Ticks and tick-borne haemoparasites from domestic animals in Lesotho

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my original work and that I have not previously in its entirety or in part submitted at any university for a degree. I furthermore cede copyright of the dissertation in favour of the North-West University.

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Date: …….....

DEDICATION

To the Diseko clan, Diseko ancestors, my guardian angel, my mother, father, younger sister, grandmother, aunt, cousins, P.A. group, Modimo Wa Boikanyo Congregation of URC, Ntwanngwe, Majaena, Mbo, Buyambo, Rakaku, Mokae and Kunene family I sincerely appreciate all the support you had for me from the start until now.

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Abstract

Ticks are amongst groups of ecto-parasites that feed on blood and transmit pathogens including protozoan parasites, bacteria, viruses which are disease causing agents in animals and humans. Lesotho is a landlocked country surrounded by the Republic of South Africa and lacks documented scientific information on ticks infesting domestic animals and the tick-borne haemoprotozoa that they harbour. The aim of this study was therefore to document information of ticks infesting domestic animals in Lesotho as well as detecting haemoparasites they are harbouring. A total of 1654 tick specimens were collected from cattle, sheep, goats, horses and dogs in five districts of Lesotho, namely Leribe, Maseru, Qacha's Neck, Mafeteng and Butha-Buthe. Ticks were identified on the basis of their morphology using microscopy and tick guides. The tick specimens were submitted to the tick museum of the ARC-Ondersterpoort Veterinary Research - where species identification was verified and voucher specimens were issued.

Successfully extracted tick DNA samples were used for amplification of cytochrome oxidase1 (COI) and the internal transcribed spacer 2 (ITS2) genes whereby PCR positive amplicons were purified, sequenced and analysed for genetic diversity and phylogenetics using MEGA 6.0 software.

Out of 1654 specimens, 132 (8%) tick samples were obtained from Leribe district with 53 from cattle, 51 from sheep and 28 from unrecorded hosts. In Maseru district 322 (19%) tick specimens were collected, with 268 from cattle and 54 from unrecorded hosts. In Qacha's Neck district 641 (39%) tick samples were collected, with 290 from cattle, 36 from dogs, 87 from horses, 2 from sheep and 226 from unrecorded hosts. In Mafeteng district all 75 (5%) tick samples were collected from cattle. Whilst in Butha-Buthe district a total of 484 (29%) tick samples were collected, with 422 from cattle, 30 from sheep, 28 from goats and 4 from unrecorded hosts. Four Ixodidae ticks were identified namely; *Rhipicephalus evertsi evertsi*, *R. microplus*, *Hyalomma rufipes* and *H. truncatum*, and one Argasidae tick, *Otobius megnini*. In Leribe district, there was a total of 93 (70%) of *R. e. evertsi a*nd 39 (30%) of *R. microplus*, in Maseru district, 181 (56%) of *R. microplus,*138 (43%) of *R. e. evertsi* and 3 (1%) of *O. megnini;* in Qacha's Neck district, 351 (55%) of *R. e. evertsi,* 215 (34%) of *O. megnini,* 39 (6%) of *R. microplus,* 26 (4%) of *H. rufipes* and 10 (2%)

of *H. truncatum*; and in Mafeteng district, 28 (37%) of *O. megnini*, 24 (32%) of *R. e. evertsi* and 13 (17%) of *R. microplus.*

From the COI multiple alignment of soft tick sequences, the average p distance (pairwise distance) value for the intraspecific divergence of soft ticks was 0.4% with an average number of nucleotide differences (nt) of 3 and an average p distance of 15.2% (95nt) the for the interspecific divergence. Both COI gene maximum likelihood (ML) and neighbour-joining (NJ) phylogenetic trees of soft ticks correctly clustered Lesotho *O. megnini* in its respective species specific *O. megnini* cluster together with other *O. megnini* species from Madagascar and South Africa.

Multiple alignments of COI sequences of hard ticks, showed an average p distance of 2.5% with an average number of nucleotide differences of 11 for intraspecific divergence of *R. e. evertsi* and 0.2% (1nt) for intraspecific divergence of *H. rufipes*. Multiple alignments of ITS2 for hard ticks showed an average p-distance of 0.8% (5 nt) for intraspecific divergence of *R*. *microplus*, 0.1% (9nt) for intraspecific divergence of *R. e. evertsi* A and D and 6.3% (42 nt) for interspecific divergence of *R. microplus* and *R. e. evertsi.* The COI ML and NJ phylogenetic trees grouped *R. e. evertsi* A and D from Lesotho in the *R. e. evertsi* species sub-cluster within the genus *Rhipicephalus* cluster. The Lesotho *H. rufipes* tick species also appeared in the genus *Hyalomma* cluster. The ITS2 gene ML and NJ phylogenetic trees showed that both *R. microplus* and *R. e. evertsi* belonged in their respective species specific clusters. In a nutshell, both COI and ITS2 gene sequence analyses have supplemented the morphological identification of Lesotho tick species collected in this study.

A total of 164 tick DNA pools from cattle were screened for the presence of *B. bigemina* and *B. bovis* DNA by PCR. None of the tested samples were positive for the presence *B. bigemina.* A total of 13 (7.9%) samples were PCR positive for the presence of *B. bovis* DNA for which 5 samples were represented by *R. microplus* species and the other eight were *R. e. evertsi* from various villages in Butha-Buthe district. Four horse DNA samples collected from Maseru district tested negative for both *B. caballi* and *T. equi.* Twenty two samples from goats (n = 6) and sheep (n = 16) which were screened for the presence of *Babesia ovis*, *B. motasi*, *Theileria ovis*

and *T. lestoquardi* tested negative for *T. ovis* and *T. lestoquardi.* One *R. e. evertsi* DNA sample from a goat and two *R. e. evertsi* samples from sheep of Qalo village tested positive (13.6%) for *B. ovis.*

This study has documented tick species infesting domestic animals in four Lesotho districts using both morphological and molecular techniques. Furthermore, the study has also documented the haemoparasites harboured by these ticks. This study is the first of it's kind in Lesotho and will hopefully contribute in formulation of control methods for both vectors and tick-borne parasitic diseases as well as open doors for detailed epidemiological studies of ticks and tick-borne diseases in domestic animals in Lesotho.

Key words: Ixodidae, Argasidae, Haemoparasites, Lesotho, COI, ITS2 and Phylogenetic tree.

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Abbreviations

AIC: Akaike Information Criterion

ARC: Agricultural Research Institute

BLAST: Basic Local Alignment Search Tool

COI: Cytochrome Oxidase1

DDW: Double Distilled Water

ELISA: Enzyme-Linked Immunosorbent Assay

GTR: General Time Reversal

TVM: Transversion Model

LAMP: Loop Mediated Isothermal Amplification

ML: Maximum Llikelihood

NCBI: National Centre Biotechnology Information

NJ: Neighbour-joining

PBMS: Peripheral Blood Mononuclear Cells

PCR: Polymerase Chain Reaction

RBL: Reverse Line Blotting

IFAT: Indirect Fluorescent Antibody Test

ITS2: Internal Transcribed Spacer 2

Chapter 1

Introduction and literature review

1.1. Ticks

Ticks are ecto-parasitic blood feeding arthropods that infest vertebrate animals such as birds, mammals, amphibians and reptiles (Parola & Raoult, 2001). They have an oval body shape with lengths ranging from 2 - 30 mm and are not separated into tagma, for instance head, thorax and abdomen cannot be distinguished. Thus the frontal (anterior) body part of a tick composes of mouthparts with sensory, cutting and immobile (the hypostome) organs, but lacks antennae. This anterior part is named capitulum (Sonenshine, 1991; Hillyard, 1996; Sonenhine & Roe, 2014). Life cycle of ticks is recognised by three feeding life stages the larval, nymphae and adult stage. Adult and nymphae forms can be easily recognized by the presence of four pairs of legs from the larval form, which only has three pairs. Genital pores are present in adults and, absent in both larval and nymphae forms (Sonenshine, 1991; Hillyard, 1996; Sonenhine & Roe, 2014). According to Olivier (1989), blood meals serve as prerequisites for egg production in most female ticks. Ticks have a circulatory system where all organs and tissues are bathed by the haemolymph (Sonenshine, 1991; Hillyard, 1996; Sonenhine & Roe, 2014). They have a variety of sensory organs that facilitate the location of hosts and communication amongst each other. Most ticks have no eyes, but if present it is doubtful that their purpose is to produce a detailed vision of the surrounding environment (Parola & Raoult, 2001).

1.2. Families of ticks

Ticks consist of three families namely Ixodidae (Hard ticks), Argasidae (Soft ticks) and Nuttalliellidae (Tick species with characteristics of both hard and soft ticks). Ixodidae ticks make up the largest part of the world's tick fauna with 702 species, followed by Argasids with 193 species and lastly Nuttalliellidae with only one species (Guglielmone *et al.,* 2010). Family Ixodidae is identified by the presence of a scutum or dorsal shield, anterior capitulum (Figure 1.1) and a body covered by a simple-striated integument (Klompen *et al*., 1996). It is further divided into groups of relatively short mouthparts, the metastriate ticks, (examples are *Dermacentor* or *Rhipicephalus* genera) and longer barbed mouthparts, the prostriate ticks, (example is *Ixodes* genera) (Francischetti *et al*., 2009). Sauer *et al*. (2000) mentions that hard

ticks are unique among other ecto-parasites in that they have relatively long attachment to their hosts which coincides with their feeding. Adult female hard ticks feed only once and die after producing eggs (Oliver, 1989; Francischetti *et al*., 2009). Hard ticks have four life stages eggs, larvae, nymphs and adults. Adult females bear a large amount of eggs (Olivier, 1989; Klompen *et al*., 1996). Mating occurs off the host and mostly through a nest-based mating strategy, but exceptions are species of males which seek their hosts on vegetation (Kiszewski *et al*., 2001). Most ixodids are exophilic ticks that inhabit moist and open areas such as forests, woodlands and grasslands, and cannot withstand dry conditions (Parola & Raoult, 2001; Jongejan & Uilenburg, 2004). Few exceptions are the genus *Ixodes*, which display an endophilc behaviour and inhabit hidden spaces such as host's nest (Parola & Raoult, 2001). Ixodid ticks consist of seven important genera: *Amblyomma, Boophilus, Dermacentor, Haemaphysalis, Hyalomma, Ixodes* and *Rhipicephalus* (Kiewra & Lonc, 2012; Estrada-Pena *et al*., 2013).

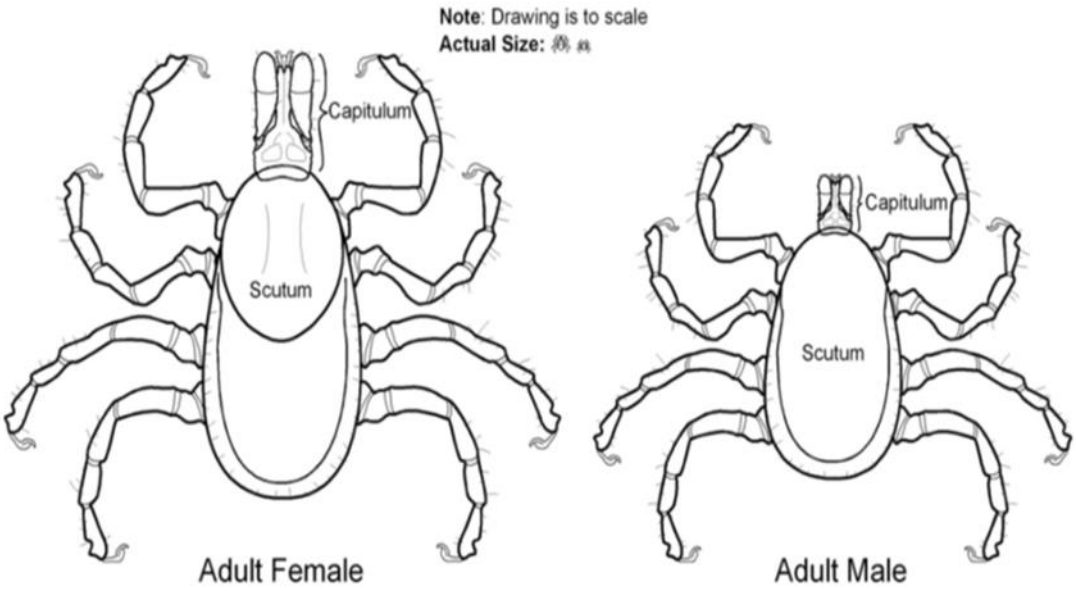

Figure 1.1. Adult female and male hard tick
(https://extension.entm.purdue.edu/publications/E-243.pdf)

Argasidae ticks are generally recognized by lack of dorsal shield or scutum and distinctive upright knob-like structure called capitulum. Their bodies are covered by a dimensionally well build integument (Pospelova-Shtrom, 1969; Vail, 2009). They have a short duration for feeding (Vail, 2009; Sauer *et al*., 2000) and adult female

soft ticks feed for multiple times (Francischetti *et al*., 2009). Soft ticks have developmental stages composed of egg, larva, nymphs and adult stages and, females lay small amounts of eggs (Klompen et al., 1996). Mating usually takes place off the hosts (Oliver, 1989). Argasidae ticks inhabits dry and hot places (Hoogstraal 1956; Olivier, 1989) such as burrows or nests which are close to their hosts (Shoneshine *et al*., 1993; Vail, 2009; Dautel & Kahl, 1999; Kiewra & Lonc, 2012) and are capable of surviving harsh environmental conditions (Less, 1947; Vail, 2009).

Family *Nuttalliellidae* is a monotypic family of ticks with a rare representative species called *Nuttalliella namaqua* (Guglielmone *et al*., 2010). The *N. namaqua* is recognized by unique characteristics such as the organ of an unknown function posterior to coxae IV, three segmented palpi, pseudoscutum, ball and socket leg joints, Heller's organ structure and lack of spiracles plates (Keirans *et al*., 1976; Latif *et al.*, 2012). *Nuttalliella namaqua* shares similar morphological traits (Figure 1.2) with ticks from family; Ixodidae and Argasidae (Bedford, 1931; El Shoura, 1990). Keirans *et al*. (1976) enumerated similar traits that relate *N. namaqua* to Ixodidae ticks. Such traits are apical position of the capitulum, pseudoscutum, absence of a ventral paired organ, coxal and supra coxal folds (Figure 1.2.A), and the similarity of dorsal (Figure 1.2.B) and ventral integuments (Figure 1.2.C). As for the case of similarities in Argasid ticks and *N. namaqua*, the shared characteristics include integument structure, unarmed coxae, hypostome structure and lack of porous areas (Keirans *et al*., 1976; Latif *et al*., 2012).

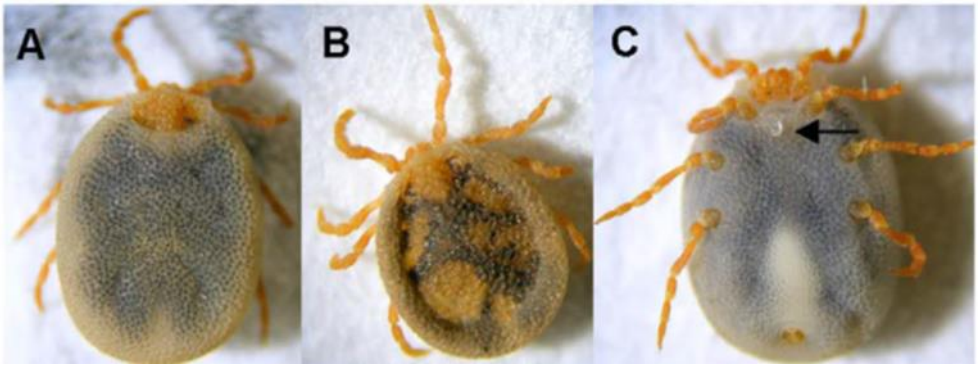

Figure 1.2. Dorsal and ventral view of *Nuttalliella namaqua.* (Latif *et al*., 2012)

Several variety of habitats have been postulated for *N. namaqua*, because of its previous collections from underneath stones, the ground, nest of the striped swallow,

an abandoned eagle's nest, rock crevices and rock face (Bedford, 1931; Keirans *et al*., 1976; El Shoura, 1990, Mans *et al*., 2011, Mans *et al*., 2014). Suggested preferable hosts for *N. namaqua* include lizzards, elephant shrews, rodents, suricates and birds (Keirans *et al*., 1976; Latif *et al*., 2012). Such multiple potential hosts set forward the notion that *N. namaqua* could be a generalist and its ecological habitat could determine its host preferences, but this is not yet concluded. About eighteen species of *N. namaqua* have been found to date in southern Africa and Tanzania (Mans *et al*., 2011).

1.3. Life cycle of ticks

During a three-host life cycle (Figure 1.3), each tick stage has its own host to feed on (Nava & Guglielmone, 2013; Estrada-Pena & De la Fuente, 2014; Estrada-Pena, 2015). Ticks do not moult on the host. Once the larva is fed and /engorged, it drops off to the ground and moults into a nymph, which later has to find a second host. Once located, it attaches to the host and feeds until engorgement, then drops off to moult into an adult stage, which also has to find the final host (Jongejan & Uilenburg, 2004). Kiewra and Lonc (2012) indicated that three-life host cycles is seen on some species of genera *Ixodes, Dermacentor, Rhipicephalus* and *Amblyomma*, and species of medical importance such as *I. ricinus, I. persulcatus, I. scapularis* and *I. pacificus*.

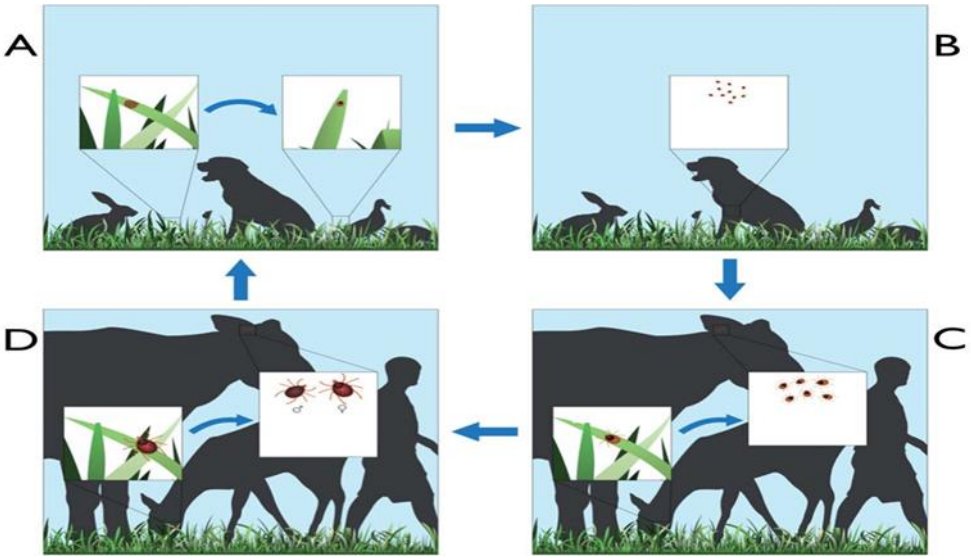

Figure 1.3. Life cycle of a three host tick (Estrada-Pena, 2015)

During two-host life cycle, tick species complete their cycle by feeding only on two hosts (Estrada-Pena & De la Fuente, 2014; Estrada-Pena, 2015). The larval stage moults only once on the host into the nymph. An engorged/fed nymph drops off from the host and moults to an adult stage, which later has to seek a new host (Jongejan & Uilenburg, 2004). Example of two-host life cycle include *Hyalomma detritum* and *R. e. evertsi* (Walker *et al*., 2014). Lastly, in one-host life cycle, tick species spend their life cycle on one host (Estrada-Pena & De la Fuente, 2014; Estrada-Pena, 2015). The one-host tick usually moults two times on the same host, which is from the larvae to the nymph and from the nymph to the adult (Jongejan & Uilenburg, 2004). For example, McCoy *et al*. (2013) explains that species such as *R.* (*Boophilus*) *microplus* which exhibits a one-host life cycle, often remains associated with one host individual for its entire life cycle. *Otobius megnini* is also an example of a one-host life cycle tick (Keirans & Pound, 2003; Walker *et al*., 2014). Such features of life cycle in ticks play an important role in transmission of pathogens, because in order for ticks to transmit pathogens successfully, they first need to acquire it from an infected host, then pass it into the next active stage of the tick and then transmit it successfully to the new host (Kahl *et al*., 2002; Estrada-Pena, 2015).

1.4. Behavioural ecology of ticks

Ticks display questing behaviour as a way of locating their prey. They either actively (hunting method) or passively (ambush method) search for their prey (Parola & Raoult, 2001; Beck & Orozco, 2015). Randolf (2004) states that example of an active host questing is seen on *Hyalomma dromedarii* which chase livestock and people resting close to their shelters. On the other side, example of passive host questing can be recognized from *Ixodes ricinus* nymphaea and adults, and nymph of *Amblyomma mixtum* which use vegetation to elevate to their hosts (Randolf, 2004; Beck & Orozco, 2015). In addition, the blood meal in ticks may serve as a source for tick's infections, for instance pathogens acquired from host's blood build up in tick's haemolymph and spread to the rest of the tissue body cells, and then wait for the second feeding to take place on the new host (Estrada-Pena, 2015). Francischetti *et al.* (2009) elaborates that adaptation of blood feeding in ticks has contributed in giving rise to complex salivary components which help the parasites to overcome defence mechanisms of their hosts against blood loss. For example, Tirloni *et al*. (2016) demonstrated that serpins contribute during the evasion of host immune

system. Beck & Orozco (2015) further highlights the significance of having an understanding of parameters, for example such as questing behaviour or feeding, which contributes in the life cycle of ticks as being crucial in designing predictive models that might help to promote efficient management of disease transmission.

1.5. Impacts of ticks on livestock

According to Rajput (2006) and Manjunathachar *et al*. (2014), the ability of ticks to parasitize wide range of vertebrates and to serve as vectors for large spectrum of pathogens increases their chances of imposing huge economic limitations on livestock production. Such economic constrains are due to direct impacts on livestock production and can be associated with tick worry, blood loss, damages to hides and skins of animals, and introduction of toxins (De Castro, 1997; Rajput *et al*., 2006). Examples of ticks that cause paralysis subsequent to toxins include *I. holocylus* and *Rhipicephalus* species (Drummond, 1983; Rajput *et al*., 2006). Other direct economic constrains by tick bites on livestock extend to reduction of body weight and milk production (Jonsson, 2006; Mapholi *et al*., 2014). Indirect limitations include outbreaks of tick borne diseases in susceptible hosts recognized by mortality, chronic morbidity, cost of veterinary diagnosis and treatment, cost of vaccines and maintenance of moving livestock (Jonsson *et al*., 2006; Mapholi *et al*., 2014).

1.6. Tick identification

Precision through identification of tick species has a primary key role in controlling tick borne diseases (Lv, 2014). Thus identification of ticks can pave a way in tracing diseases down to their host associates and interpreted habitat range, and distribution patterns, but misidentification might lead to the opposite (Anderson *et al*., 2004). Morphology based approach to describe tick species has been applied as a conventional method in most studies (Soneshine, 1991; Coporale *et al*., 1995; Mangold *et a*l., 1998; Lv, 2014). In contrast, morphological techniques to describe species have limitations on poorly preserved specimens, similar shared features among taxa (e.g. *Ixodes ricinus* and *I. paracinus*) and unreadily describable features seen after feeding (Nava *et al*., 2009; Ronaghi *et al*., 2015). For instance, *Rhipicephalus* (*B.*) *microplus* and *Haemaphysalis bispinosa* are difficult to differentiate by just using their physical features, because they both appear dark in

colour, have similar sizes and their festoon are not visible once they are engorged (Braham *et al*., 2014). In addition, these ticks have respectively unique traits of hexagonal capitulum and rectangular capitulum that cannot be seen with naked eyes (Braham *et al*., 2014).

