast Fever from morbidity and mortality and loss in production in all systems of production and also in the measures undertaken to control the vector and the disease. It was estimated that the cost of vector control with the use of acaricide ranges between US$6 and US$36 per adult animal in Kenya, Tanzania and Uganda (Minjauw & McLeod, 2003). East Coast Fever is a hindrance to introduction of exotic breeds of cattle that are susceptible to it and therefore limiting development in the livestock sector greatly. This loss is dubbed –lost potential.

Identification of *T.parva* in African elephant is thus of concern particularly because a single spill over from wildlife to livestock may have severe consequences not only on health, but also on economy. The virulence of the *T. parva* isolated from the African elephant is not known (Gachohi *et al*., 2012)

Theileria haplotype 2 from elephants of Tsavo and Narok was confirmed by the phylogeny to be T.cf velifera. T.cf velifera has been found mostly in African buffalos. *Theileria* haplotype 1, *Theileria* haplotype 3, *Theileria* haplotype 5 and *Theileria* haplotype 6 are potentially new *Theileria* species. Novel *Theileria* species have been reported in a number of wildlife hosts. For example three novel *Theileria* haplotypes have been identified in Rhinos (Otiende *et al*., 2015) and novel *Theileria* species have been identified in grant gazelles of Kenya (Hawkins *et al*., 2015).

Narok elephant population had a high haplotype or species diversity of *Theileria* compared to Tsavo elephant population; In fact all *Theileria* haplotypes from Tsavo were also represented in Narok and constituted half of the haplotypes found in Narok. The Narok elephant population was isolated

and was in conflict with people. Such isolation and conflict with humans is speculated to increase parasite diversity in several ways. First elephants

coming into conflict with humans tend to be highly stressed as assessed from elevated glucocorticoid levels in the feces compared to elephants in protected areas. Stress has immunosuppressive effects which are likely make elephants susceptible to infections from different strains of *Theileria*. Secondly, isolation and compression of elephants into small areas can lead to locally elevated host density, increased edge effects and exposure to several edge species with consequences for increased pathogen transmission within and among different host species. Tsavo ecosystem also has the largest number of elephants in Kenya.

Although *Theileria* haplotypes identified in this study clustered closely with *Theileria* and *Babesia* species of known identity. This study could not confidently confirm their species identity except for elephant *Babesia* haplotype 1 that showed no divergence to *Babesia bigemina* (**Table 4.1**). Elephant *Theileria* haplotype 2 showed no divergence to *T.cf veilifera* and elephant *Theileria* haplotype 4 that showed no divergence to *T.parva* (**Table 4.3**).

5.2 *Theileria* and *Babesia* in Wildebeests

Results from this study show that all the wildebeests were infected with *Theileria*, which is a high prevalence but consistent with infection level in other wild mammalian hosts. For example in another study the prevalence of *Theileria equi* in Grevy‘s zebra was found to be to be 100% (Hawkins *et al* 2015). Similarly high levels of infection have been reported in African buffalo populations in Uganda (Oura *et al* 2011). This study presents the first genetic identification of *Theileria* in the East African blue wildebeests.

This study specifically identified three new *Theileria* haplotypes in the wildebeest population in which the most abundant haplotype clustered in

Theileria ovis clade and the other two were within the *T.separata* cluster (**Figure 4.24**). *Theileria ovis* and *T.separata* are mainly identified in small or medium sized wild and domestic ruminants. Elsewhere, both *Theileria* ovis and *Theileria separata*, beside *Theileria lestoquardi*, are the cause of ovine theileriosis in sheep and goats (Schnittger *et al*., 2003; Razmi & Yaghfoori, 2013); however, the occurrences of *Theileria* infecting sheep and goats in Kenya are not known. In wildlife, *Theileria* sp closely related to *Theileria separata* have been identified in grey duiker (*Sylvica pragrimmia*), common tsessebe (*Damaliscus lunatas*) and sable antelope (*Hippotragus niger*) (Nijhof *et al*., 2005).