Navajas *et al*. (1992) demonstrated that molecular approach can be incorporated with morphological criteria to determine evolutionary relations of spider mites. There are numerous studies which indicate how mitochondrial DNA can be a reliable gene marker to characterize and evaluate phylogenetic relationship of organisms at various levels of taxa (Boore & Brown, 2000; Chitimia *et al*., 2010). Braham *et al*. (2014) used mitochondrial ITS2 and 16S rDNA sequences to characterize *R. (B). microplus* and *H. bispinosa* ticks in the North East India. In addition, mitochondrial cytochrome oxidase gene (COI) is recognized as the most robust, reliable and sufficient gene marker for identification of different organisms (Boehme *et al*. 2012, Sharma & Kobayashi, 2014). Folmer *et al*. (1994) sequenced mitochondrial COI gene of diverse organisms and studied their phylogenetic relations. Engdahl *et al*. (2014), for example, used COI gene to identify mosquitoes. Advantage of using COI gene is that its attributes makes it possible to identify individual species from just a small amount of tissue (Sharma & Kobayashi, 2014). Other studies suggested that combination of COI with other mitochondrial gene markers might even be more effective in identifying organisms when applied alone (Chitimia *et al*., 2010; Cakic *et al*., 2014). For instance, Ronaghi *et al.* (2015) used COI and ITS2 gene sequences to demonstrate that *R. (B.) annulatus* from two isolates of Iran are sister groups.

1.7. Tick control strategies

Effective and complete tick eradication on larger islands and continents has not yet been completely successful, with an exception to the first complete eradication of *Boophilus* species that was possible in USA (Jongejan & Uilenburg, 1994; 2004). Thus, ever since then the subject of tick control has continued to spark attention to researchers throughout the world, because many important livestock pathogen-diseases are transmitted by ticks and their demand for mitigation has remained an ultimate goal for control strategies. Control strategies of ticks include the use of acaricides, non-chemical methods (grooming, pasture management etc), endosymbiotic approach, use of biological controls, genetic manipulation,

vaccination and integrated control system (Manjunathachar *et al*., 2014). However, Ostfeld *et al*. (2006) states that methods of reducing tick abundance are by far the most promising effective methods for preventing tick borne diseases, albeit there's a need for intense scientific tests. An example is the application of fungal pathogens as biological control, (such as *Metarhizium anisopliae* and *Beauveria bassiana*) to kill ixodes ticks (Ostfeld *et al*., 2006; Alekseev, 2011). However, despite such promises, other studies have highlighted the significance of understanding epidemiology of particular tick species in tandem to their relative ecological habitat as a critical aspect that can be considered when developing effective control strategies on tick abundance (Jurisic *et al*., 2010). For example, Jurisic *et al*. (2010) showed that ecological conditions of habitats with low vegetation and appropriate access for treatment proved to have high efficacy of chemical treatments as opposed to those found in habitats with condensed vegetation and uneasy access to chemical treatment, even though they had potential of posing health risk on non-target organisms and the environment.

1.9. Distribution of ticks in southern Africa

According to Walker *et al*. (2014) most common tick species' distribution recorded throughout South Africa include; *Amblyomma herbraeum, Haemaphysalis laechi* (*elliptica*)*, Hyalomma marginatum rufipes, H. truncatum, Ixodes rubicandus, Margaropus winthemi, Ornithodorus moubata* complex, *Otobius megnini, Rhipicephalus* (*Boophilus*) *decoloratus, R.* (*Boophilus*) *macroplus, R. appendiculatus, R. evertsi evertsi* and *R. simus*. Other tick species with isolated distributions *are Argas persicus, A. walkerae* (Spicket *et al*., 2011; Walker *et al*., 2014), *Ixodes pilosus* (Galezardy & Horak, 2007; Horak *et al*., 2009; Spicket *et al*., 2011; Walker *et al*., 2014), *Margaropus winthemi* (Tonnetti *et al*., 2009; Walker *et al*., 2014), *A. marmoreum* (Galezardy & Horak, 2007; Horak *et al*., 2006; Golezardy *et al*., 2016), *R. zambeziensis* (Horak *et al*., 1992; Golezardy *et al*., 2016; Spicket *et a*l., 2011; Walker *et al*., 2014), *O. savignyi* (Spicket *et al*., 2011; Walker *et al*., 2014; Horak *et al*., 2015), *H. silacea* (Horak *et al*., 2015), *R. evertsi mimeticus* (Spicket *et al*., 2011; Horak *et al*., 2015) and *R. gertudae* (Galezardy & Horak, 2007; Spicket *et al*., 2011; Mathee *et al.*, 2015). In addition, several collections of tick species recorded outside the Free State Province include *R. follis, R. near pravus* and *R. sulcatus* (Spicket *et al.*, 2011).

1.10. Tick-borne haemoparasites

Haemoparasites refer to all the tick-borne organisms observable under a light microscope and present throughout the circulating blood of tick-vectors and/or host animals (Uilenberg, 1992, 1995). Haemoparasites of most economically significant genera include *Anaplasma, Ehrlichia* (*Cowdria*), and the protozoan parasites including *Theileria, Babesia* and *Trypanosoma* (Uilenburg, 1995; Bell-Sakyi *et al*., 2004; Pfitzer *et al*., 2011). Four dominant tick-borne diseases known to be the most important limiting factor to the health and improvements of domestic animals are anaplasmosis, babesiosis, heartwater, therileriosis and trypanosomosis (Rajput *et al*., 2006; Spickett *et al*., 2011; El-shker *et al*., 2015; Walelign & Mekuriaw; 2016). Apart from being responsible for high morbidity and mortality, tick-borne diseases also indirectly impede the introduction of more productive exotic breeds and consequently limit genetic improvements of indigenous breeds on domestic animals (Bell-Sakyi *et al*., 2004). Distribution of haemoparasites causing tick-borne diseases is widely synonymous to the presence and distribution of their tick-vector (Alekaw, 2000; Sitotaw *et al*., 2014). For instance, haemoparasites are more common to most regions of the world and infectious to all domestic animals (Uilenberg, 1992, 1995).

1.11. Bovine babesiosis: *Babesia bigemina* and *Bavesia bovis*

Bovine babesiosis is an important and fatal disease of cattle (Hunfeld *et al.* 2008). It is caused by an apicomplexan protozoa of the genus *Babesia* and is transmitted by ticks. Out of more than 100 species of *Babesia*, the two species *B. bovis* and *B. bigemina* are considered to be the most predominant species of the subtropical and tropical regions causing a massive loss in livestock throughout the world. In ticks, *Rhipicephalus* (*Boophilus*) species are the major vectors transmitting *B. bovis* and *B. bigemina* (Bock *et al*. 2004; Gupta & Kaur, 2004; Oliveira-Sequeira *et al*., 2005). In South Africa it has been found out that *R.* (*Boophilus*) *microplus* is the main vector of *B. bovis*, whereas both *R.* (*B*.) *decoloratus* and *R.* (*B*.) *microplus* are capable of transmitting *B. bigemin*a (Potgieter, 1977; De Vos, 1979; Tonnsen *et al*., 2004; 2006). Brock *et al*. (2004) explains that infections of *B. bigemina* involve mostly direct destruction of erythrocytes, whereas that of *B. bovis* have more progressive haemolytic anaemia. Gupta & Kaur (2004) further elaborates that diseases caused

by *B. bigemina* are usually less severe with rapid development, whereas that of *B. bovis* are normally severe and associated with high mortality.

Both *B. bovis* and *B. bigemina* show similar patterns of development in an adult *Rhipicephalus* (*Boophilus*) species. *Babesia* species are known to invade and infect erythrocytes than any other cells of vertebrates. During the first stage of the life cycle (Figure 1.4), sporocytes penetrate cell membranes of erythrocytes and give rise to merozoites. Unlike in other piroplasms, meroizoites (called gamont precursor in *B. bigemina*) of *Babesia* species do not develop until the second stage of the life cycle, recognized by an intake of blood by a tick, begins. Transition from host blood to tick's midgut stimulates gamonts and produce ray bodies. Ray bodies multiply within erythrocytes to form large number of haploid ray bodies now called gametes. Aggregates of gametes fuse in pair to form a zygote, which eventually infects the digestive system in tick's gut (Bock *et al.*, 2004).

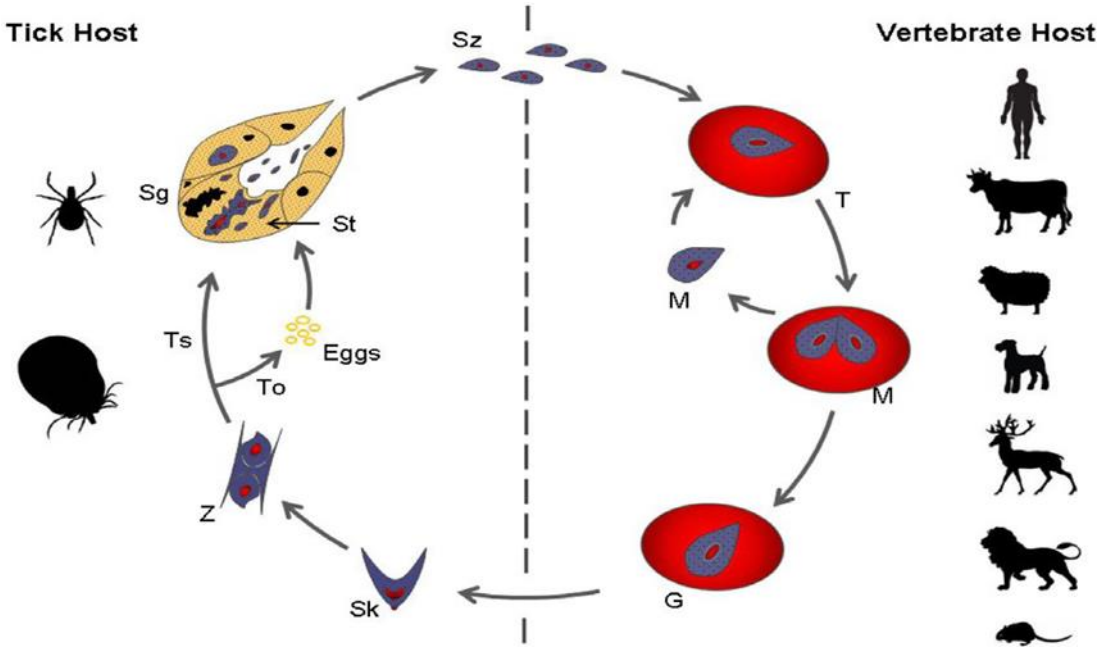

Figure 1.4. Life cycle of *Babesia* species (Schnittger *et al*., 2012)

In the gut, the zygote proliferates to basophilic cells which later multiply and give rise to kinetes. Kinetes move into tick's haemolymph. In the case of *B. bigemina*, haploid zygote is formed by one step meisosis at some developmental stage in the gut. This then leads to multiple fission of sporocytes to form polyploid kinetes. The kinetes enter haemolymph of the tick and infect different cell types and tissues, including the oocytes. This also allows transovarial transmission to take place which consequently

affect the larval stage through further developments. Kinetes then migrate to the salivary glands and form haploid merozoites, called sporozites. In many species, development of sporozoites is initiated by an attachment of an infected tick on a vertebrate host. In the case of *B. bigemina* development of sporozoite is seen in the feeding larvae, but it takes infective sporozoites about nine days to occur, which therefore only takes place in a nymphae and adult stage. However, in the case of *B. bovis*, duration of infective sporozoites is two to three days to form in a larval stage (Bock *et al*., 2004).

Bovine babesiosis can be diagnosed by microscopic methods, immunological methods, e.g. Indirect Fluorescent Antibody Test (IFAT); Enzyme-Linked Immunosorbent Assay (ELISA) and molecular methods, e.g. DNA polymerase chain reaction (PCR); Reverse Line Blot Hybridization; Real time PCR and Loop Mediated Isothermal Amplification (LAMP) (Mosqueda *et al*. 2012; Mahmoud *et al*., 2015). The use of chemical substances for treatment of babesiosis has played an essential role in controlling and preventing babesiosis in several parts of the world. For instance conventional drugs (e.g imidocarb, diminozene etc.), anti babesial drugs (e.g triclosan etc) and new drugs in research (e.g cysteine proteases) can be considered in treating certain cases of babesiosis, depending on the degree of infection (Mosqueda *et al*., 2012).

Cases of babesiosis in cattle have been reported from several regions across the globe, such as Africa, Australia, Asia, South and Central America, and United States (Zulfigar *et al*., 2012). Common tick vectors of *B. bigemina* and *B. bovis* such as *R.* (*B*) *decolaratu*s are said to be endemic in the eastern grasslands of the Free State Province, South Africa (Horak *et al*., 2015). Its distributional pattern can be linked to annual mean temperature of 500 mm. Its nymphs and adults have been collected from cattle and buffaloes, whilst larvae were from drag-sample from vegetation (Horak *et al*., 2015). There have been cases of African red water in resident cows within the Free State Province caused by *B. bigemina* and transmitted by *R.* (*B*) *decoloratus* (Horak *et al*., 2015). In addition, *R.* (*B*) *microplus* has been collected from cattle in four different localities of the Free State. Its first introduction to the eastern Free Sate was suggested to be by infested cattle moved from KwaZulu-Natal by farmers (Horak *et al*., 2015). Thus the presence of both vectors in the Free

State Province suggest that chances of severe cases of Asiatic redwater and mortality are likely to be encountered by susceptible resident animals should there be any more introduction of infected cattle (Horak *et al*., 2015).

1.12. Equine piroplasmosis: *Babesia caballi* and *Theileria equi*

Equine piroplasmosis refers to an acute, subacute or chronic tick-borne disease of Equidae e.g. horses, donkeys, mules and zebras (De Waal, 1992). Its aetiological agents are intracellular haemoprotozoan parasites of Equidae namely *Babesia caballi* and *Theileria equi* (Bashiruddin *et al*., 1999). These two haemoprotozoan parasites are said to have similar pathology, life cycles and vector relations, albeit they are biologically distinct (Scoles & Ueti, 2015). Both agents are transmitted by three genera of hard ticks *Dermacentor* (nine species), *Rhipicephalus* (eight species) and *Hyalomma* (thirteen species) (De Waal, 1992; Scoles & Ueti, 2015). Only one species of *Amblyomma* has been reported as a potential vector for *T. equi*, because its dominance on horses was related the epidemiology of *T. equi* infections in many parts of its range (Wise *et al*., 2013).

Equines can clear infections of *B. caballi* after few years, even though the parasite can still persist in the tick vector for over several generations without reinfection to an infected host. Thus in this situation tick vector can serve as a reservoir of infections. In contrast, this scenario does not comply with that of *T. equi*. There are reported instances where animal hosts may be reservoirs, whereby ticks will be obtaining and transmitting pathogens from persistently infected equines (Scoles & Ueti, 2015). Transmission of both pathogens has been reported to have occurred iatrogenically as a result of improper or unethical mixing of infected and uninfected blood (Gerstenberg *et al*.1999; Tamazali, 2013; Wise *et al*., 2013). Example will be sharing of needles among positive and negative horses, and donation of blood from a chronically infected horse to another horse, such as practises of illegal blood doping (Gerstenberg *et al*.1999; Wise *et al*., 2013). In addition, during experiments inoculation of infections which takes place through intravenous and subcutaneous paths can also be considered as another mode of transmission (Kuttler *et al*., 1986; Wise *et al*., 2013). Allsopp *et al*. (2007) provided data showing that *T. equi* parasites can be transmitted through transplacental route. Placental transmission, associated with *T. equi* infections, is reported to be likely the cause of abortion in carrier mares

(De Waal, 1992; Allsopp *et al*., 2007). There is no sufficient data on the prevalence of such transmission, but in South Africa *T. equi* has been reported to have contributed to about 11% of abortions on thoroughbred mares (De Waal *et al*, 1998; Lewis *et al*., 1999). Infections involving *B. caballi* may take about ten to thirteen days to develop clinical signs after transmission, whereas that of *T. equi* may take twelve to nineteen days (Wise *et al*., 2013). Clinical symptoms of equine piroplasmosis can be identified as fever, anemia, red urine, jaundice, edema and weight loss (De Waal, 1992; Mahmoud *et al.*, 2016).

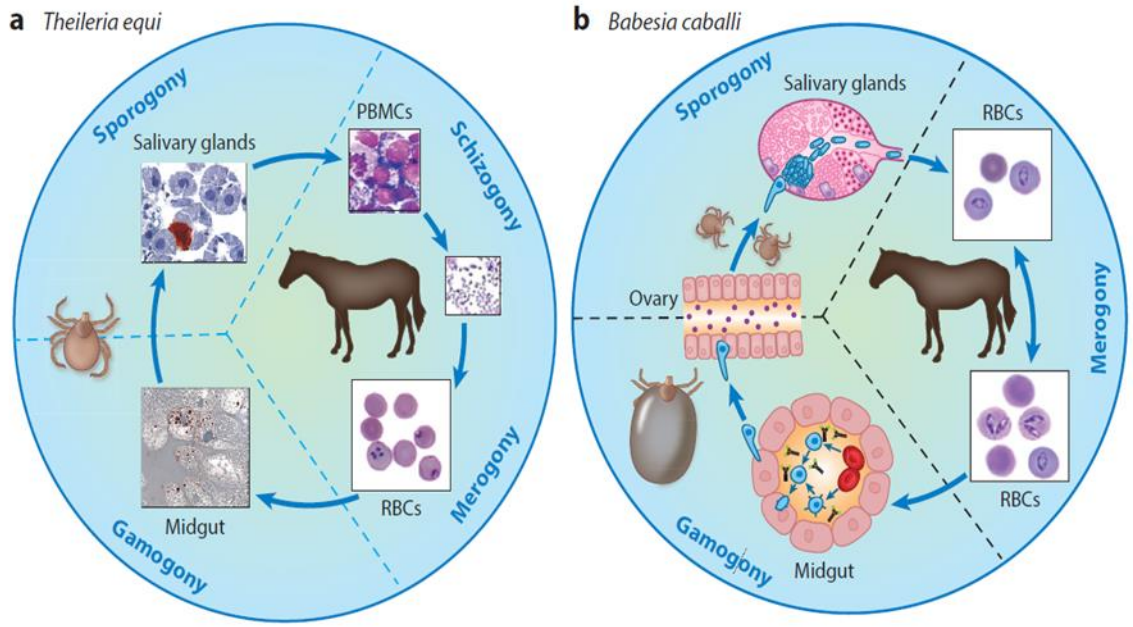

Figure 1.5. Life cycle of; (a) *Theleria equi* and (b) *Babesia caballi* (Scoles & Ueti, 2015)

Life cycle of both *T. equi* and *B. caballi* shows few features that set them apart. For example, *T. equi* has four replication stages (Figure 1.5.a), whereas *B. caballi* three (Figure 5b). The *B. caballi* directly attacks the red blood cells, whereas *T. equi* invades the peripheral blood mononuclear cells (PBMC) (Scoles & Ueti, 2015). Despite such species variations, how the pathogens are inoculated to the equid hosts is the same (Wise *et al.*, 2013). For *T. equi* (Figure 1.5.a), asexual replication of sporozoites (shizogony) occurs in the PBMS within the host to form large schizonts. Within the tick vector, the merozoites inside the parasitized erythrocytes develop into gametes, some are already developed into gametes from the PBMC (Scoles & Ueti, 2015; Wise *et al*., 2013). In the tick's midgut lumen sexual replication

follows and haploid gametes fuse to form a diploid zygote and eventually give rise to haploid sporozoites which will later be released to the host as the tick feeds (Scoles & Ueti, 2015).

In the case of *B. caballi* life cycle (Figure 5b), schizogony does not occur and sporozoites directly attach to the red blood cells. Tick vector ingests infected red blood cells and the formation of gametes takes place in the midgut. Gametes then fuse to form diploid zygote, which gives rise to motile kinetes. These motile kinetes escape the gut and enter the haemolymph to infect multiple internal organs, as well as the ovaries, and replicate asexually. In tick embryo, motile kinetes attach to the salivary glands. Once the tick has attached, then the motile kinetes attack the salivary glands and transform into sporozoites. Such infective sporozoites become inoculated into the host through saliva of the ticks during feeding periods (Scoles & Ueti, 2015).

Clinical diagnosis of equines might be achieved by blood smear, serological and PCR tests (Kumar *et al*., 2009; Tamazali, 2013). Treatment of equine piroplasmosis is often dependent on the endemic status of a particular region. For instance, in endemic regions, the goal might be to reduce the infection, whereas in non-endemic regions the goal might be to clear and eliminate the infections and its transmission risks (Tamazali, 2013). Chemotherapy, supportive therapy and tick management have been offered as a treatment for equine piroplasmosis (De Waal, 1992). Both, *T. equi* and *B. caballi* have been documented from larger parts of Europe and Russia, and are widely spread in Africa, South and Central America (De Waal, 1992; Hornok *et al*., 2007).

The geographical distribution of equine piroplasmosis is relative to the distribution of its vector (Gummow *et al*., 1996; De Waal, 1992). For example, in southern US, South America, equine piroplasmosis has been reported to be transmitted by *Amblyomma cajennense*. In south-east of Russia, *D. niveus* is regarded an important vector for both equine piroplasms. In Japan, *Haemaphysalis longicornis* has been shown to be able to transmit both equine piroplasms (Scoles & Ueti, 2015). In northern Thailand, Kamyingkird *et al*. (2014) detected occurrence of *T. equi* and *B. caballi* in equids, though the animals appeared to be asymptomic to equine piroplasmosis clinical signs. Qablan *et al*. (2013) reported equine piroplasmosis to be

enzootic in Jordan of the eastern Mediterranean, where horses showed to be more susceptible to *T. equi*, while *B. caballi* showed no host specificity.

In South Africa, *R. e. evertsi* of the ixodid tick is reported to be the main tick vector tick for *T. equi* and *B. caballi* to horses (De Waal & Potgieter, 1987), while *Hyalomma truncatum* has been reported to transmit *B. caballi* to horses and rodents (Gummow *et al*., 1996). Studies in the north-east of the Free State Province, South Africa, detected the presence of both *T. equi* and *B. caballi* using serological tests on blood samples collected from horses (Motloang *et al*., 2008). Serological studies conducted in the Northern and Eastern Cape Province of South Africa, detected antibodies of both *T. equi* and *B. caballi* from blood samples of horses, but the prevalence rates of equine piroplasmosis could not correlate with the known distribution of tick vectors, namely, *R. e. evertsi* and *H. truncatum*. Thus, these survey findings suggested possible existence of other tick vector(s) (Gummow *et al*., 1996). According to Kumar *et al.* (1999), distribution of *B. caballi* is thought to be widespread as opposed to that of *T. equi* within South Africa.

1.13. Ovine babesiosis: *Babesia ovis* and *Babesia motasi*

Ovine babesiosis is described as a haemoparasitic tick-borne disease of small ruminants. Its causative agents are *Babesia ovis*, *B. motasi* and *B. crassa* (Ferrer *et al*., 1998; Uilenberg, 2001; Aktas *et al*., 2005). Known tick vectors for *B. ovis* include *Dermacentor marginalis, Hyamaphysalis punctate*, *Hyalomma anatolicum excavatum, R. bursa* and *R. turanicus* (Friedhoff, 1997; Guan *et al*., 2002). In addition, findings by Ramzi *et al*. (2002) proposed that *H marginatum* and *R. sanquineus* could be potential vector agents for *B. ovis* due to their high prevalence in sheep from Mshhad area, Iran. Ticks of the genus *Haemaphysalis* are known to be a vector for *B. motasi* (Ferrer *et al*., 1998; Yin & Luo, 2007; Torino & Caracappa, 2012). According to Guan *et al*. (2002) there has been several reports from regions where ovine babesiosis was present, which showed that the main tick vectors are *D. silvarum*, *H. longicornis* and *H. qinghaiensis*.

The *B. ovis* is said to be the most pathogenic agent of babesiosis in sheep (Friedhoff, 1997; Guan *et al*., 2002) and *B. motasi* is moderate (Friedhoff, 1997;

Aktas *et al.,* 2005). Infections by *B. ovis* can cause severe anaemia, fever, icterus (Aktas *et al*., 2005), mortality as a result of haemoglobinuria and acute pneumonia (Habela *et al*., 1990; Torina & Caracappa, 2012). Studies on histopathology showed clinical symptoms of *B. ovis* to be purulent encephalitis, interstitial pneumonia, exudative, haemorrhagic pericarditis and central necrotic hepatitis and lobular necrosis of the renal tubules (Torina & Caracappa, 2012). Acute form of *B. motasi* may have clinical symptoms which range from anorexia, fever, fast and tachycardia, pale mucous membranes, jaundice to haemoglobinuria. Its chronic form is recognized by loss of weight, cough and oedema (Torino & Caracappa, 2012). Microscopic diagnostics and serological tests have been used for acute infections (Shahzad *et al*., 2013), but to date PCR and PCR-based reverse line blotting (RBL) are used to diagnose ovine *Babesia* species (Atkas *et al.*, 2005; Altay *et al*., 2012). Cases of ovine babesiosis have been reported from Pakistan (Shahzad *et al*., 2013), Iran (Ramzi *et al*., 2002), China (Yin & Luo, 2007; Tian *et al*., 2013) and Spain (Ferrer *et al*., 1998).

1.14. Ovine theileriosis: *Theileria ovis* and *Theileria lestoquardi*

Ovine theileriosis is a haemoprotozoal disease occurring on sheep and goats, and is caused by *T. ovis, T. lestoquardi, T. separate* and *Theileria* sp. China (Preston, 2001; Nagore *et al*., 2004; Jianxung and Yin, 1997; Torina & Caracappa, 2012). The most involved species through the occurrence of ovine theileriosis in sheep are said to be *T. ovis, T. lestoquardi* and *T. separate* (Sayin *et al*., 2009; Shahzad *et al*., 2013). Laboratory experiments by Jansen and Neitz (1956) have successfully illustrated that transmission of *T. ovis* in sheep by *R. evertsi evertsi*. Torina and Caracappa (2012) states that tick species of the genus *Hyalomma* are potential tick vectors for *T. lestoquardi*. Infections by *T. ovis* are either low or non-pathogenic (Friedhoff, 1997; Rjeibi *et al.*, 2014) and those by *T. lestoquardi* are virulent (Ahmed *et al*., 2003; Rjeibi *et a*l., 2014). General clinical signs of ovine theileriosis are characterized by fever, weight loss, low production and subsequent death (Shahzad *et al*., 2013). Ovine theileriosis can be diagnosed by clinical symptoms, microscopic examinations (Kirvar *et al*., 1998; Durrani *et al*., 2011), serological tests such as IFAT (Leemans *et al*., 1997; El Imam & Taha, 2015) and ELISA (Gao *et al*., 2002; El Imam & Taha, 2015), and DNA-based tests such as PCR (Almeria *et al*., 2001; El Imam & Taha, 2015), RLB (Gubbels *et al*., 1999;El Imam & Taha, 2015), LAMP

(Notomi *et al*., 2000; El Imam & Taha, 2015) and RFLP (Spitalska *et al*., 2004; El Imam & Taha, 2015). Presence of *T. ovis* and *T. lestoquardi* as causative agents of ovine theileriosis has been recorded in Iran (Jalali *et al*., 2014; Shayan *et al*., 2016). The presence of *T. lestoquardi* has been detected from south-eastern Europe, northern Africa, the Near and Middle East and India (Levine, 1985; Durrani *et al*., 2011; El Hussein *et al*., 1993; Ali *et al*., 2017), and in China (Yin *et al*., 2007). *T. ovis* has been reported to infect sheep from different countries (Altay *et al*., 2005; Durrani *et al*., 2011). In South Africa, *T. ovis* has been reported from sheep in 1929 (Bigalke *et al*., 1926; Stoltsz, 1989) and by De Kock and Quilan (1926) (Jansen & Neitz, 1956.

Despite extensive research conducted worldwide as well as on the African continent and southern Africa including South Africa on ticks and tick-borne diseases, there is lack of such studies in the Mountain Kingdom of Lesotho. The current study was thereofere formulated to initiate ticks and tick-borne protozoan disease research in Lesotho. As a start-up, in this study tick infesting domestic animals are identified and then screened for the presence of DNA of protozoan parasites of veterinary and economic importance that they are possibly harbouring.

Chapter 2

Problem statement, hypothesis, aim and objectives

2.1. Problem statement

Ticks are capable of exhorting a significant loss on livestock production and economic growth through high infestations which cause anaemia and skin damage (De Castro, 1997; Norval *et al.*, 1992; Mbati *et al*., 2002) and the high cost of control measures (George *et al*., 2004; Young *et al*., 1988; Ogore *et al*., 1999). Furthermore, they act as vectors of various disease causing pathogens (McCoster, 1979; Colwell *et al.*, 2011) including viruses (Labuda *et al*., 1993), bacteria and protozoan parasites (Alekaw, 2000). Their impact on livestock is rated second after mosquitoes in terms of vectors of pathogens of either medical or veterinary importance (Day, 2011; Sandor *et al.*, 2014; Smith & Wall, 2013; Rogovskyy *et al*., 2017). It is apparent that haemoparasites associated with tick borne diseases have also become predominant limiting factors at various degrees in many countries that are highly dependent on production and productivity of livestock (Central Statistics Authority, 2009).

Lesotho is a landlocked country which is completely surrounded by the Republic of South Africa. Occurrence and distribution of various ticks of domestic animals has been widely reported in South Africa, whereby recorded species include *Rhipicephalus simus* (Horak *et al*., 1987; Walker *et al*., 2000; Golezardy *et al*., 2016)*, R. evertsi mimeticus, R. zambeziensis, R. appendiculatus* (Spickett *et al*., 2011), *R. sanguineus* (Horak *et al*., 2009), *R. decoloratus, R. microplus* (De Vos, 1979; Tonnesen *et al*., 2004; Nyangiwe *et al*., 2013)*, R. e. evertsi, Hyalomma* species (Marufu *et al*., 2011), *H. truncatum* (De Waal, 1990; Gummow *et al*., 1996), *Haemaphysalis silacea* (Horak *et al*., 2015), *H. elliptica* (Apanaskevich *et al*., 2007; Penzhorn, 2011), *Amblyomma hebraeum* (Spickett *et al*., 2011; Howell *et al*., 1978; Golezardy *et al*., 2016), *Ornithodoros moubata* (Penrith, 2009; Matthee *et al.*, 2013), *Otobius meginini,* and *Ixodes species* (Horak *et al*., 2009).

However, there is lack of documented scientific information on ticks of domestic animals in various districts of Lesotho. Moreover, tick-borne haemoprotozoan diseases of economic importance in domestic animals including bovine (Potgieter & Els, 1977 ;Tonnesen *et al.*, 2006; Mtshali & Mtshali, 2013) and canine babesiosis

(Apanaskevich *et al*., 2007; Penzhorn, 2011), equine piroplasmosis (De Waal, 1990; Gummow *et al*., 1996; Motloang *et al*., 2008), theileriosis (Thompson *et al*., 2008) and bovine besnoitiosis (Dubey *et al.*, 2013) have also been widely reported in South Africa and other southern African countries (Makala *et al*., 2003; Norval *et al*., 1983; Tonnesen *et al.*, 2006; Horak *et a*l., 2009; Simuunza *et al*., 2011). Despite this numerous literature on tick-borne haemoparasites and diseases they cause in domestic animals in southern African region, the same cannot be said about Lesotho. There is absolutely lack of documented published scientific information on tick transmitted haemoprotozoa infecting domestic animals including dogs, cattle, donkeys, horses, goats and sheep in districts of the Mountain Kingdom of Lesotho.

Identification of tick species plays a key role in basic regulations for tick borne disease (Lv *et al*., 2014). Anderson *et al*. (2004) elaborates that tick identification makes it possible to trace down diseases according to potential pathogens involved and, their interpreted host, habitat range and distribution patterns. Traditional morphological identification of tick species has limitations consequent to poor specimen preservation or changes of body forms seen after feeding or few unique traits shared among similar species (Anderson *et al*., 2004; Nava *et al*., 2009; Dergousoff & Chilton, 2007; Zhang & Zhang, 2014). Thus, combination of morphological and molecular approaches can be an effective means of identifying ticks to their species level (Najavas *et al*., 1992; Zhang & Zhang, 2014). The cytochrome oxidase I (COI) gene is regarded as the most utilized mitochondrial gene marker for many organisms and has also been used in several studies, with other gene markers, to identify and characterize ticks (Chitimia *et al*., 2010; Cakic *et al*., 2014; Lv *et al*., 2014; Ronaghi *et al*., 2015).

The advent of molecular technology has brought about development of various DNA-based diagnostic assays (O'Brein *et al*., 1991; Hwang & Kim, 1999; Persing, 1991; Salki *et al*., 1988; Figueroa *et al*., 1992; Mukabana *et al*., 2002; Gariepy *et al*., 2012). Amongst DNA-based assays, PCR is the most widely adopted for detection of haemoparasite infections in domestic animals and vectors in the world including African continent as well as southern Africa (Oliveira-Sequeira *et al*., 2005; Mtshali & Mtshali, 2013; Caccio *et al*., 2000; El-Ashker *et al*., 2015; Duarte *et al*., 2008; Carret

et al., 1999; Bashiruddin *et al*., 1999; Motloang *et al*., 2008; Mahmoud *et al*., 2015; Ellis *et al*., 2000; Kiehl *et al*., 2010).

Therefore, this study has sought to fill in the information gap by documenting ticks infesting domestic animals in various districts of Lesotho. Morphological and molecular techniques have been used to identify ticks occurring in domestic animals. Furthermore, this study has used PCR to detect haemoparasites harboured by these ticks.

2.2. Hypothesis

There is correlation between the diversity of tick species and the occurrence of blood parasites of in the Lesotho.

2.3. Aim of the study

To identify ticks infesting domestic animals in Lesotho and detect haemoparasites infecting ticks collected from domestic animals in Lesotho.

2.4. Objectives of the study

_ To identify ticks infesting domestic animals of Lesotho by morphological analysis using microscopy.

_ To conduct molecular characterization of ticks infesting domestic animals in Lesotho by PCR, sequencing and phylogenetic analysis of COI and ITS2 genes.

_ To detect the presence of *Babesia bigemina* and *Babesia bovis* in ticks collected from cattle in Lesotho by PCR.

_ To detect the presence of *Theileria equi* and *Babesia caballi* in ticks collected from equines in Lesotho by PCR.

_ To detect the presence of *B. ovis* and *B. matasi* in ticks collected from sheep and goats in Lesotho by PCR.

_To detect the presence of *T. ovis* and *T. lestoquardi* in ticks collected from sheep and goats in Lesotho by PCR.

Chapter 3

Materials and methods

3.1. Study area

Lesotho is a country found in the southern part of Africa and covers about 30, 355 km^2 of landmass (Figure 3.1.) (Flannery, 1977). It is situated within the southern African plateau at an elevation of about 1, 388 m and 3, 482 m above sea levels, between latitudes 28° and 31°S, and longitudes 27° and 30°E (Cauley, 1986; Ministry of Energy, Meteorology and Water Affairs, Lesotho, 2013). Lesotho is divided into four agro-ecological zones based on the climate and elevation; Lowlands, Senqu Rivers Valley, Foot-Hills and Mountains (Cauley, 1986). These zones differ in terms of climates and ecological features. However, Lesotho has generally temperate climate with alpine characteristics. The fact that Lesotho is located at high sea levels causes the air temperatures to be lower than in other countries with similar latitudes. Mean summer temperatures are around 25°C and mean winter temperatures are about 15°C. The highest summer temperature ever recorded was 38.5°C and the lowest winter temperature was -21°C (Ministry of Energy, Meteorology and Water Affairs, 2013).

The average temperature ranges between 28°C in summer and -2°C in winter (Ministry of Natural Resources, Lesotho, 2007). Snowfalls are seen between May and September. Rainfalls occur between October and April with annual precipitation of 85%. Mountain regions make up about 24% of this country (Lesotho Meteorological Services, 2007) and most of the communities reside in these mountain zones (Lesotho Meteorological Services, 2000). The Mountain zones are characterized by high livestock numbers, food insecurity, high population degradation of rangelands and extreme cold conditions (Lesotho Meteorological Services, 2000). Highest population pressure is found in the lowland zones of Lesotho (Bureau of Statistic and Planning, 2007). In Lesotho, rainfall is sporadic whereas drought and winter can be quite severe (Flannery, 2007).

Figure 3.1. Map of Lesotho showing different districts

3.2. Collection of tick samples from domestic animals of Lesotho

Veterinary centers of Lesotho provided the study with tick samples collected from domestic animals such as cattle, horses, sheep, goats and dogs. The tick specimens were obtained from the five districts of Lesotho; Leribe, Maseru, Qacha's Neck, Mafeteng and Butha-Buthe. In the first batch of collection, all the tick samples from Leribe, Maseru and Qacha's Neck were kept in 70% glycerol plus 30% ethanol containers. In the second batch of collection, tick samples from Mafeteng and few from Maseru districts were kept in 70% glycerol plus 30% ethanol containers, those from Butha-Buthe were not stored in any form of medium/liquid (were just kept in fridge within containers). The lodging of tick samples in 70% glycerol and 30% was for long term preservation (e.g. tick samples from Leribe, Maseru, Qacha and Mafeteng), but not DNA extraction purposes. Those stored under no liquids (e.g. tick samples from Butha-Buthe and four tick samples from Maseru horses) were later to be used for molecular work.

3.3. Morphological identification of ticks

All the ticks of the study from the five districts were identified morphologically using dissecting microscope (Olympus SZ51, Tokyo, Japan) and multi-purpose

microscope (Nikon AZ100M, Tokyo, Japan), and a tick guides of Walker *et al*. (2013) and Latif (2013). In addition, saved individual tick specimens representing tick species which were identified morphologically were sent to tick museum of the ARC – Ondersterpoort Veterinary Institute for further species identification confirmation and for generation of voucher specimens.

3.4. Extraction of DNA from tick specimens

In the first batch of tick collection, DNA extraction was conducted from 148 ticks of Qacha's Neck, 25 ticks from Leribe and 24 ticks from Maseru. For the second batch of tick collection, DNA was extracted from 186 tick samples of Butha-Buthe district (Bovine n = 164; Goats n = 6; Sheep n = 16) and four tick specimens from a horse of Maseru district. Methods used for extraction were salting out method adopted from Diallo *et al*. (1997) with few modifications. The extraction procedure was as follows:

On the first day the tick samples were crushed with a metal pistil inside a 1.5 ml Eppendorf tube. Then 480 µl of extraction buffer and 10 µl of Proteinase K were added on the crushed samples. The mixture was then placed in the tissue lyser for 10 minutes to mix the contents thoroughly. The mixed contents were then placed in an incubator at a temperature of 56°C for 1hour. After an hour, 10 µl of Proteinase was added to the mixture and incubated at 56°C for overnight. On the second day, incubated contents were centrifuged at 12 000 rpm for 5 minutes and the upper layer was pippeted into new Eppendorf tube. In the new tube, 180 µl of 5M NaCl was added to the upper layer. The contents were vortexed for 30 seconds and centrifuged at 13 500 rpm for 5 minutes at 4°C to precipitate the DNA. The upper layer was transferred to a new tube. A volume of 440 µl was added to the new tube, slowly inverted 50 times, centrifuged for 15 minutes at 4°C to precipitate the DNA. After the centrifugation, the upper layer was discarded, washed with 250 µl of 70% ethanol vortexed briefly and centrifuged at 15 000 rpm for 5 minutes. The ethanol step was then repeated, but this time the upper later was discarded and the tick sample was air dried for an hour at room temperature to allow the 75% ethanol to evaporate. Lastly the DNA was dissolved with 200 µl of double distilled water.

3.5. Amplification of tick DNA by PCR

Successfully extracted tick DNA samples (Qacha's Neck n = 8 and Butha-Buthe n = 12) which were identified on the basis of morphological traits, were subjected to PCR

amplification of COI and ITS2 genes. Different PCR kits were used, for instance for Qacha's Neck tick DNA samples a Phire Tissue Direct kit (Thermo Fisher Scientific, South Africa) was used and AmpliTaq Gold 360 kit (Thermo Fisher Scientific, South Africa) was used in DNA samples from Butha-Buthe tick DNA. Twenty selected tick DNA samples on which COI gene was amplified, using LCO1490 and HC02198, and Cox-F and Cox-R primers (Table 3.1), included species of male *H. rufipes* (Cattle n = 1), nymph of *O. megnini* (Cattle n = 1) and *R. e. evertsi* (Cattle n = 5) from Qacha's Neck, as well as female *R. e. evertsi* (Goat n = 1; Sheep n = 2; Cattle n = 6) and *R. microplus* (Goat n = 1; Cattle n = 2) from Butha-Buthe district. PCR reactions using Phire Tissue Direct Kit (Thermofisher, USA) were performed at a total volume of 20 µl which was made up of; 10 µl Taq, 3 µl forward primer, 3 µl reverse primer, 1 µl double distilled water (DDW) and 3 µl DNA. The cycling conditions were adopted from Phire Tissue Direct Kit with initial denaturation of 98°C for 5 minutes, followed by 40 cycles for denaturation of 98°C for 5 seconds, annealing temperature of 48°C for 5 seconds and extension temperature of 72°C for 20 seconds. A final extension was performed at 72°C for 1 minute and kept at 4°C.

The PCR reactions for AmpliTaq Gold 360 kit (Applied Biosystems, USA) were performed at a volume of 25 µl using; 12.5 µl of AmpliTaq mastermix, 1.5 µl forward primer, 1.5 µl reverse, double distilled water 7.5 µl and DNA of 2 µl. The cycling conditions were adopted form AmpliTaq Gold 360 manual and included an initial denaturation of 95°C of 10 minutes, followed by 40 cycles with denaturation temperature of 95°C for 30 seconds, annealing temperature of 47°C for 60 seconds and extension of 72°C for 2 minutes. A final extension was performed at 72°C for 10 minutes and kept at 4°C for hold. Positive and negative controls were added in each reaction. Five µl of PCR amplicons were examined through 1% agarose gel electrophoresis to confirm amplification efficiency.

The ITS2 gene was amplified from the tick's DNA samples of Butha-Buthe district using ITS2-forward and ITS2-reverse primers (Table 3.1). Species involved were female *R. e. evertsi* (Goat n = 1; Sheep n = 2; Cattle n = 6) and female *R. microplus* (Goat n = 1; Cattle n = 2). Only AmpliTaq Gold 360 kit, according the manufacture's guide was used. PCR reactions were as follows 12.5 µl AmpliTaq, 7.5 µl double distilled water, 1.5 µl forward primer, 1.5 µl reverse and DNA of 2 µl at a final volume

of 25µl. The cycling conditions included initial temperature set at 95°C for 10 minutes for x1 cycle, denaturation at 95°C for 30 seconds; Annealing at 50°C for 30 seconds and the final extension step was set at 72°C for 60 seconds and 35 cycles. The final hold step was at 72°C for 7 minutes. Five microliters of PCR amplicons were electrophoresed on a 1% agarose gel which was stained with ethidium bromide and visualized under UV light.

Table 3.1. PCR primer pairs for amplification of tick DNA

Primer	Primer sequences	Product size
LCO1490	: 5'-GGTCAACAAATCATAAAGATATTGG-3'	710bp (Folmer *et al*., 1994)
HCO2198	: 5'TAAACTTCAGGGTGACAAAAAATCA-3'	
Cox-F	F: 5'-GGAACAATATATTTAATTTTTGG-3'	732 bp (Chitimia *et al*., 2010)
Cox-R	R: 5'-ATCTATCCCTACTGTAAATATATG-3'	
ITS2-Forward	5´-YTGCGARACTTGGTGTGAAT-3´	950 bp
ITS2-Reverse	5´-TATGCTTAARTTYAGSGGGT-3´	(Abdigourdarzi *et al*., 2011)

3.6. Purification of PCR products

All the positive PCR amplicons of the study were purified using the QIAquick Gel Extraction Kit protocol according to manufacturer's instructions (Qiagen, USA) as follows: the DNA fragment from the agarose gel was cut using a scalpel and transferred into new 1.5 ml Eppendorf tube. A volume of 500 µl QG buffer was added to each tube. The tube containing sliced gel and QG buffer were incubated for ten minutes at a temperature of 50°C until the gel dissolved completely and was vortexed on every 2 minutes to during the incubation to help dissolve the gel. The final colour of the dissolved mixture was yellow and in instances where the mixture was orange or violet, a 10 µl of 3 M sodium acetate was added. Following that 100 µlof isopropanol was added to the mixture. A total volume of 800µl of the mixture was transferred to the QIquick spin column with the collection tube and centrifuged for one minute at a speed of 13 000 rpm. The flow-throw through was discarded and the QIAquick column was placed in new collection tubes. To wash, 750 µl of buffer PE was added to QIAquick column and centrifuged at 13 000 rpm for 1 minute. The QIAquick columns were placed into clean 1.5 ml microcentrifuge tube. For the final step of eluting DNA, 50 µl of buffer EB was added to the center of the QIAquick membrane and centrifuged for 1 minute at 13 000 rpm. The purified PCR amplicons

were sent to Inqaba Biotechnical Company, Pretoria and some to Obihiro University of Agriculture and Veterinary Medicine, Japan for sequencing.

3.7. Analysis of tick gene sequences

After sequencing, the generated nucleotide sequences of COI and ITS2 for Lesotho ticks were subjected to BLASTn (https://www.ncbi.nlm.nih.gov/) in order to verify whether they match with subsequent tick sequences in the NCBI database. The forward and reverse nucleotide sequences of COI and ITS2 from ticks of Lesotho were assembled using Geneious 10.1.3 to obtain a suitable consensus for each tick sequence. Each consensus sequence was put on BLASTn to verify whether they could generate relatively similar hits of COI and ITS2 tick sequences from the NCBI database. Matching sequences for ticks were downloaded from the Genbank (https://www.ncbi.nlm.nih.gov/) (Table 3.2). Separate multiple alignments for COI sequences representing soft (*Otobius megnini* from the study) and hard (*Hyalomma rufipes* and *Rhipicephalus evertsi evertsi* from the study) tick species along with their matching tick sequences from NCBI were aligned using Clustal W1.6 or Muscle within MEGA 6.0 to evaluate their similarities on the bases of their conserved regions. Visual default parameters such as open and extended gaps were deleted. Multiple alignments for ITS2 nucleotide sequences was performed on MEGA 6.0 using Clustal W1.6 or MUSCLE (Edgar, 2004) and visual default parameters such as open and extended gaps were deleted. Species involved were *R. e. evertsi* and *R. microplus* from the study. The genetic distance for intra- and inter-species variations of soft and, hard ticks was analyzed by the Kimura two parameter model added on MEGA 6.0 (Kimura, 1980; Braham *et al.,* 2014; Chao *et al.,* 2011; 2017).