Wildlife is considered important reservoir of up to 77% of the livestock diseases (Cleaveland *et al*., 2001). Since most of the *Theileria* species are multi-host parasites, wildebeest in their large populations (Serneels & Lambin, 2001; Homewood *et al*., 2001) coupled by the high *Theileria* prevalence are likely to provide an inexhaustible pool which poses perennial risks of theileriosis in livestock. Several species of *Theileria* have recently been identified in wild mammalian species in Kenya, of which majority have been novel species or haplotypes (Githaka *et al*., 2013; Githaka *et al*., 2014; Hawkins *et al*., 2015; Otiende *et al*., 2014). Increasing concern on zoonotics and zoophilic diseases is driving the interests in understanding the diversity of pathogens harbored by wildlife. This interest, coupled by the availability of robust molecular tools has resulted in the plethora of studies on the detection of novel *Theileria* species and/or haplotypes.

In the present study, two haplotypes (haplotype 7 and 9), were shared between the resident and migratory wildebeests. This suggests that migration

of this massive population is playing a central role in disease spread and in homogenizing the distribution of *Theileria* haplotypes across spatially distinct host populations in the Serengeti-Maasai Mara ecosystem. The population of resident wildebeest is estimated to be 31, 000 whereas the migratory population that mostly resides on the Serengeti is estimated to be 1.2 Million (Serneels & Lambin, 2001; Homewood *et al*., 2001). Migratory wildebeest is the carrier of the wildebeest-derived Malignant Catarrhal fever, which is a lethal viral disease in cattle, and a major constraint in livestock farming among the Maasai pastoralist communities (Barnard *et al*., 1994).

Although the *Theileria* haplotypes from wildebeest clustered closely with known *Theileria* species, this study could not confidently confirm their species identity except for haplotype 9 which clustered with *Theileria* ovis. This haplotype had limited sequence divergence from *T. ovis*. The *Theileria* separata cluster which contained haplotypes 7 and 8 displayed greater sequence divergence within the cluster suggesting that there is either greater genetic heterogeneity in this species or that the cluster contains distinct species that are closely related to T. separata. This study could not confirm the relationship between our haplotype 7 and 8 with previously confirmed species of *Theileria* (*Theileria gorgonis*) known to infect blue wildebeest due to the lack of sequence data for this species in GenBank (Burridge, 1975). Support for heterogeneity hypothesis within a cluster comes from studies on genetic variation of *T. parva* infecting buffalo and cattle, which have shown that these two hosts can maintain almost independent variants of *T. parva* even when opportunities for cross transmission in sympatry do occur (Oura *et al*., 2011; Elisa *et al*., 2014).

5.3 *Theileria* and *Babesia* in Impalas and Reedbucks

This study confirmed presence of *Theileria* in impalas and reedbucks of Kenya. This is the first genetic identification of piroplasm in this wildlife host species. The prevalence of *Theileria* in impalas and reedbucks was 100% this is in consistence with prevalence of *Theileria* in other wildlife host like Grevy‘s zebras (Hawkins *et al* 2015).

Phylogenetic relationship showed that *Theileria* haplotypes isolated from impalas and reedbucks formed distant clades with other known *Theileria* species. These results suggest that they are a potentially new species of *Theileria*. *Theileria* species from reedbuck was found to be similar to *Theileria* that had previously been isolated from a waterbuck in Kenya (Githaka *et al*., 2014). It also formed a distinct cluster with *Theileria luwenshuni* although the sequence divergence between the two was estimated to be 0.041 thus indicating a high rate of nucleotide heterogeneity. This result is in consistence with results from other studies where variation and nucleotide heterogeneity in the 18s r RNA gene of *Theileria* has been observed even among *Theileria* species in the same clade (Chaisi *et al*., 2013, 2014).