Table 3.2. Source of tick specimens used in the analysis of the study results

Type of Gene	Species	Geographic Origin	Accession number
COI for soft ticks	*Otobius megnini*	Madagascar, Africa	KC769589
	Otobius megnini	South Africa, Africa	KJ133592
	Ornithodoros moubata	Tanzania, Africa	AB073679
	Ornithodoros moubata	South Africa, Africa	KJ133594
	Argas walkerae	South Africa, Africa	KJ133585
	Argas walkerae	South Africa, Africa	KJ133584
COI for hard ticks	*Hyalomma rufipes*	Zimbabwe, Africa	AF132823
	Hyalomma truncatum	Ethiopia, Africa	AJ437088
	Hyalomma marginatum	France, Europe	KX000635
	Hyalomma turanicum	Israel, Asia	KT989638
	Hyalomma dromedarii	Ethiopia, Africa	AJ437081
	Hyalomma dromedarii	Ethiopia, Africa	AJ437082
	Rhipicephalus evertsi evertsi	Uganda, Africa	AB934398
	Rhipicephalus evertsi evertsi	Kenya, Africa	AF132835
	Rhipicephalus evertsi mimeticus	Kenya, Africa	AF132836
	Rhipicephalus australis	Bunya, Austalia	KC503255
	Rhipicephalus turanicus	China, Asia	KF688136
	Rhipicephalus bursa	Iran, Asia	KM494914
	Rhipicephalus turanicus	China, Asia	KU880575
	Rhipicephalus annulatus	North America	KX228540
	Rhipicephalus annulatus	North America	KX228542
	Rhipicephalus geigy	Burkina Faso, Africa	KC503263
	Rhipicephalus microplus	Benini, Africa	KY678120
	Rhipicephalus geigyi	Burkina Faso, Africa	KY678125
	Rhipicephalus sanguineus	Iran, Asia	KM494915
ITS2 for hard ticks	*Rhipicephalus microplus*	China, Asia	JF758642
	Rhipicephalus microplus	China, Asia	JQ625705.1
	Rhipicephalus microplus	Nigeria, Africa	MF373428
	Rhipicephalus microplus	Nigeria, Africa	MF373429
	Rhipicephalus microplus	Nigeria, Africa	MF373427
	Rhipicephalus microplus	China, Asia	KC203365
	Rhipicephalus evertsi evertsi	Zambia, Africa	DQ849266
	Rhipicephalus evertsi evertsi	Kenya, Africa	U97701.1
	Rhipicephalus evertsi mimeticus	Namibia, Africa	AF271279

3.8. Phylogenetic analysis of tick samples from domestic animals

The JModeltest2 (Darriba *et al*., 2012) was used to choose the best fitting evolutionary model to conduct Neighbor-Joining (NJ) and Maximum Likelihood (ML) phylogenetic analysis, on the basis of Akaike Information Criterion (AIC) selection criterion. Mega 6.0 software was used to construct Neighbor-Joining and Maximum Likelihood trees (Tamura *et al*., 2013). Selected Bootstrap replications for all the constructed phylogenetic trees were performed at 10 000. The best models suggested by JModeltest2 for the phylogenetic analysis of COI nucleotide sequences on Maximum Likelihood was General Time Reversal (GTR+I+R) for hard ticks and GTR+I for soft ticks. An outgroup for hard ticks was *Dermacentor andersoni* (KX360398) and for soft ticks, *Ixodes ricinus* (AY945438). For Neighbor-Joining approach, the model which was selected for all analysis was kimura 2-Parameter with bootstrap of 10 000 (Tamura *et al*., 2013). For ITS2 nucleotide sequences of hard ticks, the best suggested models included transversion model (TVM+G) and TVM+I by JModeltest2, but GTR+G was selected for a maximum likelihood approach, because it was the closest and, in addition, MEGA 6.0 does not have TVM+G or TVM+I models. For Neighbor-Joining, the selected model was Kimura 2-Parameter (Tamura *et al*., 2013). *Dermacentor nuttali* (KF281880) was used as outgroup.

3.9. PCR for detection *B. bigemina* and *B. bovis* from tick DNA

PCR conditions were adopted from Fiqueroa *et al*., (1993); Mtshali *et al*., 2013; 2014). In the 1st round of nested PCR (nPCR) for amplification of *B. bigemina* Substrate glycoprotein (Gps45) gene the BIG1 forward and BIG2 reverse primers were used (Table 3.3). The 2nd round of nPCR employed nBIG1 forward and nBIG2 reverse primers (Table 3.3). Thermal cycling conditions for both 1st and 2nd rounds of of nPCR for *B. bigemina* were similar and were performed in a total volume of 25 µl made up of 12.5 µl of Dream taq (Thermofisher, USA), 1.5 µl of forward primer, 1.5 µl of reverse primer, 7.5 µl of DDW and 2 µl of DNA template. For 2nd round of nPCR, 1 µl of 1st round PCR amplicon was used as DNA template and the DDW was adjusted accordingly. The thermal cycling conditions included; initial denaturation of 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing temperature of 55°C for 45 seconds and extension of 72°C for 1 minute. A final extension was performed at 72°C for 7 minutes and kept at 4°C for hold.

For *B. bovis* a portion of rhoptry-associated protein1 (RAP-1) gene was targeted using BOV1 forward and BOV2 reverse primers in the 1st round of nPCR (Table 3.3.). The 2nd round of nPCR was conducted using nBOV1 forward and nBOV2 reverse primer sets to amplify the RAP-1 gene of *B. bovis* (Table 3.3). Thermal cycling conditions for both 1st and 2nd round nPCR for *B. bovis* were similar and performed in a total volume of 25 µl with 12.5 µl of Dream taq (Thermofisher, USA), 1.5 µl of forward primer, 1.5 µl of reverse primer, 7.5 µl of double distilled water (DDW) and 2 µl of DNA template. For 2nd round of nPCR, 1µl of 1st round PCR amplicon was used as DNA template and the DDW was adjusted accordingly. The thermal cycling conditions included; initial denaturation of 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing temperature of 55°C for 45 seconds and extension of 72°C for 1 minute. A final extension was performed at 72°C for 7 minutes and kept at 4°C for hold. Positive and negative controls were added in each reaction. Five microlitres of PCR amplicons were electrophoresed on a 1% agarose gel which was stained with ethidium bromide and visualized under UV light.

Table 3.3. The nPCR Primer pairs for *B. bigemina* and *B. bovis*

Primer	Primer sequences	Product size
BIG1	forward: 5'-CATCTAATTTCTCTCCATACCCCTCC-3'	278 bp (Fiqueroa *et al*., 1993;
BIG2	reverse: 5'-CCTCGGCTTCAACTCTGATGCCAAAG-3'	Mtshali *et al*., 2013; 2014)
nBIG1	forward: CGCAAGCCCAGCACGCCCCGGTGC	170 bp (Fiqueroa *et al*., 1993;
nBIG2	reverse: CCGACCTGGATAGGCTGTGTGATG	Mtshali *et al*., 2013; 2014)
BOV1	forward: 5'-CACGAGGAAGGAACTACCGATGTTGA-3'	360bp (Fiqueroa *et al*., 1993;
BOV2	reverse: 5'-CCAAGGAGCTTCAACGTACGAGGTCA-3'	Mtshali *et al*., 2013; 2014)
nBOV1	forward: TCAACAAGGTACTCTATATGGCTACC	298 bp (Fiqueroa *et al*., 1993;
nBOV2	reverse: CTACCGAGCAGAACCTTCTTCACCAT	Mtshali *et al*., 2013; 2014)

3.10. PCR for detection of *B. caballi* and *T. equi* from tick DNA

The PCR primers targeting the Bc48 gene of *Babesia caballi* merozoite rhoptry protein were used to amplify *B. caballi* DNA (Table 3.4). The primers targeting the EMA1 gene were used to amplify *T. equi* DNA and there was no positive control for *T. equi* (Table 3.4). PCR was performed in a total volume of 10 µl containing 3.1 µl

amplitaq gold, 1 µl forward primer, 1 µl reverse primer, 2.9 µl double distilled water and 2 µl template. The cycling conditions were initial denaturation temperature of 94°C for 10 minutes, followed by cycles of 35 at a denaturation step of 94°C for 45 seconds, annealing temperature of 58°C for *B. caballi* and 57°C for *T. equi* for 1 minute, and extension temperature of 72°C for 2 minutes and a final extension temperature of 72°C for 7 minutes and hold temperature of 4°C. Five microlitres of PCR amplicons were electrophoresed on a 1% agarose gel which was stained with ethidium bromide and visualized under UV light.

Table 3.4. The PCR primers for *B. caballi* and *T. equi*

Primer	Primer sequences	Product size
BC 48	F: 5'-CGGCTGCTATGGTTATTCAG-3' R: 5'-AGAGTGCAACCGAGCAATGC-3'	659bp (Bashiruddin *et al*., 1999)
EMA1	F: 5'-GCATCCATTGCCATTTCGAG-3' R: 5'-TGCGCCATAGACGGAGAAGC-3'	664bp (Bashiruddin *et al*., 1999)

3.11. PCR for detection of *B. ovis* and *B. motasi* DNA from ticks

There were no positive controls available for PCR assays performed for detection of *B. ovis* and *B. motasi* from DNA of ticks collected from goats and sheep, for this reason blind PCR assays without positive controls were conducted. Forward and reverse primer sets targeting the 18S rRNAgene were used to amplify *B. ovis* (Table 3.5). PCR conditions were optimised for this study and were performed in a total volume of 10ul with 3.1 µl amplitaq gold, 1 µl forward primer, 1 µl reverse primer, 2.9 µl double distilled water and 2 µl template. The cycling conditions were initial denaturation temperature of 94°C for 10 minutes, followed by cycles of 38 at a denaturation step of 94°C for 45 seconds, annealing temperature of 63°C for *B. ovis* for 1 minute, and extension temperature of 72°C for 2 minutes. A final extension temperature of 72°C for 7 minutes and hold temperature of 4°C. A semi-nested PCR was performed was performed, where primer sets for forward (P1, P4) and reverse (P2) targeting 18S rRNA gene were used to amplify *B. motasi* (Table 3.5). Primer P1 and P2 were catch all primers for *Babesia-Theileria* species (Shayan *et al.,* 2008) and, Primer P2 and P4 were specifically for amplifying *B. motasi,* see table 3.6 (Shayan *et al.,* 2008). The first PCR was conducted in a volume of 25µl using *Taq* DNA polymerase kit (New England BioLabs, USA): 2.5 µl of 10X standard *Taq*

reaction buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of forward primer, 0.5 µl of reverse primer, 0.125 µl of *Taq* DNA polymerase, 17.875 µl double distilled water and 3 µl DNA template. The cyclic conditions were as follows: initial denaturation of 95°C for 30 seconds, number of cycles of 35 for denaturation step of 95°C for 30 seconds and annealing temperature of 55°C for 1 minute and extension temperature of 68°C for 1 minute, the final extension temperature was 68°C for minutes and kept on hold to infinity at 4°C. The second PCR for *B. motasi* was performed at a final volume of 25 µl: The first PCR was conducted at a volume of 25 µl using *Taq* DNA polymerase kit (New England BioLabs, USA): 2.5 µl of 10X standard *Taq* reaction buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of forward primer, 0.5 µl of reverse primer, 0.125 µl of *Taq* DNA polymerase, 19.875 µl double distilled water and 1 µl DNA template. The cycling conditions did not change, was similar as the first PCR. Five microlitres of PCR amplicons were electrophoresed on a 1% agarose gel which was stained with ethidium bromide and visualized under UV light.

Table 3.5. The PCR primer pairs for *B. ovis* and *B. motasi*

Primer	Primer sequences	Product size
B. ovis	Bbo-F: 5'-TGGGCAGGACCTTGGTTCTTCT-3' Bbo-R: 5'-CCGCGTAGCGCCGGCTAAATA-3'	549bp (Aktas *et al*., 2005)
B. motasi	P1: 5'-CACAGGGAGGTAGTGACAAG-3'	389-402bp (Shayan *et al.,* 2008)
	P2: 5'-AAGAATTTCACCTATGACAG-3'	426-430bp (Shayan *et al.,* 2008)
	P4: 5'-CGCGATTCCGTTATTGGAG-3'	205bp (Shayan *et al*., 2008)

3.12. PCR for detection of *T. ovis* and *T. lestoquardi* DNA from ticks

Primer pairs targeting the 18S rRNA genes *T. ovis* and *T. lestoquardi* (Table 3.6) were used in the PCR of the current study. PCR conditions were optimized in this study and were performed in a total volume of 10ul with 3.1 µl amplitaq gold, 1 µl forward primer, 1 µl reverse primer, 2.9 µl double distilled water and 2 µl template. The cycling conditions were initial denaturation temperature of 94°C for 10 minutes, followed by cycles of 35 at a denaturation step of 94°C for 45 seconds, annealing temperature of 52°C for *T. ovis* and 60°C for *T. lestoquardi* for 1 minute, and extension temperature of 72°C for 2 minutes, a final extension temperature of 72°C for 7 minutes and hold temperature of 4°C. Five microlitres of PCR amplicons were

electrophoresed on a 1% agarose gel which was stained with ethidium bromide and visualized under UV light.

Table 3.6. The PCR primers for *T. ovis* and *T. lestoquardi*

Species	Primer sequences	Product size
T. ovis	P4 18S rRNA antisense: 5'-CTAAGAATTTCACCTCTGACA-3'	228bp
	P5 18S rRNA sense: 5'-CTTTACGAGTCTTTGCATTG-3'	(Shayan *et al*., 2016)
T. lestoquardi	Tl-1-1-Forwad: 5'-GACCAGCCTCTTCTCCAACATT-3'	785bp
	Tl-1-1-Reverse: 5'-CAGGTTTAGTGACTGGAGTGGTC-3'	(Ali *et al*., 2017)

Chapter 4

Results

4.1. Tick samples

A total of 1654 tick specimen were collected from five districts in Lesotho. Female ticks (n = 877) were the most prevalent followed by males (n = 455) and the nymphae (n = 374) were the least. From Leribe district (coordinates: 28°56'15.37"S and 28°15'29.74"E), a total of 132 (8%) tick samples (males: 31; females: 101) were obtained from different domestic animals whereby 53 were from cattle, 51 from sheep and 28 were from unrecorded hosts (Table 4.1). Maseru district (coordinates: 29°17’42.38”S; 27°30’20.98”E) was represented by 322 (19%) tick samples (males: 117; females: 154; nymphae: 51) from cattle (n = 268) and unrecorded hosts (n = 54) (Table 4.2). The largest tick samples number was collected from Qacha’s Neck district (coordinates: 30° 6'41.24"S, 28°40'44.32"E) which included a total n = 641 (39%) (males: 98; females: 47; nymphae: 215) from four known hosts namely, cattle (n = 290), dogs (n = 36), horses (n = 87) and sheep (n = 2), and the remaining 226 tick samples were from unrecorded hosts (Table 4.3). In Mafeteng district (29°38’26.52”S; 27°09’21.19”E) a total of n = 75 (5%) tick samples (males: 2; females: 10; nymphae: 63) all collected from cattle (Table 4.4). Lastly Butha-Buthe district (28°48’22.22”S; 28°23’50.58”E) had a total of n = 484 (29%) tick samples (males: 133; females: 306; nymphae: 45) from cattle (n = 422) ovine and caprine (sheep 30; goats: 28) and unrecorded hosts (n = 4) (Table 4.5).

4.2. Morphological identification of ticks

Of the collected tick samples, three genera were identified namely, *Hyalomma* (Plate 4.1 – 4.2), *Otobius* (Plate 4.3) and *Rhipicephalus* (Plate 4.4, 4.5 and 4.7). Note that in this study individual tick specimens representing each identified tick species were submitted to the tick museum of the ARC – Onderstepoort Veterinary Institute for voucher specimens provided on Plate 4.1 to Plate 4.7. The most abundant tick species through all the districts was *R. e. evertsi* 52% (n = 864) (Plate 4.7) and the least was *H. rufipes* 4% (n = 26) (Plate 4.1 – 4.2) and *H. truncatum 2%* (n = 10) (Plate 4.6).

In Leribe district only one genus, namely, *Rhipicephalus* (n = 132) was identified (Table 4.1) with two species i.e. *R. e. evertsi* 70% (n = 93) (Plate 4.7) and *R. microplus* 30% (n = 39) respectively (Plate 4.4 - 4.5). *R. e. evertsi* was identified by a dark brown scutum and conscutum, and uniform orange colour on both males and females' legs (Latif, 2013; Walker *et al*., 2014), while *R. microplus* was distinguished by four plus four (or 4 + 4) columns of teeth on the hypostome (Walker *et al*., 2014). Fifteen *R. e. evertsi* were collected from cattle, 50 were from sheep and the remaining 28 were from unrecorded hosts. Thirty eight *R. microplus* tick specimens were obtained from cattle and only one from sheep (Table 4.1). Thus, *R. e. evertsi* 70% (n = 93) was more abundant than *R. microplus* 30% (n = 39) (Table 4.1).

Table 4.1. Tick species identified in Leribe district by morphological features

Tick species	Hosts			Sex		Total
	B	O (S)	U	Male	Female	
R. e. evertsi	15	50	28	31	62	93
R. microplus	38	1	**	**	39	39
						Total = 132

B = Bovine | O (S) = Ovine: Sheep | Hosts or Sex not given = ** | U = Tick samples were collected by state veterinarians and they did not write hosts on data sheet

In Maseru district, a total of 322 tick specimens was identified from two genera designated *Rhipicephalus* and *Otobius* (Table 4.1). The genus *Otobius* was represented by *O. megnini* (Plate 4.3) which is a soft tick from family Argasidae. It was identified by grey and violin-shaped body in both adults and nymphae, and simple ventrally situated mouthparts. Additionally nymphae were identified by absence of genital openings (Walker *et al*., 2014). Only three ticks of this species were recorded from unknown hosts (the state veterinarians did not record the host during collection of ticks). Two species under the genus *Rhipicephalus* were identified as *R. e. evertsi* (Plate 4.6) and *R. microplus* (Plate 4.4). All the *R. e. evertsi* (n = 88) were identified from bovine hosts and 50 were from unrecorded hosts. A sum of 180 *R. microplus* tick specimens were identified from cattle and one was from an unrecorded host (Table 4.2). The *R. microplus* was the most abundant species at

56% (n = 181) followed by *R. e. evertsi* at 43% (n = 138) and the least was *O. megnini* at 1% (n = 3) (Table 4.2).

Table 4.2. Tick species identified in Maseru district by morphological features

Tick species	Centre	Hosts			Sex			Total
		B	O (S)	U	Male	Female	Nymph	
R. e. evertsi	LAC	64	**	**	52	10	2	64
	*	24	**	50	52	21	1	74
R. microplus	LAC	32	**	**	6	26	**	32
	*	148	**	1	7	97	45	149
O. megnini	*	**	**	3	**	**	3	3
								Total = 322

B = Bovine | O(S) = Ovine: Sheep | LAC=Lesotho College of Agriculture | Centre or Village not given = * | Host or Sex not found ** | U = Tick samples were collected by state veterinarians and they did not write hosts on data sheet

In Qacha's Neck district, a total of 641 tick specimens represented by three genera *Hyalomma*, *Otobius* and *Rhipicephalus* were recorded (Table 4.3). Similar to Maseru, only *O. megnini* was identified (Plate 4.3). The genus *Hyalomma* was represented by two species; *H. rufipes* (Plate 4.1 - 4.2) and *H. truncatum* (Plate 4.6). Under the genus *Rhipicephalus*, two species, *R. microplus* (Plate 4.4 - 4.5) and *R. e. evertsi* (Plate 4.7) were identified. The *H. rufipes* was recognised by annulated legs and a large, robust and shiny-black scutum in males, whilst females by their wide genital pore with a broad posterior margin (Latif, 2013). According to Walker *et al.* (2014) *H. truncatum* can be recognized by its unusual smooth, shiny and dark conscutum which has a large single concave or depressed area in the posterior region. Twenty six and 10 tick specimens of *H. rufipes* and *H. truncatum*, respectively were obtained from unrecorded hosts from Qacha's Neck district (Table 4.3). For *R. microplus* ticks, 31 specimens were identified from cattle, 3 from dogs and 5 from unrecorded hosts (Table 4.3). A total of 113 tick specimens of *O. megnini* was collected from cattle, 24 from dogs, 1 from sheep and 77 from unrecorded hosts (Table 4.3). The *R. e. evertsi* ticks were collected from cattle (n = 146), dogs (9), horses (n = 87), sheep (n = 1) and from unrecorded hosts (n = 108) (Table 4.3). The *R. e. evertsi* was the most abundant species at 55% (n = 351) followed by *O. megnini*at 34% (n = 215). *R. microplus* at 6% (n = 39), *H. rufipes* at 4% (n = 26) and *H. truncatum* at 2%(n = 10) were the least abundant (Table 4.3).

Table 4.3. Tick species identified in Qacha's Neck district by morphological features

Tick species	Hosts					Sex			Total
	B	C (D)	E (H)	O (S)	U	Male	Female	Nymph	
H. rufipes	**	**	**	**	26	20	6	**	26
H. truncatum	**	**	**	**	10	**	10	**	10
R. microplus	31	3	**	**	5	**	39	**	39
O. megnini	113	24	**	1	77	**	**	215	215
R. e. evertsi	146	9	87	1	108	152	199	**	351
									Total = 641

B = Bovine | C = Canine: Dogs | E (H) = Equine: Horses | O (S) = Ovine: Sheep | Host or Sex not found = ** | U = Tick samples were collected by state veterinarians and they did not write hosts on data sheet

In Mafeteng district, a total of 75 tick specimens were collected and were represented by two genera *Otobius* and *Rhipicephalus* (Table 4.4). Like in Leribe and Qacha's Neck districts *O. megnini* (Plate 4.3) *R. e. evertsi* (Plate 4.7) and *R. microplus* (Plate 4.4 – 4.5) were recorded. Of the specimen obtained from cattle *O. megnini* was the most abundant species at 37% (n = 28) followed by *R. e. evertsi* at 32% (n = 24) and lastly *R. microplus* at 17% (n = 13) were the least abundant (Table 4.4).

Table 4.4. Tick species identified in Mafeteng district by morphological features

Tick species	Village	Hosts	Sex			Total
		B	Male	Female	Nymph	
O. megnini	Tsa Kholo	28	**	**	28	28
R. e. evertsi	Tsa Kholo	24	2	2	20	24
R. microplus	*	13	**	8	5	13
						Total = 75

B = Bovine | Village not given = * | Host or Sex not found = **

In Butha-Buthe district, an overall total of 484 tick specimens were collected which consisted of, one genus, *Rhipicephalus* (Table 4.5), represented by two species *R. microplus* (Plate 4.4 - 4.5) and *R. e. evertsi* (Plate 4.7). Tick specimens identified as *R. microplus* from cattle were 216 and from goats were 16, whilst 1 specimen was from an unrecorded host (Table 4.5). The *R. e. evertsi* specimens identified from cattle were 206, whilst 12 were from goats, 30 from sheep and 3 were from unrecorded hosts (Table 4.5). Images representing overall tick specimen which were identified morphologically to species level are provided below (Plate 4.1 to - 4.7). The *R. e. evertsi,* with 52% (n = 251) tick species, had the highest abundance as compared to *R. microplus* which was represented by 48% (n = 233) of specimens (Table 4.5).

Table 4.5. Tick species identified in Butha-Buthe district by morphological features

Tick species	Centre	Village	Hosts				Sex			Total
			B	O		U	Male	Female	Nymph	
				G	S					
R. microplus	Selomo Resource Centre	Manamela	**	15	**	**	1	14	**	15
	Selomo Resource Centre	Qalo	20	1	**	**	1	20	**	21
	Ngoajane Resource Centre	Ha Montlobo	4	**	**	**	2	2	**	4
	Nqabene Resource Centre	Ha Khabile	24	**	**	**	1	23	**	24
	Nqabene Resource Centre	Belo	10	**	**	**	*	10	**	10
	Nqabene Resource Centre	*	8	**	**	**	*	8	**	8
	Resource Centre	*	4	**	**	**	*	4	**	4
	Matsoaing Resource Centre	Matsoaing	29	**	**	**	7	22	**	29
	*	*	117	**	**	1	3	115	**	118
R. e. evertsi	Selomo Resource Centre	Qalo	15	5	**	**	10	10	**	20
	Selomo Resource Centre	Linakeng	**	**	3	**	2	1	**	3
	Ngoajane Resource Centre	Ha Montlobo	10	**	**	**	6	4	**	10
	Ngoajane Resource Centre	Seboche	8	**	**	**	2	6	**	8
	Nqabene Resource Centre	Belo	8	**	**	**	8	*	**	8
	Nqabene Resource Centre	Ha Khabile	8	**	**	**	3	5	**	8
	Nqabene Resource Centre	*	7	**	**	**	4	3	**	7
	Ngabene Resource Centre	Ha Majara	**	**	3	**	1	2	**	3
	Resource Centre	*	7	**	**	**	4	3	**	7
	Matsoaing Resorce Centre	Matsoaing	12	**	**	**	6	6	**	12
	*	*	3	**	**	**	*	3	**	3
	*	Phutong	**	**	3	**	1	2	**	3
	*	*	128	7	21	4	71	43	45	159
										Total = 484

B = Bovine │ O = Ovine; G = Goat; S = Sheep │ Centre or Village not given = * │ Host or Sex not found = **│ U = Tick samples were collected by state veterinarians and they did not write hosts on data sheet

Plate 4.1. *Hyalomma rufipes* male (voucher number: OP5136), (A) dorsal view and, (B) ventral view. Images not according to scale, the original scale was1000 µm

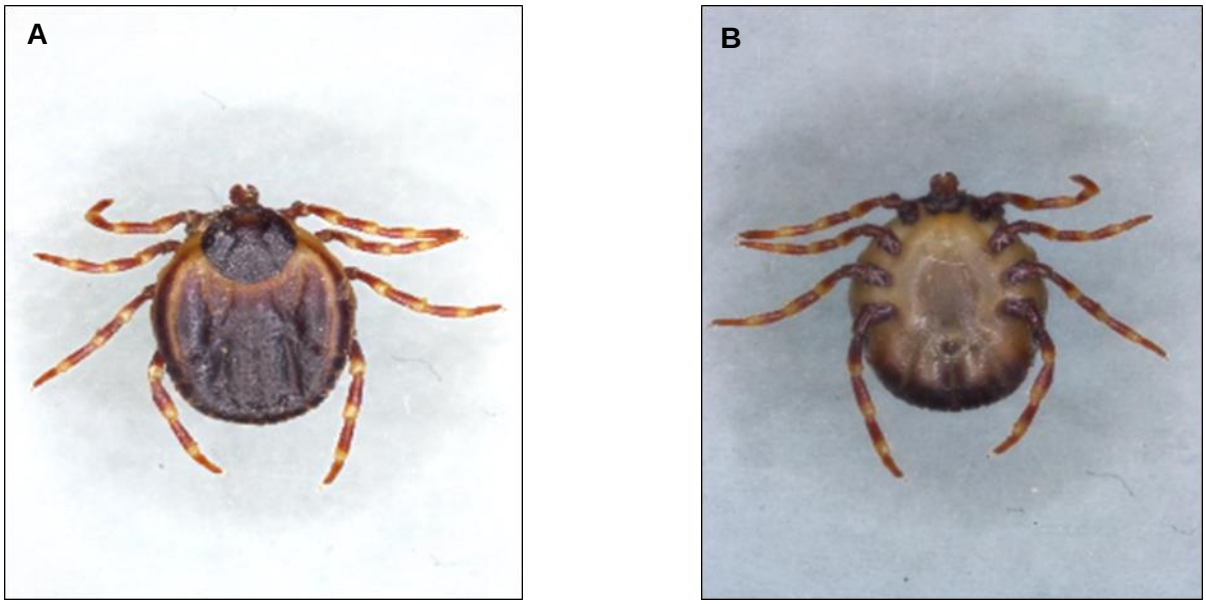

Plate 4.2. *Hyalomma rufipes* female (voucher number: OP5135) (A) dorsal view and (B) ventral view. Images not according to scale, the original scale was 500 µm.

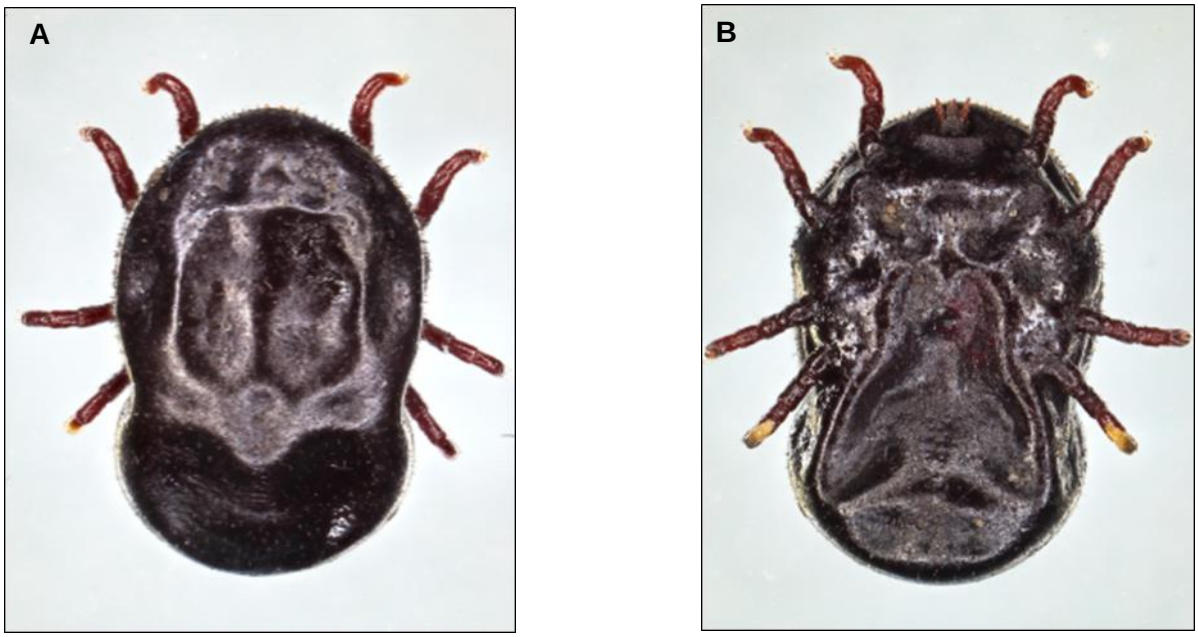

Plate 4.3. *Otobius megnini* nymph (voucher number: OP5127) (A) dorsal view and (B) ventral view. Images not according to scale, the original scale was 500 µm.

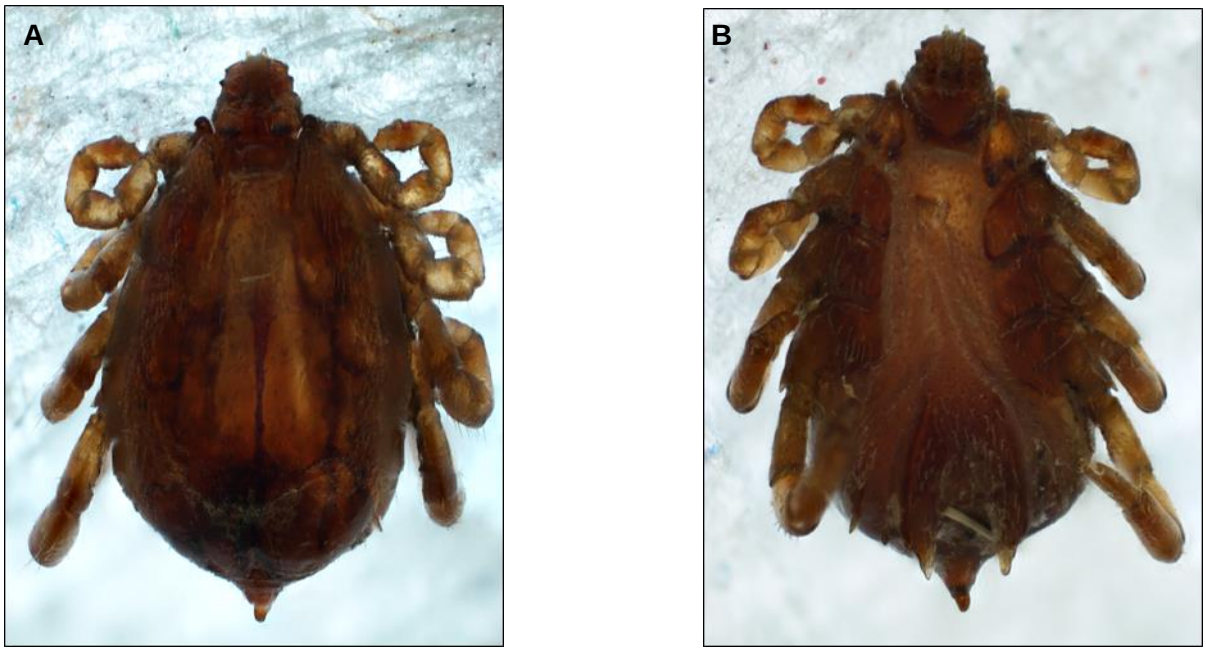

Plate 4.4. *Rhipicephalus microplus* male (voucher number: OP5120), (A) dorsal view and (B) ventral view. Images not according to scale, the original scale was 100 µm.

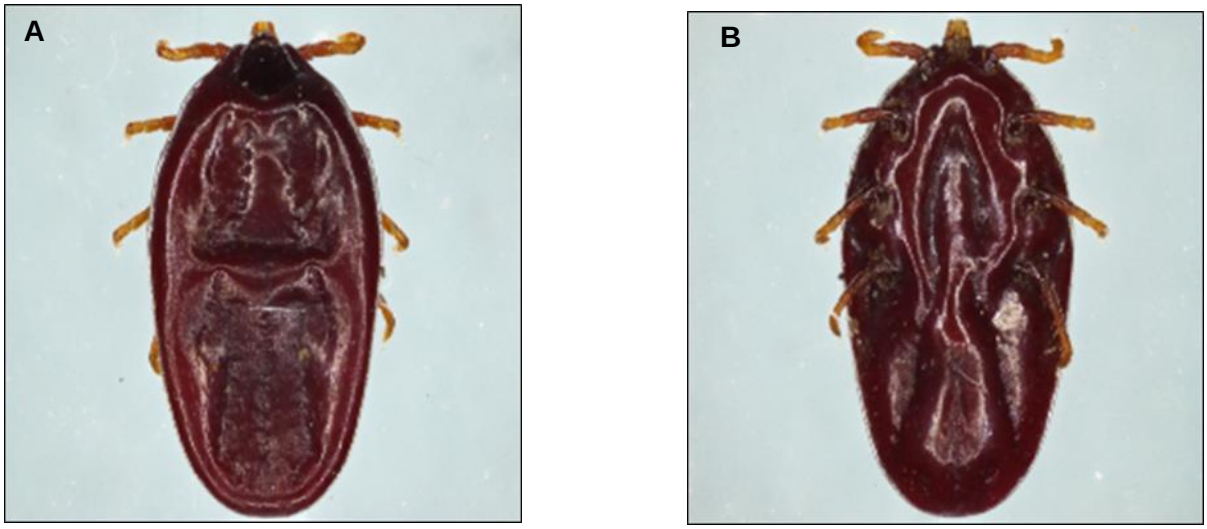

Plate 4.5. *Rhipicephalus microplus* female (voucher number: OP5089), (A) dorsal view and (B) ventral view. Images not according to scale, the original scale was 500 µm.

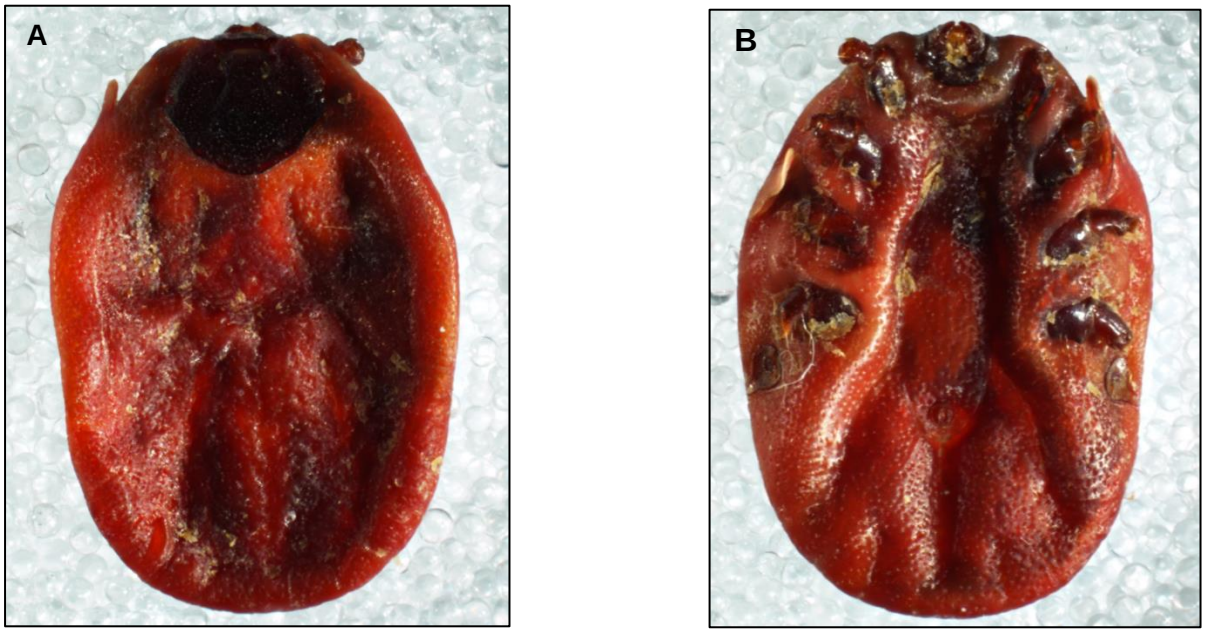

Plate 4.6. *Hyalomma truncatum* female (voucher number: OP5134), (A) dorsal view and (B) ventral view. Images not according to scale, the original scale was 500 µm.

Plate 4.7. *Rhipicephalus evertsi evertsi*, (A) Male (voucher number: OP5117) and female (voucher number: OP5122) dorsal view and (B) Male and female ventral view. Images not according to scale, the original scale was 1000 µm.

4. 3. Amplification of tick COI and ITS2 genes and sequence identification

A total number of twenty selected tick DNAs (8 from Qacha's Neck and 12 from Butha-Buthe) were used for molecular analysis on the basis of COI mitochondrial gene. DNA was not successfully extracted from the rest of the ticks collected from Maseru, Leribe, Mafeteng and Qacha's Neck districts, this was due to the fact that they were stored in a mixture of 30% glycerol and 70% ethanol after collection which appeared to have affected the quality of DNA. Two different primer sets with slightly different band sizes for COI gene were used (Table 3.1). PCR amplification of COI gene from Qacha's Neck samples was observed a710 bp and 732 bp (Table 3.1) product bands for *H. rufipes* (L2), *O. megnini* (L8) and *R. e. evertsi* (L3, L4, L6, L7 & L9) (Figure 4.1), positive COI gene PCR for Butha-Buthe tick specimen was observed as evidenced by a product size of 710 bp for *R. e. evertsi* (L2, L3, L4, L5, L5, L6, L7) and *R. microplus* (L12) using only one set of primers which target COI gene (Figure 4.2).

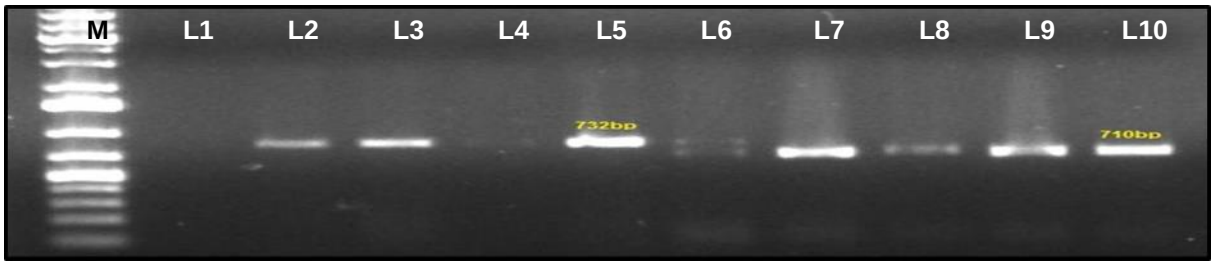

Figure 4.1. Agarose gel illustrating COI PCR amplicons from representative individuals of *H. rufipes* (Lane 2), *O. megnini* (Lane 8) and *R. e. evertsi* (Lane 3, 4, 6, 7 & 9). Lane 1 isnegative control and Lane 5 and 10 are positive control tick DNA. Lane M is a 1kb DNA ladder.

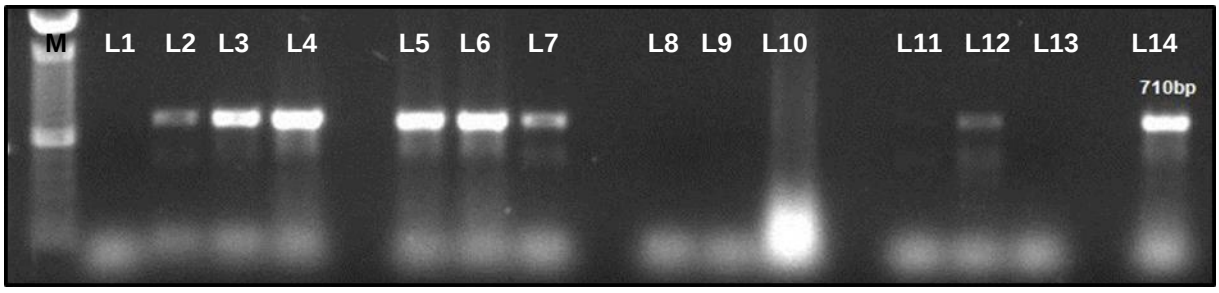

Figure 4.2. Agarose gel illustrating COI PCR amplicons from representative individuals of *R. e. evertsi* (Lane 2 - 7 & Lane 11 - 13) and *R. microplus* (Lane 8 - 10). Negative control was Lane 1 and Positive control for ticks was Lane 14. Lane M is a 100 bp DNA ladder.

For analysis of ITS2 gene, positive PCR amplifications were recorded at expected amplicon sizes ranging from 900 to 950 bp for *R. e. evertsi* (L2 - L7 and L11 - L13) and *R. microplus* (L8 - L10) (Figure 4.3).

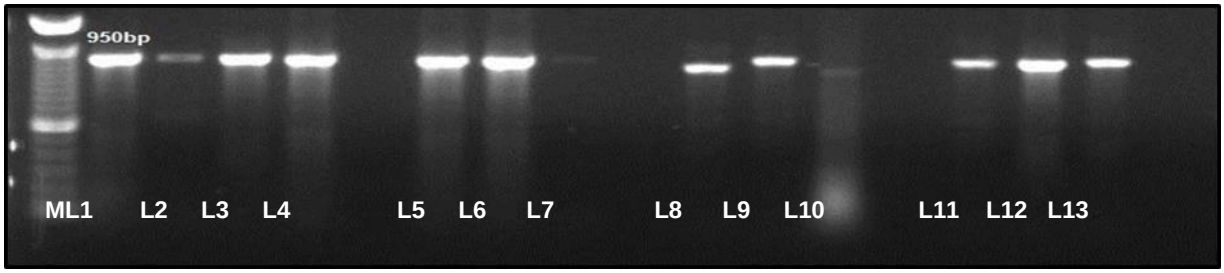

Figure 4.3. Agarose gel illustrating ITS2 PCR amplicons from representative individuals of *R. e. evertsi* (Lane 2 - 7 & Lane 11 - 13) and *R. microplus* (Lane 8 - 10). Positive control for ticks was Lane 1 and Negative control was Lane 14. Lane M is a 100 bp DNA ladder.

4. 4. COI and ITS2 gene sequence analyses

The sequences of tick COI and ITS2 genes were separately inserted on nucleotide basic local alignment search tool (BLASTn) to determine their compatibility with other related tick sequences available on the National Centre for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 3.2). Note that in this study, *R. e. evertsi* is referred to *as* A and D, they same species with similar nucleotide sequences, but were retrieved from hosts of different villages, whereby A is from Qalo village, Butha-Buthe and D is from Ha Majara village, Butha-Buthe. For COI BLASTn results, *O. megnini* sequence (Qacha's Neck) had 99% identity with *O. megnini* (KC769589), *R. e. evertsi* A (Qalo village, Butha-Buthe) and *R. e. evertsi* D (Ha Majara village, Butha-Buthe) had 96% and 97% identity respectively with *R. e. evertsi* (AF132835), and *H. rufipes* (Butha-Buthe) had 98% identity with *H. rufipes* (AF132823) (Table 4.6).

The ITS2 BLASTn results were as follows: *R. microplus* (Butha-Buthe) showed 95% identity with *R. microplus* (JF758642 & JQ758642). The *R. e. evertsi* A and D (Butha-Buthe) showed 95% and 96% identity with *R. e. evertsi* (DQ849266) respectively. Similarly *R. e. evertsi* A (Qalo village, Butha-Buthe) and *R. e. evertsi* D (Ha majara village, Butha-Butha), were 95% and 96% identical to R. e. evertsi (U97701 & AF271279), respectively (Table 4.7).

The multiple sequence length of COI for soft tick species (*O. megnini* from Lesotho versus (vs) NCBI soft ticks) had a length of 622 bp and was relatively conserved and similar throughout the beginning, middle and last portions of the aligned sequences (Figure 4.4). The COI multiple length of *H. rufipes* and *R. e. evertsi* from Lesotho vs hard tick species from NCBI was 457 bp (Figure 4.5.a – 4.5.b) and several segments throughout the alignment were relatively conserved and similar, but dominated by less similar segments in the middle portion of the alignment. The ITS2 multiple alignment of *R. microplus* and *R. e. evertsi* from Lesotho versus *Rhipicephalus* species from the NCBI had a total length of 662 bp and recognized relatively large conserved and similar segments of the alignments in the beginning, middle and end sites (Figure 4.6.a – 4.6.b). For COI multiple alignment of soft tick sequences, the average p distance (pairwise distance) value for the intraspecific divergence of soft ticks was 0.4% with an average number of nucleotide differences (nt) of 3 and for interspecific divergence, the average p distance was 15.2% (95nt) (Table 4.8). The minimum and maximum p distance and number of nucleotide differences of the intraspecific divergence of soft ticks was 0.2 - 0.6% (1nt) (Table 4.8). The minimum and maximum p distance of interspecific divergence of soft ticks was 0 - 3.7% (1-133nt) (Table 4.8).

Multiple alignments of COI sequences of hard ticks, showed an average p distance of 2.5% with an average number of nucleotide differences of 11 for intraspecific divergence of *R. e. evertsi* and 0.2% (1nt) for intraspecific divergence of *H. rufipes* (Table 4.9). The minimum and maximum p distance value for intraspecific divergence of *R. e. evertsi* was 0 - 3.1% (0 - 14nt) and *H. rufipes* was 0.2% (1nt) (Table 4.9). The average p distance was 10.8% (49nt) for interspecific divergence of *Rhipicephalus* species, 0 - 1.8% (0 - 8nt) for *Hyalomma* species and 0 - 14.1% (0 - 64nt) for both *Hyalomma* and *Rhipicephalus* species (Table 4.9). The minimum and maximum p distance of interspecific divergence for *Rhipicephalus* species was0 - 16% (0 - 73nt) and for *Hyalomma* species was 0 - 3.7% (0-17nt), and for both *Hyalomma* and *Rhipicephalus* species was 0 - 22.8% (0 - 104nt) (Table 4.9).