It was interesting to find 2 different haplotypes of *Theileria* unique and specific to impala and reedbuck in the same ecosystem, exposed to the same habitat and share grazing grounds with a possibility of cross species parasite transmission via shared vectors. Secondly there was limited *Theileria* diversity in Laikipia ecosystem which was interesting because in this study site impalas and reedbuck mingle with a large number of wild ungulates amounting to up to 25 different species (Hooge *et al*., 2015). This result suggests that these *Theileria* haplotypes are host specific and thus rarely shared among wild ungulates.

Identification of potentially new species of *Theileria* in reedbuck that formed a distinct clade with *Theileria luwenshuni*, a species that has been reported to infect small ruminants and highly pathogenic to sheep and goats particularly in China (Yin *et al*., 2008) raises the question as to whether reedbucks is a source of novel *Theileria luwenshuni.* This could be negatively influencing livestock since in Laikipia ecosystem, impalas and reedbucks share grazing pastures with small domesticated ruminants like goats and therefore host ticks which would parasitize domesticated ruminants (Walker *et al*., 2000).

5.4 Selection pressures on *Theileria* and *Babesia* harbored by populations of African elephants, Wildebeests, Impalas and Reedbucks in Kenya

There was a dual influence of host species and individual animals to the molecular variation of the *Theileria* 18S rRNA gene and less influence of spatial isolation. These results suggest that spatial isolation of hosts does not influence so much the evolution of *Theileria* species infecting wildlife (Odongo *et al*., 2006). This could result from the fact ticks that transmit the diseases are ubiquitous and most are host generalist. This means that other hosts that are widespread and intersect isolated ecosystems which may play an important role in long distance transmission of *Theileria* and therefore limiting the influence of genetic drift on divergence of isolated *Theileria* from divergent host populations. However *Babesia* infecting elephants had a haplotype distribution that was divergent across host populations. These results suggest an important role played by host species and individual animals in the molecular evolution. This could be caused by the fact that some strains of *Theileria* are common on some host species than others. The influence of individual animals to molecular variation in *Theileria*18S r RNA suggests a role of individual immunity in influencing strain infection patterns across individuals within a given species (Katzer *et al*., 2007, McKeever 2009).

5.5 General Discussion

The results from this study have indicated presence of *Theileria* in African elephants, wildebeests, impalas and reedbucks in Kenya. *Babesia* was only identified in African elephant‘s thus African elephants had both *Theileria and Babesia.*

The prevalence of *Theileria* in impala, reedbuck and wildebeest was high. The prevalence and diversity of *Theileria* and *Babesia* was higher in elephants compared to other large mammals like rhino or giraffe (Oosthuizen, Allsopp, Troskie, Collins, & Penzhorn, 2009, Otiende *et al*., 2014).) . The pattern of infection however similar to that found in buffalo, suggesting buffalo and elephants are important reservoirs for *Theileria and Babesia* species infecting wild and domestic animals (Oura *et al*., 2011).

Theileria and *Babesia* haplotypes identified in this study were host specific except for only one haplotype that was shared by elephants in Narok and reedbucks in Laikipia that is haplotype 6 (**Figure 4.30**). This suggests a spatial phenomenon in distribution of *Theileria* species in that different ecosystems can have similar strains of the parasites. Two haplotypes of *Theileria* that is haplotype 7, GenBank accession number, KT163244 and haplotype 9, GenBank accession number, KT163246 were shared between the resident and migratory wildebeests (**Figure 4.24**). This suggests that migration of this massive wildebeest population is playing a central role in disease spread and in homogenizing the distribution of *Theileria* haplotypes across spatially distinct host populations in the Serengeti-Maasai Mara ecosystem.