Multiple alignments of ITS2 for hard ticks showed an average p distance of 0.8% (5 nt) for intraspecific divergence of *R*. *microplus*, 0.1% (9nt) for intraspecific divergence of *R. e. evertsi* A and D and 6.3% (42 nt) for interspecific divergence of *R. microplus* and *R. e. evertsi* (Table 4.10). The minimum and maximum p distance for intraspecific divergence of *R. microplus* was 0 - 1.4% (0 - 9nt), *R. e. evertsi* A and D 0 – 2.6% (0 - 17nt), and interspecific divergence of both *R. microplus* and *R. e. evertsi* was0 - 12.2% (0 - 81nt) (Table 4.10).

Table 4.6. BLASTn results for COI nucleotide sequences of ticks from Lesotho

Lesotho tick specimen	NCBI Matching tick species	Description	Geographical origin	Accession Number	Query cover	E value	Identity
Otobius megnini	*Otobius megnini*	COI gene partial sequence	Madagascar, Africa	KC769589	97%	0.0	99%
***Rhipicephalus evertsi evertsi* A**	*Rhipicephalus evertsi evertsi*	COI gene partial sequence	Kenya, Africa	AF132835	95%	0.0	96%
***Rhipicephalus eversti evertsi* D**	*Rhipicephalus evertsi evertsi*	COI gene partial sequence	Kenya, Africa	AF132835	93%	0.0	97%
Hyalomma rufipes	*Hyalomma rufipes*	COI gene partial sequence	Zimbabwe, Africa	AF132823	98%	0.0	98%

Table 4.7. BLASTn results for ITS2 nucleotide sequences of ticks from Lesotho

Lesotho tick specimen	Matching tick species	Description	Geographical origin	Accession Number	Query cover	E value	Identity
Rhipicephalus microplus	*Rhipicephalus microplus*	5.8S - ITS2 partial sequence	China, Asia	JF758642	99%	0.0	95%
	Rhipicephalus microplus	5.8S - ITS2 partial sequence	China, Asia	JQ758642	98%	0.0	95%
	Rhipicephalus microplus	5.8S - ITS2 partial sequence	China, Asia	KC203366	93%	0.0	95%
	Rhipicephalus microplus	5.8S - ITS2 partial sequence	Nigeria, Africa	MF373428	61%	0.0	99%
	Rhipicephalus microplus	5.8S - ITS2 partial sequence	Nigeria, Africa	MF373429	61%	0.0	99%
	Rhipicephalus microplus	5.8S - ITS2 partial sequence	Nigeria, Africa	MF373427	61%	0.0	99%
***Rhipicephalus evertsi evertsi* A**	*Rhipicephalus e. evertsi*	5.8S - ITS2 partial sequence	Zambia, Africa	DQ849266	94%	0.0	95%
	Rhipicephalus e. evertsi	5.8S - ITS2 partial sequence	Kenya, Africa	U97701	99%	0.0	95%
	Rhipicephalus evertsi mimeticus	5.8S - ITS2 partial sequence	Namibia, Africa	AF271279	93%	0.0	95%
***Rhipicephalus evertsi evertsi* D**	*Rhipicephalus e. evertsi*	5.8S - ITS2 partial sequence	Zambia, Africa	DQ849266	94%	0.0	96%
	Rhipicephalus e. evertsi	5.8S - ITS2 partial sequence	Kenya, Africa	U97701	99%	0.0	96%
	Rhipicephalus evertsi mimeticus	5.8S - ITS2 partial sequence	Namibia, Africa	AF271279	92%	0.0	96%

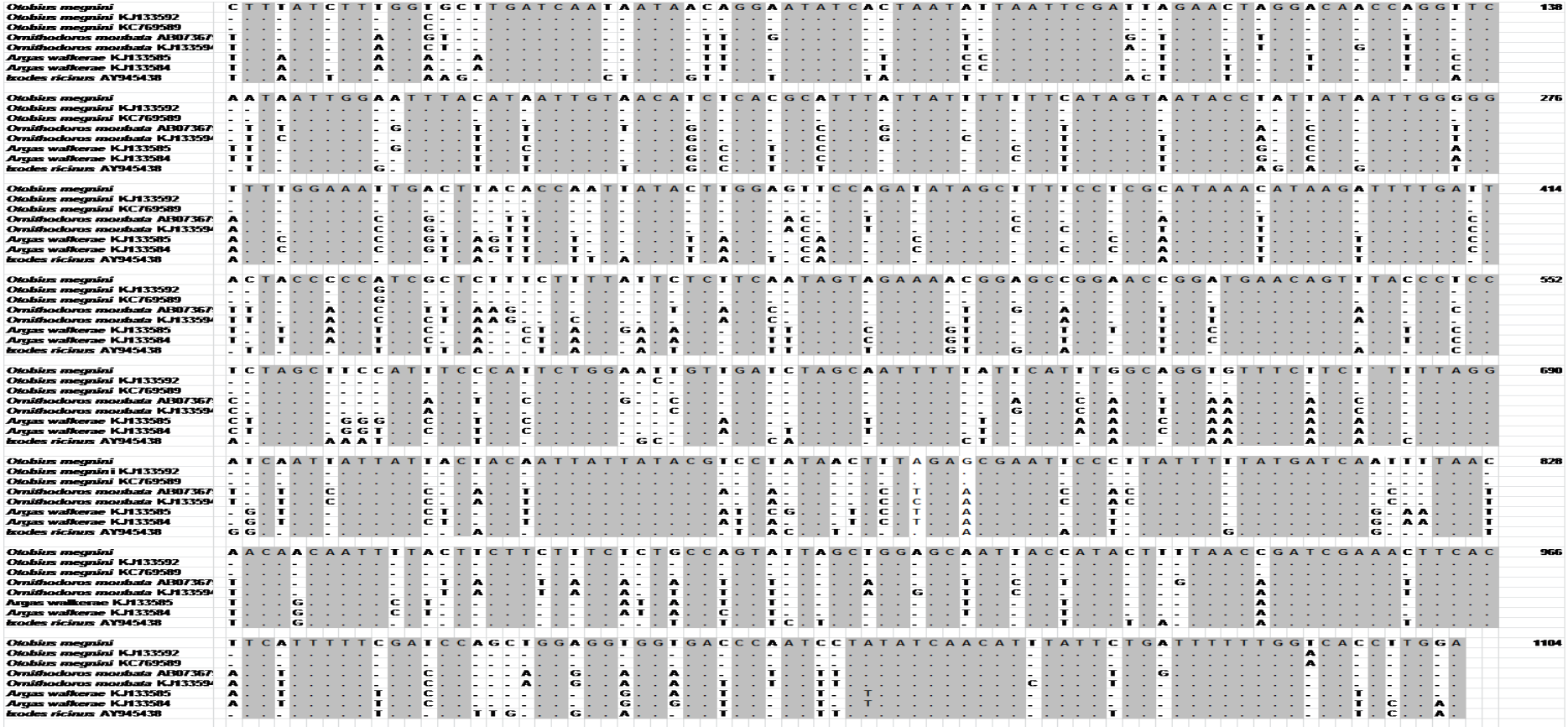

Figure 4.4. Alignment of nucleotide sequences of Soft ticks based on COI gene (622bp). The sequences represent *O. megnini* from Lesotho against other soft ticks. The gray shaded areas indicate conserved regions and the dots (.) represent the similarities shared between the sequence of this study and those from other soft ticks.

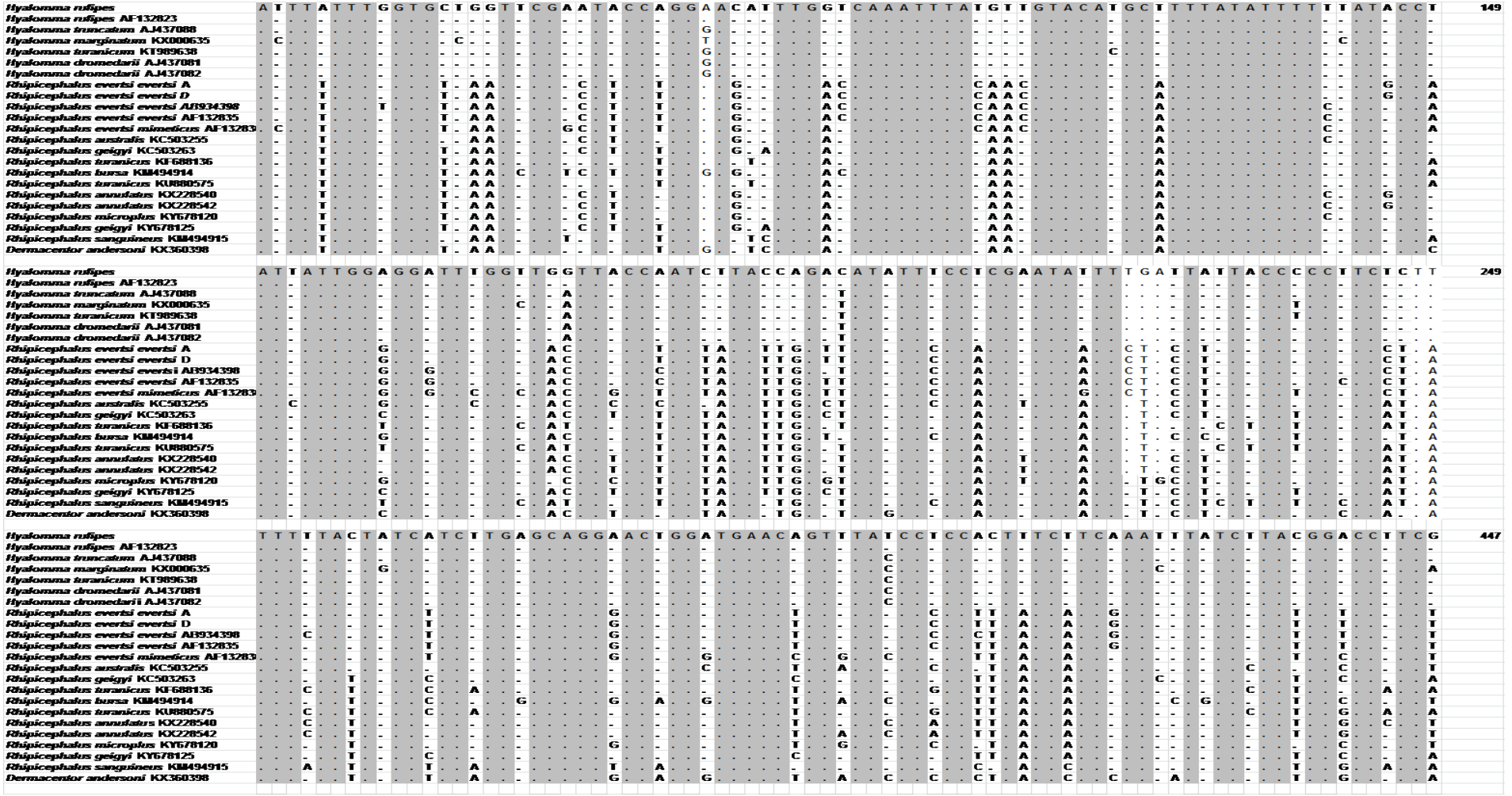

Figure 4.5.a. Alignment of nucleotide sequences of hard ticks based on COI gene (475 bp). The sequences represent *H. rufipes* and *R. e. evertsi* from Lesotho against other hard ticks. The gray shaded areas indicate conserved regions and the dots (.) represent the similarities shared between the sequence of this study and those from other soft ticks...

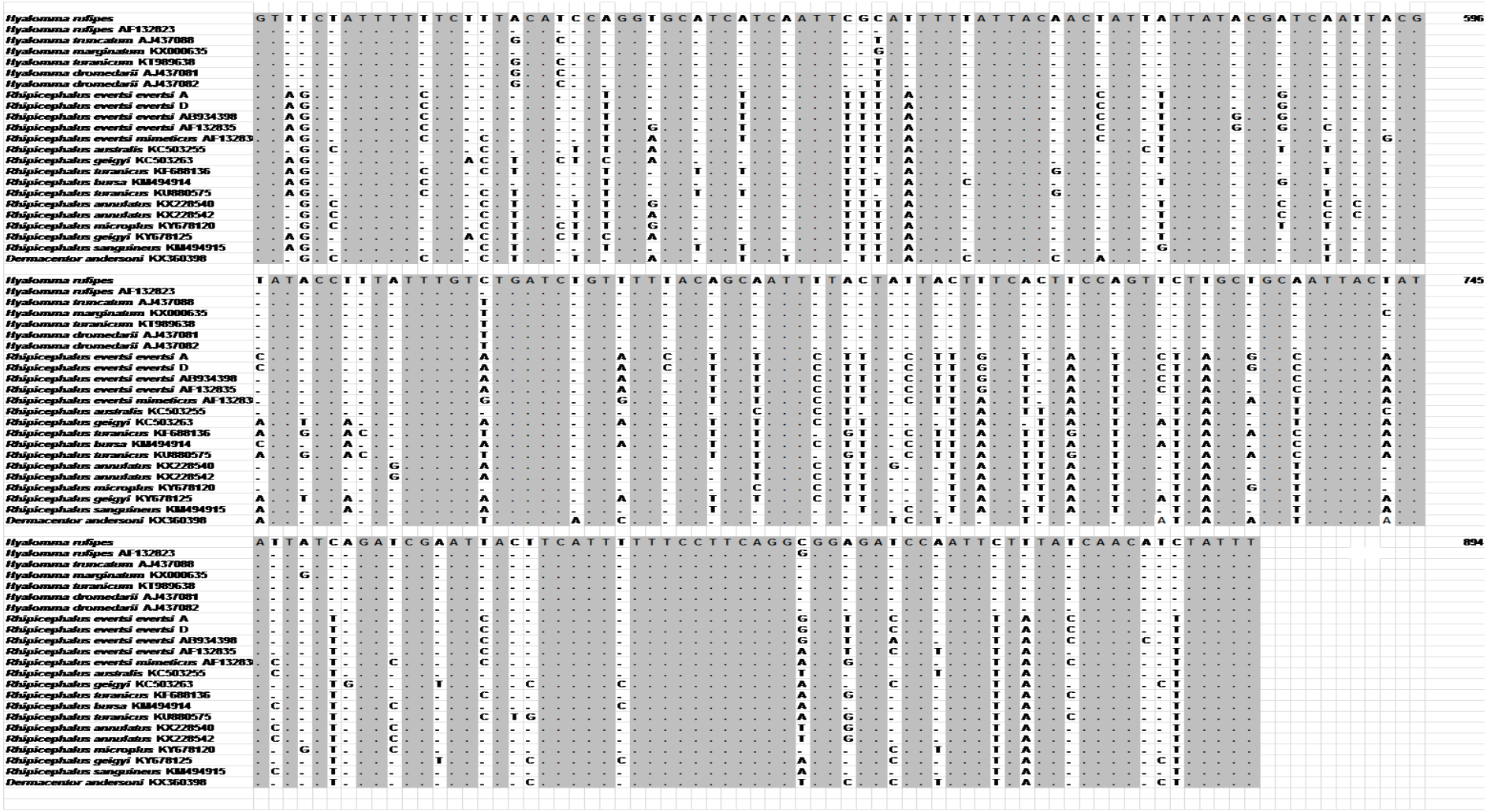

Figure 4.5.b. Alignment of nucleotide sequences of hard ticks based on COI gene (457 bp). The sequences represent *H. rufipes* and *R. e. evertsi* from Lesotho against other hard ticks. The gray shaded areas indicate conserved regions and the dots (.) represent the similarities shared between the sequence of this study and those from other soft ticks.

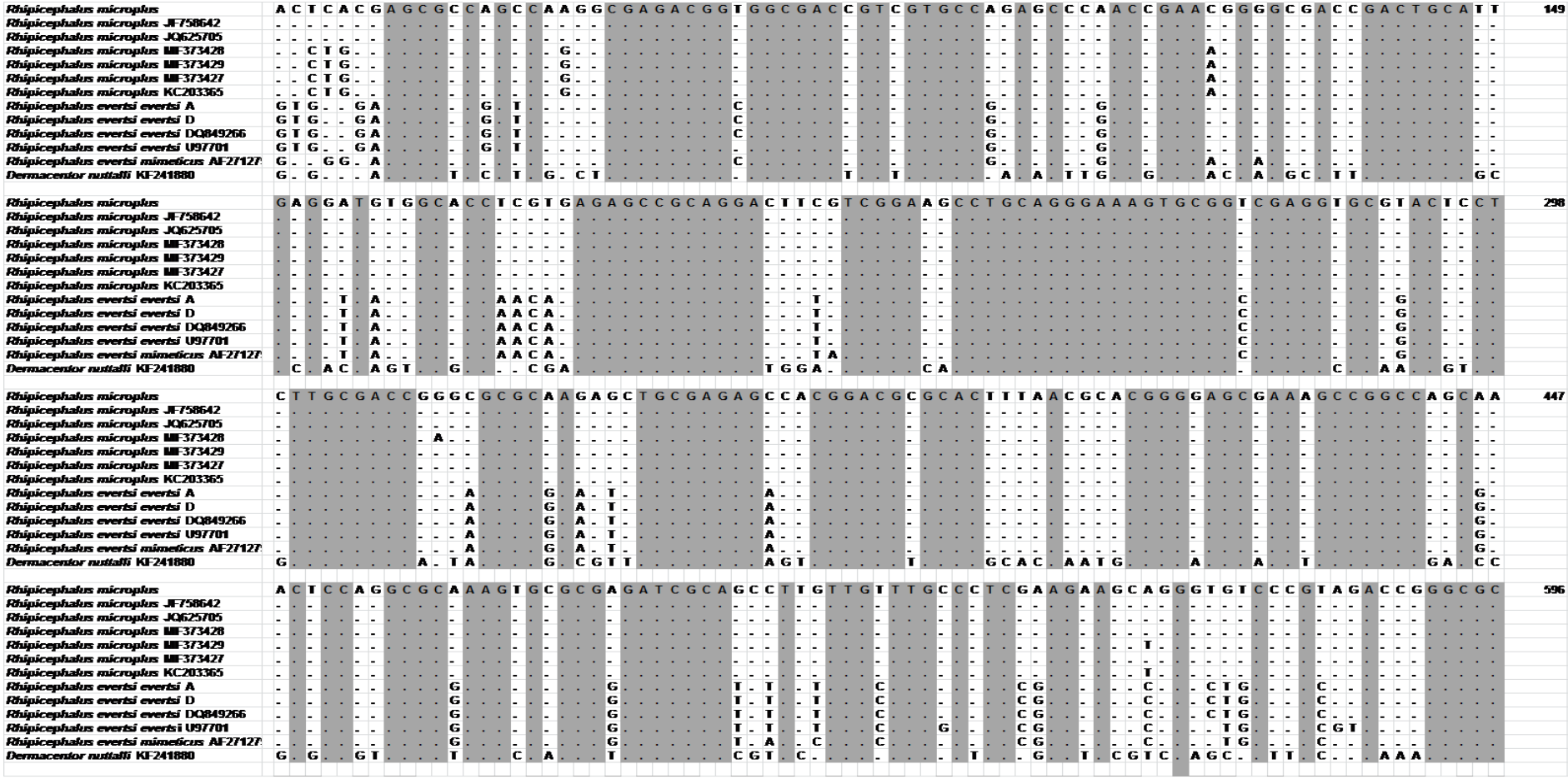

Figure 4.6.a. Alignment of nucleotide sequences of hard ticks based on ITS2 gene (662 bp). The sequences represent *R. e. evertsi* and *R. microplus* from Lesotho against other hard ticks. The gray shaded areas indicate conserved regions and the dots (.) represent the similarities shared between the sequence of this study and those from other soft ticks.

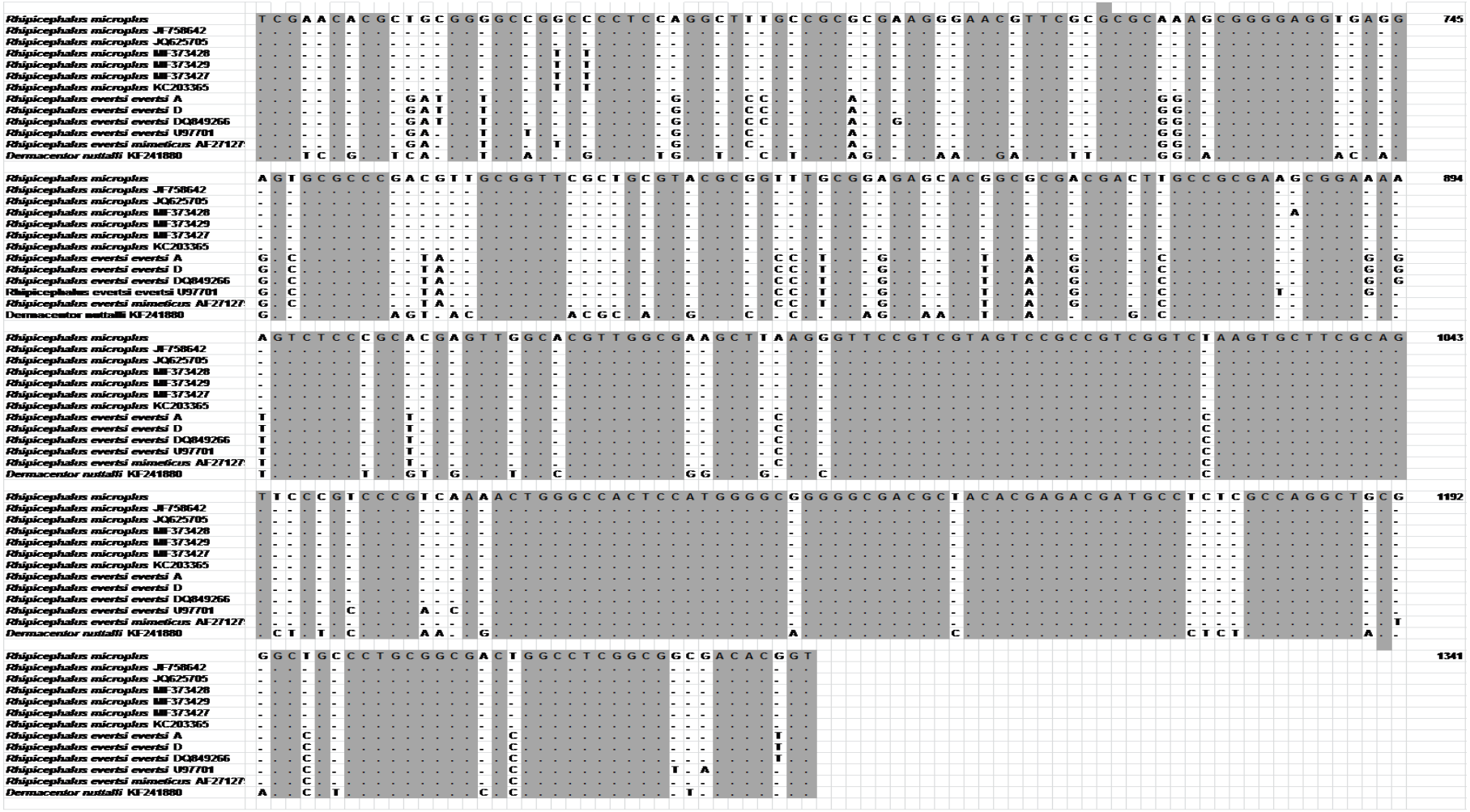

Figure 4.6.b. Alignment of nucleotide sequences of hard ticks based on ITS2 gene (662 bp). The sequences represent *R. e. evertsi* and *R. microplus* from Lesotho against other hard ticks. The gray shaded areas indicate conserved regions and the dots (.) represent the similarities shared between the sequence of this study and those from other soft ticks.

Table 4.8. Pairwise (p) distance and nucleotide differences (nt) of Lesotho *O. megnini* COI with other soft ticks from different countries

COI species of soft ticks	1	2	3	4	5	6	7	8
1. ***Otobius megnini***		4	3	114	109	131	131	126
2.*Otobius megnini* KJ133592	0.006		1	116	110	133	133	126
3. ***Otobius megnini* KC769589**	0.005	0.002		115	109	132	132	127
4. ***Ornithodoros moubata* AB073679**	0.183	0.186	0.185		29	115	117	128
5. ***Ornithodoros moubata* KJ133594**	0.175	0.177	0.175	0.047		120	118	131
6. ***Argas walkerae* KJ133585**	0.211	0.214	0.212	0.185	0.193		13	117
7. ***Argas walkerae* KJ133584**	0.211	0.214	0.212	0.188	0.190	0.021		111
8. ***Ixodes ricinus* AY945438**	0.203	0.203	0.204	0.206	0.211	0.188	0.178	

Pairwise (p) distance = Black shaded areas | Nucleotide differences (nt) = gray shaded areas

Table 4.9 Pairwise (p) distance and nucleotide differences (nt) of COI of *Hyalomma* & *Rhipicephalus* species of Lesotho with other hard ticks from different countries

COI species of hard ticks	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. *H. rufipes*		1	8	16	10	8	8	93	93	94	95	100	78	90	88	95	91	84	83	80	88	83	23
2. *H. rufipes* AF132823	0.002		9	17	11	9	9	92	92	93	95	100	78	90	88	95	91	84	83	81	88	83	90
3. *H. truncatum* AJ437088	0.018	0.020		14	2	0	0	95	95	96	97	100	81	89	90	95	93	83	82	80	87	84	90
4. *H. marginatum* KX000635	0.035	0.037	0.031		14	14	14	102	102	103	104	101	87	94	92	101	95	92	91	89	94	87	86
5. *H. turanicum* KT989638	0.022	0.024	0.004	0.031		2	2	97	97	98	99	100	83	89	90	95	93	85	84	82	87	84	94
6. *H. dromedarii* AJ437081	0.018	0.020	0.000	0.031	0.004		0	95	95	96	97	100	81	89	90	95	93	83	82	80	87	84	88
7. *H. dromedarii* AJ437082	0.018	0.020	0.000	0.031	0.004	0.000		95	95	96	97	100	81	89	90	95	93	83	82	80	87	84	86
8. *R. e. evertsi* A	0.204	0.201	0.208	0.223	0.212	0.208	0.208		0	14	14	35	59	54	57	40	59	55	54	54	52	63	86
9. *R. e. evertsi* D	0.204	0.201	0.208	0.223	0.212	0.208	0.208	0.000		14	14	35	59	54	57	40	59	55	54	54	52	63	74
10. R. e. evertsi AB934398	0.206	0.204	0.210	0.225	0.214	0.210	0.210	0.031	0.031		12	37	58	60	60	47	62	56	55	58	58	64	74
11. *R. e. evertsi* AF132835	0.208	0.208	0.212	0.228	0.217	0.212	0.212	0.031	0.031	0.026		35	54	56	62	44	64	54	54	52	54	63	77
12. *R. evertsi mimeticus* AF132836	0.219	0.219	0.219	0.221	0.219	0.219	0.219	0.077	0.077	0.081	0.077		55	56	59	44	61	54	51	56	54	61	72
13. *R. australis* KC503255	0.171	0.171	0.177	0.190	0.182	0.177	0.177	0.129	0.129	0.127	0.118	0.120		53	71	58	73	36	32	26	51	64	78
14. R. geigyi KC503263	0.197	0.197	0.195	0.206	0.195	0.195	0.195	0.118	0.118	0.131	0.123	0.123	0.116		57	47	58	46	44	46	2	54	73
15. *R. turanicus* KF688136	0.193	0.193	0.197	0.201	0.197	0.197	0.197	0.125	0.125	0.131	0.136	0.129	0.155	0.125		60	3	60	60	67	55	36	69
16. *R. bursa* KM494914	0.208	0.208	0.208	0.221	0.208	0.208	0.208	0.088	0.088	0.103	0.096	0.096	0.127	0.103	0.131		62	54	51	57	45	56	69
17. *R. turanicus* KU880575	0.199	0.199	0.204	0.208	0.204	0.204	0.204	0.129	0.129	0.136	0.140	0.133	0.160	0.127	0.007	0.136		61	61	69	56	37	75
18. *R. annulatus* KX228540	0.184	0.184	0.182	0.201	0.186	0.182	0.182	0.120	0.120	0.123	0.118	0.118	0.079	0.101	0.131	0.118	0.133		4	30	44	58	69
19. *R annulatus* KX228542	0.182	0.182	0.179	0.199	0.184	0.179	0.179	0.118	0.118	0.120	0.118	0.112	0.070	0.096	0.131	0.112	0.133	0.009		29	42	58	71
20. *R. microplus* KY678120	0.175	0.177	0.175	0.195	0.179	0.175	0.175	0.118	0.118	0.127	0.114	0.123	0.057	0.101	0.147	0.125	0.151	0.066	0.063		44	64	68
21. *R. geigyi* KY678125	0.193	0.193	0.190	0.206	0.190	0.190	0.190	0.114	0.114	0.127	0.118	0.118	0.112	0.004	0.120	0.098	0.123	0.096	0.092	0.096		52	67
22. *R. sanguineus* KM494915	0.182	0.182	0.184	0.190	0.184	0.184	0.184	0.138	0.138	0.140	0.138	0.133	0.140	0.118	0.079	0.123	0.081	0.127	0.127	0.140	0.114		61
23. *D. andersoni* KX360398	0.197	0.197	0.188	0.206	0.193	0.188	0.188	0.162	0.162	0.168	0.158	0.171	0.160	0.151	0.151	0.164	0.151	0.155	0.149	0.153	0.147	0.133	

Pairwise (p) distance = Black shaded areas | Nucleotide differences (nt) = gray shaded areas

Table 4.10. Pairwise (p) distance and nucleotide differences (nt) of ITS2 of *Rhicephalus microplus* & *R. e. evertsi* of Lesotho with other *Rhipicephalus* species

ITS2 species of hard ticks	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *R. microplus*		0	0	9	8	7	8	69	69	70	73	65	185
2. *R. microplus* JF758642	0.000		0	9	8	7	8	69	69	70	73	65	185
3. *R. microplus* JQ625705	0.000	0.000		9	8	7	8	69	69	70	73	65	185
4. *R. microplus* MF373428	0.014	0.014	0.014		3	2	3	77	77	78	81	67	190
5. *R. microplus* MF373429	0.012	0.012	0.012	0.005		1	0	75	75	76	79	65	187
6. *R. microplus* MF373427	0.011	0.011	0.011	0.003	0.002		1	75	75	76	79	65	188
7. *R. microplus* KC203365	0.012	0.012	0.012	0.005	0.000	0.002		0	75	76	79	65	187
8. *R. e. evertsi* A	0.104	0.104	0.104	0.116	0.113	0.113	0.113		75	1	16	20	175
9. *R. e. evertsi* D	0.104	0.104	0.104	0.116	0.113	0.113	0.113	0.000		1	16	20	175
10. *R. e. evertsi* DQ849266	0.106	0.106	0.106	0.118	0.115	0.115	0.115	0.002	0.002		17	21	176
11. *R. e. evertsi* U97701	0.110	0.110	0.110	0.122	0.119	0.119	0.119	0.024	0.024	0.026		26	177
12. *R. evertsi mimeticus* AF271279	0.098	0.098	0.098	0.101	0.098	0.098	0.098	0.030	0.030	0.032	0.039		176
13. *D. nuttalli* KF241880	0.279	0.279	0.279	0.287	0.282	0.284	0.282	0.264	0.264	0.266	0.267	0.266	

Pairwise (p) distance = Black shaded areas | Nucleotide differences (nt) = gray shaded areas

4.5. Phylogenetic analysis of ticks

The phylogenetic trees of *O. megnini* with other six soft ticks were constructed using ML (Figure 4.7) and NJ (Figure 4.8) methods with COI gene sequence alignments with *Ixodes ricinus* (Accession number: AY945438) as an outgroup. Both COI gene sequence ML and NJ trees of *O. megnini* from Lesotho and other soft ticks from GenBank database formed three clades (Figure 4.7 and Figure 4.8). Within the first clade, *O. megnini* of Lesotho grouped together with other two *O. megnini* from Madagascar (KJ133592) & South Africa (KC769589) with supporting bootstrap values of 100 in ML tree (Figure 4.7) and NJ tree (Figure 4.8), whereas other soft ticks from NCBI database [Tanzania (AB073679) and South Africa (KJ133594, KJ133585, and KJ133584)] formed two separate clades (Figure 4.7 and Figure 4.8).

The phylogenetic trees of *R. e. evertsi*, *H. rufipes* and other 19 hard ticks were constructed using ML (Figure 4.9) and NJ (Figure 4.10) methods based on COI gene sequence alignments with *Dermacentor andersoni* (Accession no: KX360398) as outgroup. The analyses of COI sequence alignments for ML and NJ trees for *R. e. evertsi* and *H. rufipes* of Lesotho and others formed a monophyletic group of six major clades (Figure 4.9 and Figure 4.10). The *R. e. evertsi* A and D of Lesotho and that of the NCBI database [Uganda (AB934398) and Kenya (AF132835, AF132835)] were grouped together in the first upper clade with supporting bootstraps of 99 for ML tree (Figure 4.9) and 100 for NJ tree (Figure 4.10). Within the first upper clade of *R. e. evertsi,* the two *R. e. evertsi* A and D of Lesotho and other *R. e. evertsi* from Kenya and Uganda (AF132835 and AB934398 respectively) further formed a monophyletic group which isolated them by bootstrap values of 99 ML/100 NL and 70 ML/71 NL (Figure 4.9 and Figure 4.10). In the sixth bottom clade of COI for ML and NL trees of *Rhipicephalus* and *Hyalomma* species, sequences of *H. rufipes* from Lesotho and Zimbabwe (AF132823) were grouped together with other *Hyalomma* species (Figure 4.9 and Figure 4.10). Their supporting bootstrap values were 98 for ML and 97 for NJ (Figure 4.9 and Figure 4.10).

R. e. evertsi and *R. microplus* with other 9 *Rhipicephalus* ticks and one outgroup (*Dermacentor nuttalli* KF241880) were represented by ML (Figure 4.11) and NJ (Figure 4.12) trees regarding ITS2 sequence alignment. The phylogenetic trees gave two major branches which constituted of two separate groups of *R. microplus* and *R.*

e. evertsi (Figure 4.11 and Figure 4.12). They were supported by bootstrap values of 100/100 (ML/NJ) and 93/98 (ML/NJ) (Figure 4.11 and Figure 4.12). Within first major upper branch, all the *R. microplus* together with that of Lesotho formed a cluster of its own with two small (Figure 4.11 and Figure 4.12). The *R. microplus* from Lesotho and two *R. microplus* of China (JF758642 and JQ625705) further grouped together separately from the other three *R. microplus* of Nigeria (MF373428; MF373427 and MF373429) and one from China (KC20336). These two clusters of *R. microplus* were supported by relative bootstrap values of 98/95 (ML/NJ) and 94/98 (ML/NJ) (Figure 4.11 and Figure 4.12). The second isolated major group made up of *R. e. evertsi* species at the bottom, showed that two *R. e. evertsi* A and D from Lesotho where grouped with *R. e. evertsi* from Kenya (U97701) and further branched from *R. e. evertsi* of Zambia (DQ849266) (Figure 4.11 and Figure 4.12). This branching of *R. microplus* of Lesotho, including Kenya (U97701), from that of Zambia (DQ849266) was supported by bootstrap values of 99/99 (ML/NJ) and 95/99 (ML/MJ) respectively (Figure 4.11 and Figure 4.12).

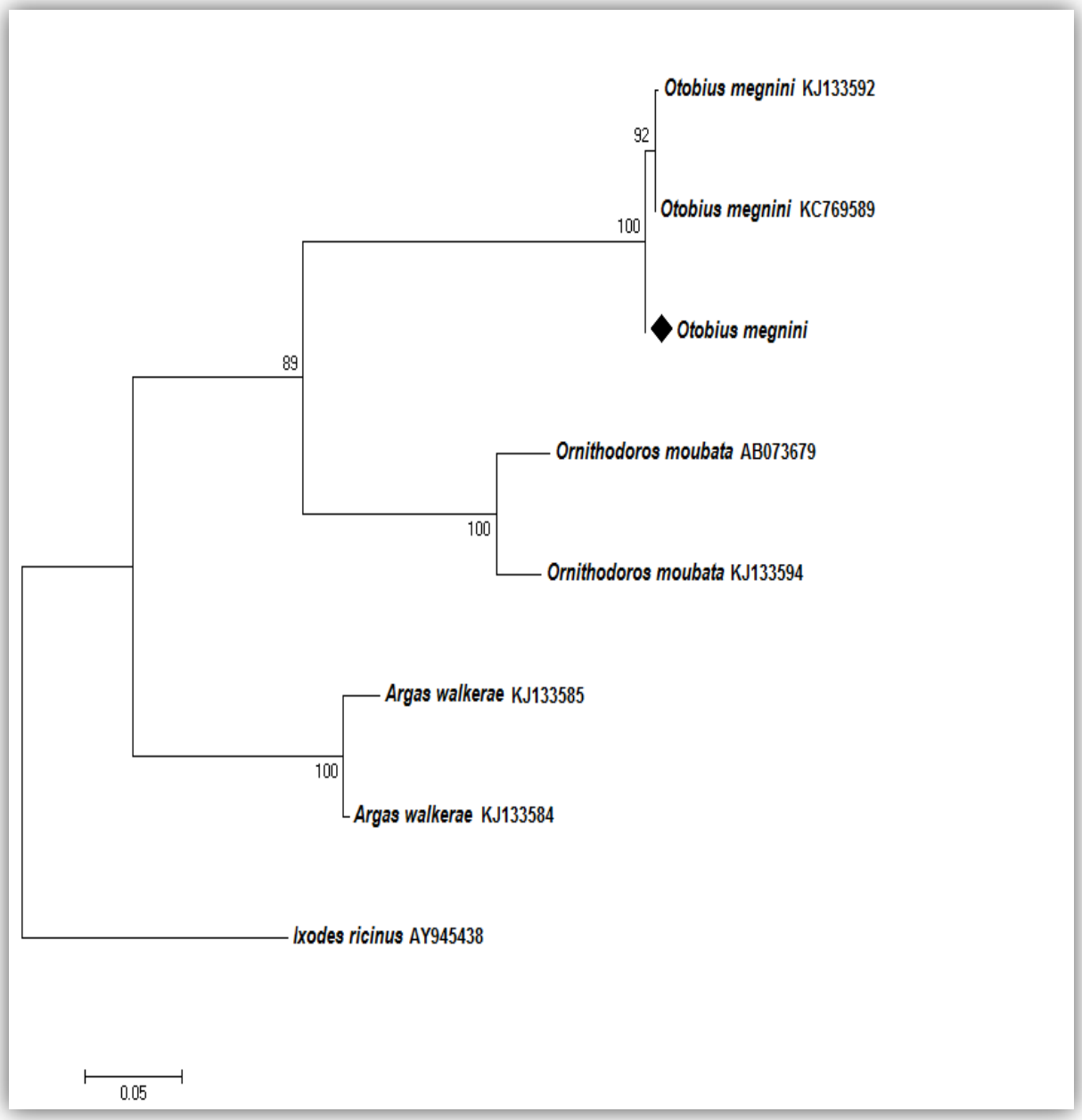

Figure 4.7. Phylogenetic tree of *Otobius megnini* of Lesotho with other soft tick sequences from the GenBank (NCBI) database based on COI gene sequences inferred on MEGA 6.0 software. Maximum likelihood (ML) tree was constructed by using *Ixodes ricinus* as outgroup with 10 000 bootstrap replicates. Bootstrap values of each tree branch are placed on the tree nodes.

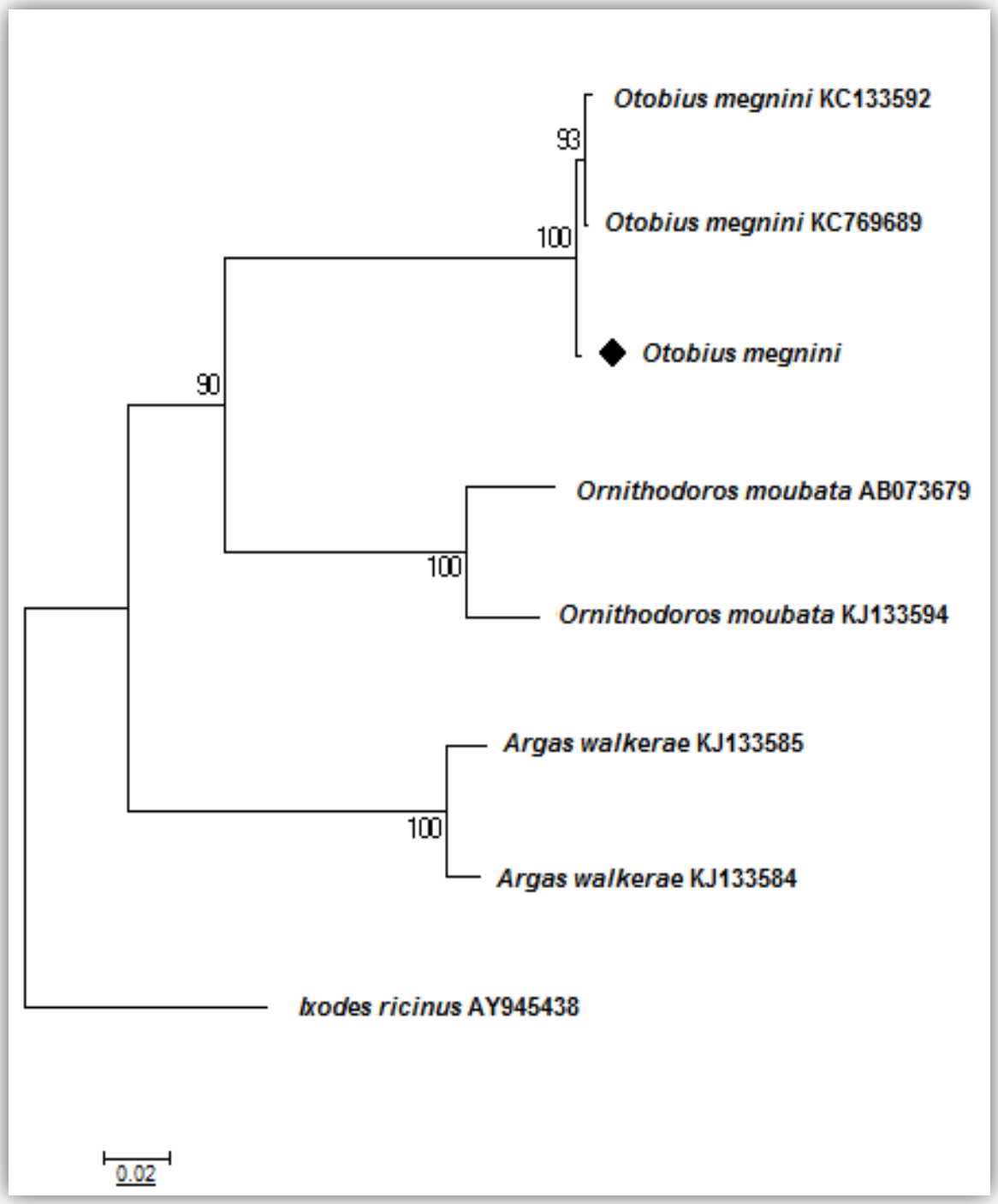

Figure 4.8. Phylogenetic tree of *Otobius megnini* of Lesotho with other soft tick sequences from the GenBank (NCBI) database based on COI gene sequences inferred on MEGA 6.0 software. Neighbour-joining tree was constructed by using *Ixodes ricinus* as outgroup with 10 000 bootstrap replicates. Bootstrap values of each tree branch are placed on the tree nodes.

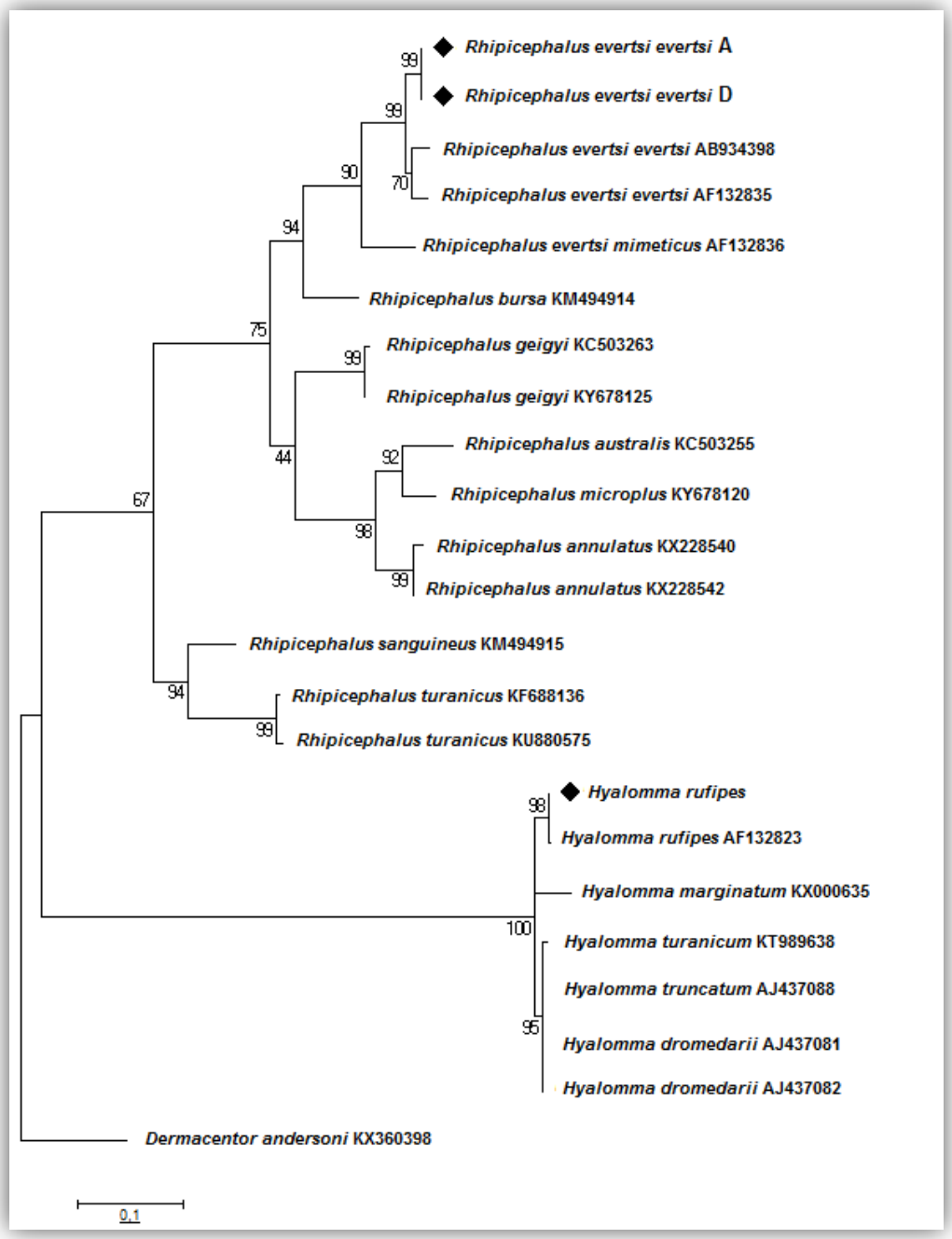

Figure 4.9. Phylogenetic trees of *Rhipicephalus microplus* and *Hyalomma rufipes* from Lesotho with other hard tick sequences from the GenBank (NCBI) database based on COI gene sequences inferred on MEGA 6.0 software. Maximum likelihood (ML) tree was constructed by using *Dermacentor andersoni* as outgroup with 10 000 bootstrap replicates. Bootstrap values of each tree branch are placed on the tree nodes.