A comparison of phylogenetic relationships among known *Theileria* species and those isolated from elephants, impala, and wildebeest revealed that

elephants had a lot of unique *Theileria* haplotypes some of which were shared with reedbuck.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

From the findings of this study it was concluded that;

1. Elephants were the only species infested with both *Theileria and Babesia* while wildebeests, impalas and reedbuck were only infested with *Theileria.*
2. Nine potentially new species of *Theileria* were identified in elephants, wildebeest, impalas and reedbucks in Kenya, while one novel *Babesia* species was identified in elephants in Kenya.
3. *Theileria* species haplotype 6 was shared between elephants and impalas. While *Theileria* species haplotype 7 and 9 (KT163244 and KT163246) respectively were shared between resident and migratory wildebeest.
4. *Theileria* prevalence was high in wildebeest, impalas and reedbuck where‘s in elephants it was low. However *Babesia* prevalence was high in elephants.
5. Elephants were found to be infested with *Theileria parva* and *Babesia bigemmina*, piroplasm that are commonly found in Cattle and African buffalo.
6. Narok ecosystem was found to have high *Theileria* species diversity as opposed to Laikipia and Tsavo ecosystem. While *Babesia* species diversity was high in Tsavo ecosystem as compared to that of Narok ecosystem.

6.2 Recommendations

Following the findings of this study I recommend that;

1. More molecular identification study of piroplasms should be done using a larger and random sample size of elephants, wildebeest, impalas and reedbuck from all conservation areas to characterize the species of piroplasms infecting them.
2. KWS should implement preventive measure in elephant translocation as *Babesia* and *Theileria* infestation can lead to mortality in this big mammal.
3. Livestock and tick samples from communities neighboring the National parks and reserves should be sampled.

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APPENDICES

Appendix I: Blastn search results with results with 18S rRNA sequences of *Babesia* species obtained from the African Elephant

#	Location	Haplotype ID	Sample ID	Highest Blast Match	Acession #	E Value	% Similarity
1	Tsavo	Haplotype 1	EEHV4	*Babesia bigemina* Isolate Umiam 18S RNA gene, partial sequence	KF112076.1	0.0	100%
2	Tsavo	Haplotype 1	EEHV40	*Babesia bigemina* Isolate Umiam 18S RNA gene, partial sequence	KF112076.1	0.0	100%
3	Tsavo	Haplotype 1	EEHV48	*Babesia bigemina* Isolate Umiam 18S RNA gene, partial sequence	KF112076.1	0.0	100%
4	Tsavo	Haplotype 1	Elephant	*Babesia bigemina* Isolate Umiam 18S RNA gene, partial sequence	KF112076.1	0.0	100%
5	Tsavo	Haplotype 2	EEHV19	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
6	Tsavo	Haplotype 2	EEHV 29	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial	HQ840969.1	4e-142	92%

				Sequence			
7	Tsavo	Haplotype 2	EEHV 11	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
8	Tsavo	Haplotype 2	EEHV12	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
9	Tsavo	Haplotype 2	EEHV 30	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
10	Tsavo	Haplotype 2	EEHV 35	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
11	Tsavo	Haplotype 2	EEHV 34	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
12	Tsavo	Haplotype 2	EEHV 33	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
13	Tsavo	Haplotype 2	EEHV 39	*Babesia orientalis* strain DaYe18S	HQ840969.1	4e-142	92%

				ribosomal RNA gene, Partial Sequence			
14	Tsavo	Haplotype 2	EEHV 49	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
15	Narok	Haplotype 2	ELE 5	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
16	Narok	Haplotype 2	ELE 8	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
17	Narok	Haplotype 2	ELE 9	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
18	Narok	Haplotype 2	ELE 12	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
19	Narok	Haplotype 2	ELE 17	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%

20	Narok	Haplotype 2	ELE 18	*Babesia orientalis* strain DaYe 18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
21	Narok	Haplotype 2	ELE 30	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
22	Narok	Haplotype 2	ELE 34	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
23	Narok	Haplotype 2	ELE 35	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%
24	Narok	Haplotype 2	ELE 40	*Babesia orientalis* strain DaYe18S ribosomal RNA gene, Partial Sequence	HQ840969.1	4e-142	92%

Appendix II: Blastn search results with 18S rRNA sequences of *Theileria* species obtained from the African Elephant, Wildebeest, Impala and Reedbuck

#	Location	Haplotype 1D	Sample ID	Highest Blast Match	Acesssion #	E value	% Similarity
1	Tsavo	Haplotype 1	EEHV 14	*Theileria sp. ex Syncerus caffer* MCO-2011 clone V8b 18S ribosomal RNA gene, partial sequence	HQ895982.1	0.0	100%
2	Narok	Haplotype 1	ELE 30	*Theileria sp. ex Syncerus caffer* MCO-2011 clone V8b 18S ribosomal RNA gene, partial sequence	HQ895982.1	0.0	100%
3	Tsavo	Haplotype 2	EEHV 16	*Theileria cf. velifera (Syncerus caffer)* clone H4a 18S ribosomal RNA gene, partial sequence	JN572701.1	0.0	100%
4	Narok	Haplotype 2	ELE 31	*Theileria cf. velifera (Syncerus caffer)* clone H4a 18S ribosomal RNA gene, partial sequence	JN572701.1	0.0	100%
5	Tsavo	Haplotype 3	EEHV 15	*Theileria cf. mutans* 3 18S	GU733377.1	0.0	98%

				ribosomal RNA gene, partial sequence			
6	Narok	Haplotype 3	EEHV 28	*Theileria cf. mutans* 3 18S ribosomal RNA gene, partial sequence	GU733377.1	0.0	98%
7	Tsavo	Haplotype 3	EEHV 41	*Theileria cf. mutans* 3 18S ribosomal RNA gene, partial sequence	GU733377.1	0.0	98%
8	Narok	Haplotype 3	ELE 27	*Theileria cf. mutans* 3 18S ribosomal RNA gene, partial sequence	GU733377.1	0.0	98%
9	Narok	Haplotype 4	ELE 4	*Theileria parva* isolate KNP102 18S ribosomal RNA gene, partial sequence	HQ684067.1	0.0	100%
10	Narok	Haplotype 4	ELE 27	*Theileria parva* isolate KNP102 18S ribosomal RNA gene, partial sequence	HQ684067.1	0.0	100%
11	Narok	Haplotype 5	ELE 36	*Theileria sp.* HN11 18S ribosomal RNA gene, partial sequence	FJ668378.1	0.0	96%
12	Narok	Haplotype 6	ELE 33	*Theileria sp.* NG-2013b isolate	KF597086.1	0.0	100%

				waterbuck 15 clone 4 18S ribosomal RNA gene, partial sequence			
13	Laikipia	Haplotype 6	Reedbuck 1	*Theileria sp.* NG-2013b isolate waterbuck 15 clone 4 18S ribosomal RNA gene, partial sequence	KF597086.1	0.0	100%
14	Laikipia	Haplotype 6	Reedbuck 2	*Theileria sp.* NG-2013b isolate waterbuck 15 clone 4 18S ribosomal RNA gene, partial sequence	KF597086.1	0.0	100%
15	Laikipia	Haplotype 6	Reedbuck 24	*Theileria sp.* NG-2013b isolate waterbuck 15 clone 4 18S ribosomal RNA gene, partial sequence	KF597086.1	0.0	100%
16	Laikipia	Haplotype 6	Reedbuck	*Theileria sp.* NG-2013b isolate	KF597086.1	0.0	100%

			16	waterbuck 15 clone 4 18S ribosomal RNA gene, partial sequence			
17	Laikipia	Haplotype 6	Reedbuck 7	*Theileria sp.* NG-2013b isolate waterbuck 15 clone 4 18S ribosomal RNA gene, partial sequence	KF597086.1	0.0	100%
18	Maasai Mara	Haplotype7	WB012 Migratory	*Theileria sp. ex Damaliscus lunatus* clone TS23_6 18S ribosomal RNA gene, partial sequence	HQ179765.1	0.0	96%
19	Maasai Mara	Haplotype7	KWS025 Resident	*Theileria sp. ex Damaliscus lunatus* clone TS23_6 18S ribosomal RNA gene, partial sequence	HQ179765.1	0.0	96%
20	Maasai Mara	Haplotype 8	WB 37 Migratory	*Theileria sp. ex Damaliscus lunatus* clone TS23_6 18S ribosomal RNA gene, partial	HQ179766.1	6e-176	94%

				sequence			
21	Maasai Mara	Haplotype 8	WB45 Migratory	*Theileria sp. ex Damaliscus lunatus* clone TS23_6 18S ribosomal RNA gene, partial sequence	HQ179766.1	6e-176	94%
22	Maasai Mara	Haplotype 8	WB046 Migratory	*Theileria sp. ex Damaliscus lunatus* clone TS23_6 18S ribosomal RNA gene, partial sequence	HQ179766.1	6e-176	94%
23	Maasai Mara	Haplotype 9	WB06 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
24	Maasai Mara	Haplotype 9	KWS024 Resident	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
25	Maasai Mara	Haplotype 9	KWS027 Resident	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
26	Maasai Mara	Haplotype 9	KWS028 Resident	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial	KM924444.1	0.0	99%

				sequence			
27	Maasai Mara	Haplotype 9	WB05 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
28	Maasai Mara	Haplotype 9	WB09 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
29	Maasai Mara	Haplotype 9	WB10 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
30	Maasai Mara	Haplotype 9	WB06 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
31	Maasai Mara	Haplotype 9	KWS02 Resident	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
32	Maasai Mara	Haplotype 9	KWS011 Resident	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
33	Maasai	Haplotype 9	WB49	*Theileria ovis* isolate TOSHA01	KM924444.1	0.0	99%

	Mara		Migratory	18S ribosomal RNA gene, partial sequence			
34	Maasai Mara	Haplotype 9	WB40 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
35	Maasai Mara	Haplotype 9	WB33 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
36	Maasai Mara	Haplotype 9	WB50 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
37	Maasai Mara	Haplotype 9	WB47 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
38	Maasai Mara	Haplotype 9	WB48 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
39	Maasai Mara	Haplotype 9	WB34 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%

40	Maasai Mara	Haplotype 9	WB41 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
41	Maasai Mara	Haplotype 9	WB36 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
42	Maasai Mara	Haplotype 9	WB39 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
43	Maasai Mara	Haplotype 9	WB44 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
44	Maasai Mara	Haplotype 9	WB42 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
45	Maasai Mara	Haplotype 9	WB43 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	KM924444.1	0.0	99%
46	Maasai Mara	Haplotype 9	WB51 Migratory	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial	KM924444.1	0.0	99%

				sequence			
47	Laikipia	Haplotype 10	Impala 21	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	FJ668378.1	0.0	96%
48	Laikipia	Haplotype 10	Impala 39	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	FJ668378.1	0.0	96%
49	Laikipia	Haplotype 10	Impala 18	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	FJ668378.1	0.0	96%
50	Laikipia	Haplotype 10	Impala 20	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	FJ668378.1	0.0	96%
51	Laikipia	Haplotype 10	Impala 25	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	FJ668378.1	0.0	96%
52	Laikipia	Haplotype 10	Impala 27	*Theileria ovis* isolate TOSHA01 18S ribosomal RNA gene, partial sequence	FJ668378.1	0.0	96%

Appendix III: Publications from this research work

Publications from this research work

1. **Wamuyu Lucy**, Vincent Obanda, Daniel Kariuki, Francis Gakuya, Moni Makanda, Moses Otiende and Sheila Ommeh. 2015. “Molecular Detection and Characterization of *Theileria* Infecting Wildebeest (*Connochaetes Taurinus*) in the Maasai Mara National Reserve, Kenya.” *Pathogens 4* (3): 626–38. doi:10.3390/pathogens4030626.
2. Vincent Obanda, **Wamuyu Lucy**, Patrick Chiyo and Sheila Ommeh. 2016. *(Submitted)* "High species diversity and putatively new species of *Babesia* and *Theileria* in African elephants." *Parasites and Vectors.*