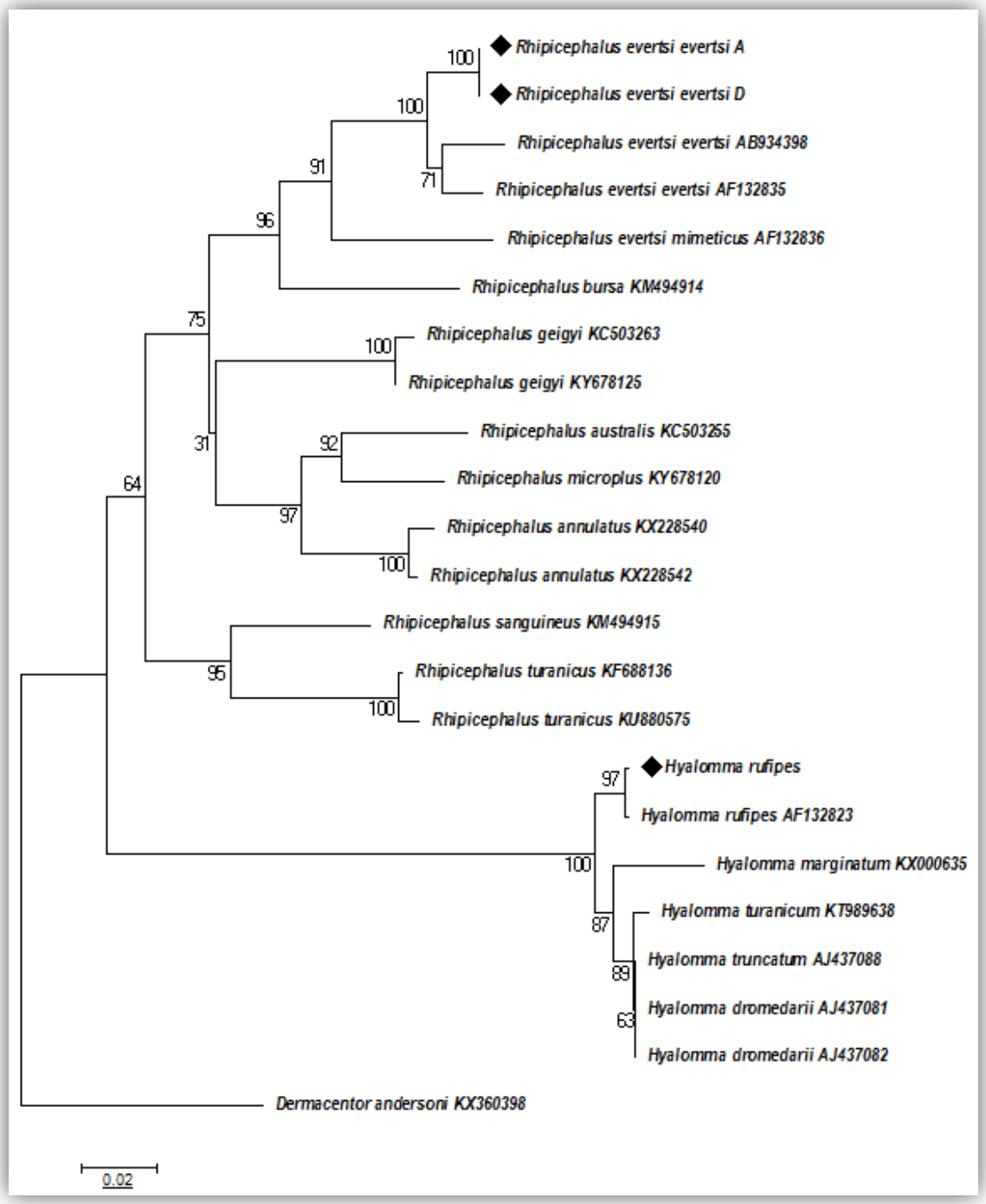

Figure 4.10. Phylogenetic relation of *Rhipicephalus microplus* and *Hyalomma rufipes* from Lesotho with other hard tick sequences from the GenBank (NCBI) database based on COI gene sequences inferred on MEGA 6.0 Software. Neighbor-joining (NJ) tree was constructed by using *Dermacentor andersoni* as outgroup with 10 000 bootstrap replicates. Bootstrap values of each tree branch are placed on the tree nodes.

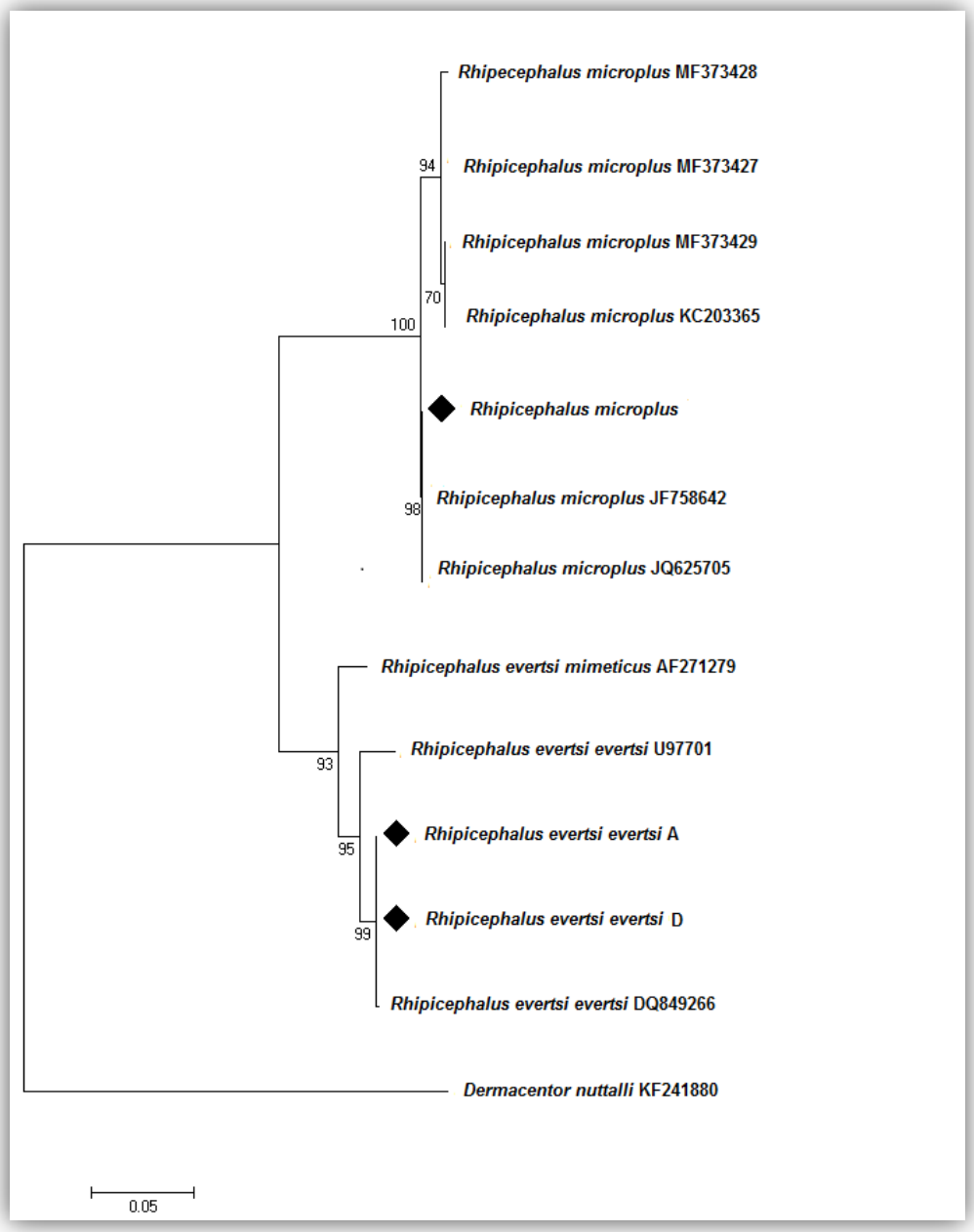

Figure 4.11. Phylogenetic relation of *Rhipicephalus microplus* and *Rhipicephalus evertsi evertsi* from Lesotho with other hard tick sequences from the GenBank (NCBI) database based on the ITS2 gene sequences inferred on MEGA 6.0 Software. Maximum likelihood (ML) tree was constructed by using *Dermacentor nuttalli* as outgroup with 10 000 bootstraps replicates. Bootstrap values of each tree branch are placed on the tree nodes.

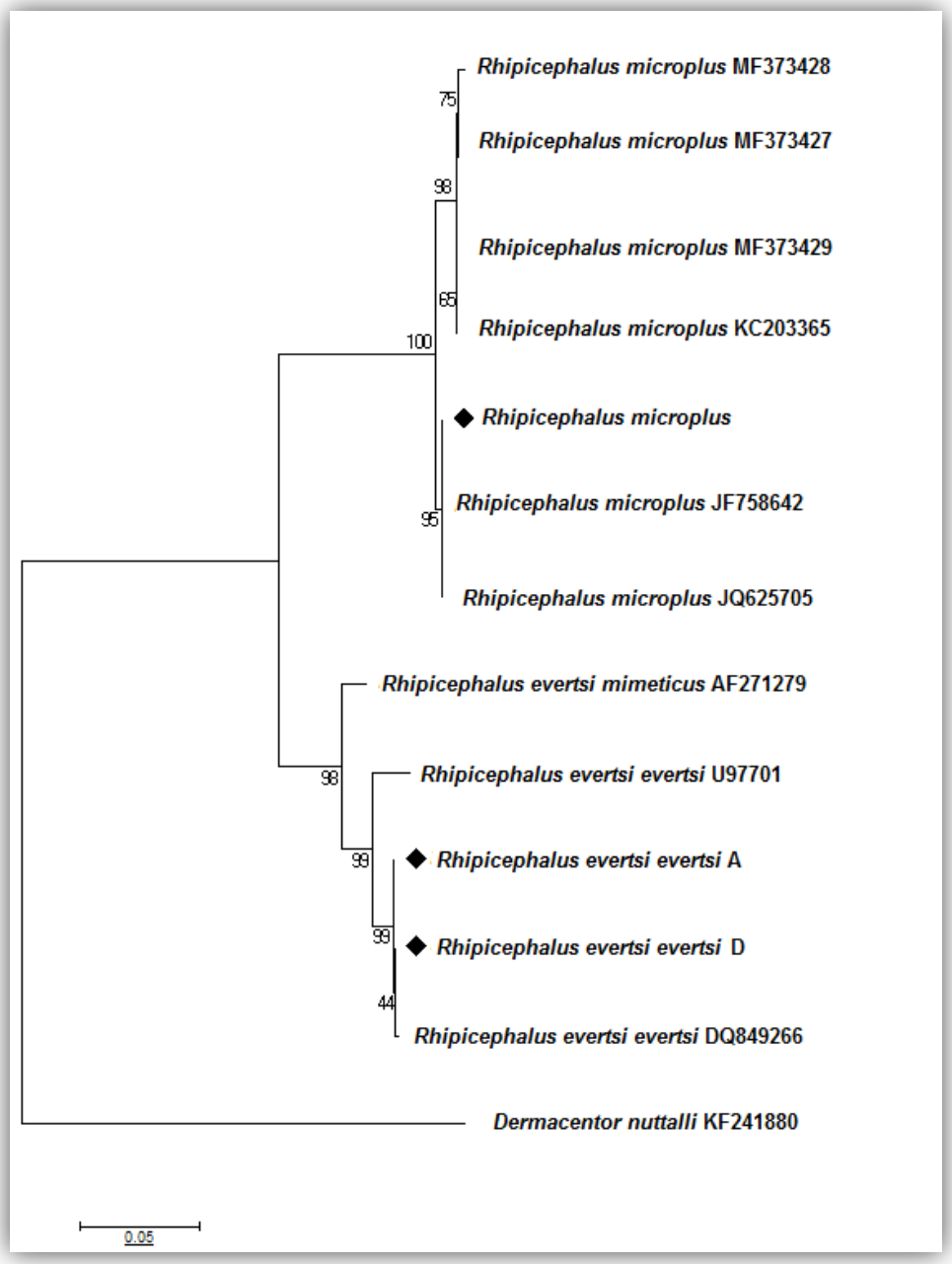

Figure 4.12. Phylogenetic relation of *Rhipicephalus microplus* and *Rhipicephalus evertsi evertsi* from Lesotho with other hard tick sequences from the GenBank (NCBI) database based on the ITS2 gene sequences inferred on MEGA 6.0 Software. Neighbor-joining (NJ) tree was constructed by using *Dermacentor nuttalli* as outgroup with 10 000 bootstraps replicates. Bootstrap values of each tree branch are placed on the tree nodes.

4.6. Detection of tick-borne haemoparasites from ticks

Out of 186 ticks DNA samples obtained from domestic animals of Lesotho (Butha-Buthe district), 164 ticks from cattle were screened for detection of *B. bigemina* and *B. bovis* by PCR assays. There were no tick DNA samples from cattle which tested positive for *B. bigemina* (Figure 4. 13). A total of 13 (7.9%) tick DNA samples from cattle tested positive for the presence of *B. bovis* DNA by PCR with product size of 298 bp (Table 3.4) (Figure 4.14). Five tick DNA samples were represented by *R. microplus* species from various villages in Butha-Buthe district, i.e. *R. microplus* DNA samples which tested positive for *B. bovis* were from cattle of Ha Khabile village (x2 tick DNA samples), Belo village (x1 tick DNA sample), Ha Mantlobo village (x1 tick DNA sample) and Matsoaing village (x1 tick DNA sample) (Table 4.5). The other eight tick DNA samples collected from cattle of Butha-Buthe district were that of *R. e. evertsi* tick species from various villages. For example, *R. e. evertsi* DNA samples which tested positive for *B. bovis*, were from cattle from Ha Khabile village (x2 tick DNA samples), Seboche village (x2 tick DNA samples), Resource centre (x2 tick DNA samples), Ha Mantlobo village (x1 tick DNA sample) and Matsoaing village (x1 tick DNA sample) (Table 4.5).

Four horse DNA samples collected from Maseru district tested negative for *B. caballi* and *T. equi* (Figure 4.15). The remaining twenty two samples from goats (n = 6) and sheep (n = 16) were screened for the presence of *Babesia ovis*, *B. motasi*, *Theileria ovis* and *T. lestoquardi*. Out of 22 ovine tick DNA samples, one *R. e. evertsi* DNA sample from goat and two *R. e. evertsi* samples from sheep of Qalo village (Table 4.5) tested positive (13.6%) for *B. ovis* with product size of 549 bp and all the tick DNA samples belonged to *R. e. evertsi* (Figure 4.16). The first PCR for *B. motasi* using catch all primer P1 and P2 for *Theileria-Babesia* species tested positive for 4 (18.2%) DNA samples with amplicon sizes ranging between 389 to 402 bp (Figure 4.17). These DNA samples were from *R. e. evertsi* collected from goats in Qalo village, *R. microplus* from goats of Manamela village, and the last two *R. e. evertsi* were from sheep of Qalo and Ha Majara villages (Table 4.5). The second PCR for species specific primers P2 and P4 for *B. motasi* did not amplify at 205 bp (Shayan *et al*., 2008), but was around 500 - 520 bp (Figure 4.18). None of *T. ovis* and *T. lestoquardi* were positively detected from all the 22 tick DNA samples collected from ovine and caprine livestock (Figure 4.19).

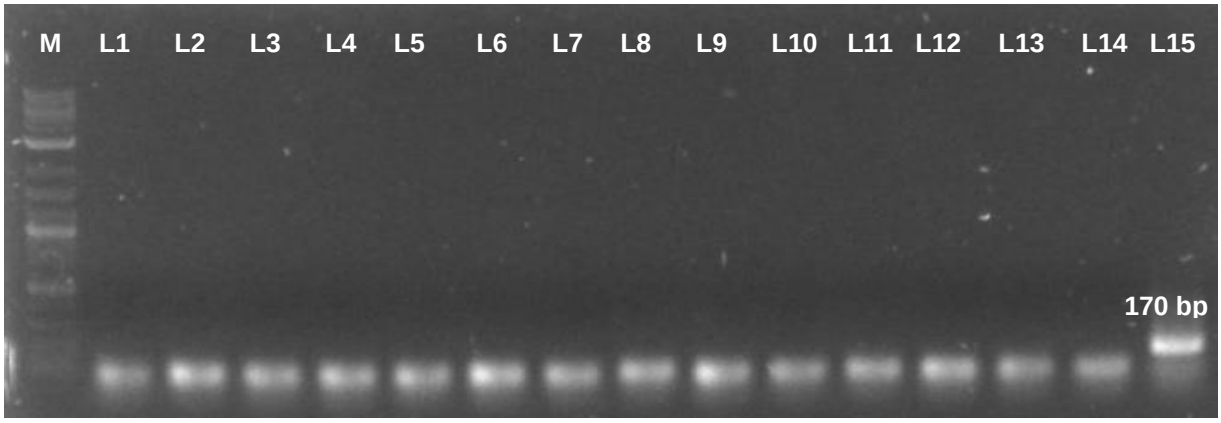

Figure 4.13. Agarose gel illustrating amplification PCR results for *B. bigemina*. Primers BIG1 and BIG2 targeting gene of *B. bigemina* were used. L1 is negative control, L15 is positive control for *B. bigemina* and L2 - 14 are tick DNA pools from cattle. Lane M is a 100 bp DNA ladder.

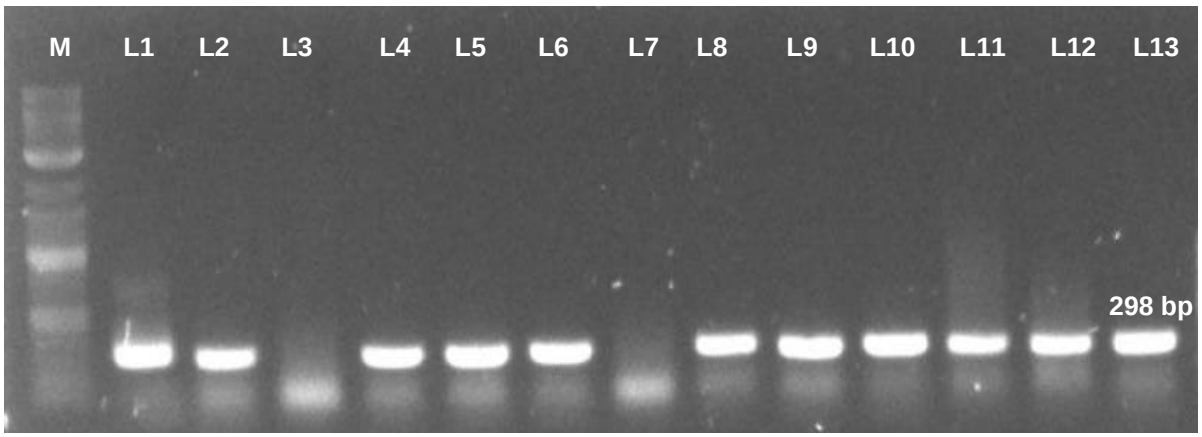

Figure 4.14. Agarose gel illustrating PCR results for *B. bovis*. Primers BOV1 and BOV2 targeting gene of *B. bovis* were used. The L3 is negative control, L13 is positive control for *B. bovis* and L1, 2, 4 – 12 are tick DNA from cattle. Lane M is a 100 bp DNA ladder.

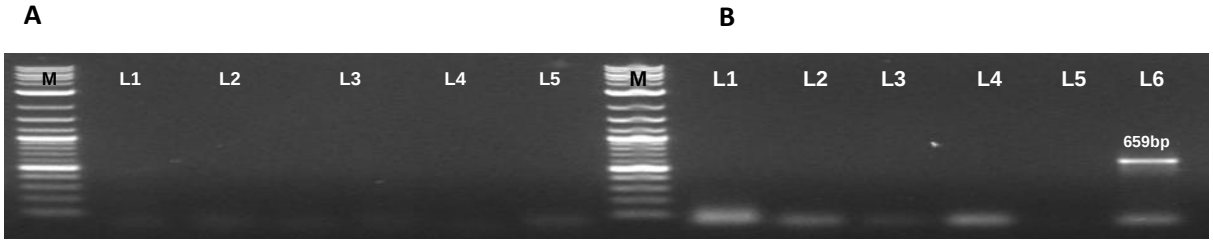

Figure 4.15. Agarose gel showing PCR results for *T. equi* and *B. caballi*. (A) primers EMA1-F and -R were used for *T. equi*. L1 is negative control and L2, 3, 4, 5 are DNAs of tick collected from horses. (B) primers Bc48-F and -R were used for *B. caballi*. The L1 is negative control, L2, 3, 4, 5 are DNAs of ticks collected from horses and L6 is a positive control for *B. caballi*. Lane M is a 1 kb DNA ladder.

Figure 4.16. Agarose gel showing PCR results for *B. ovis*. Primers Bbo-F and -R were used for *B. ovis*. The L1 is a negative control and L2-17 are tick DNA samples collected from sheep and goats. The L9, 13 and 14 were positive for *B. ovis* species. Lane M is a 1kb DNA ladder.

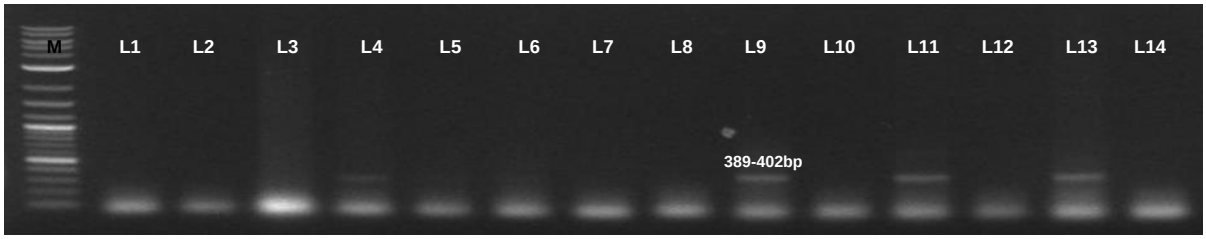

Figure 4.17. Agarose gel showing PCR results for *Babesia-Theileria* species using catch all primers P1 and P2. The L1 is a negative control and L2 - 14 are tick DNA from ovine. The L4, 9, 11 and 13 were positive for *Babesia-Theileria* species. Lane M is a 1kb DNA ladder.

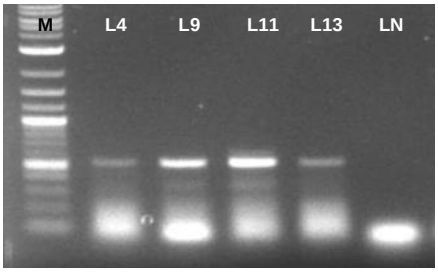

Figure 4.18. Agarose gel showing PCR results for *B. motasi*. Specific primers P2 and P4 for *B. motasi*. LN is a negative control and L4, 9, 11 and 13 are positive amplicons for *Babesia-Theileleria* catch all primers. Lane M is a 1kb DNA ladder.

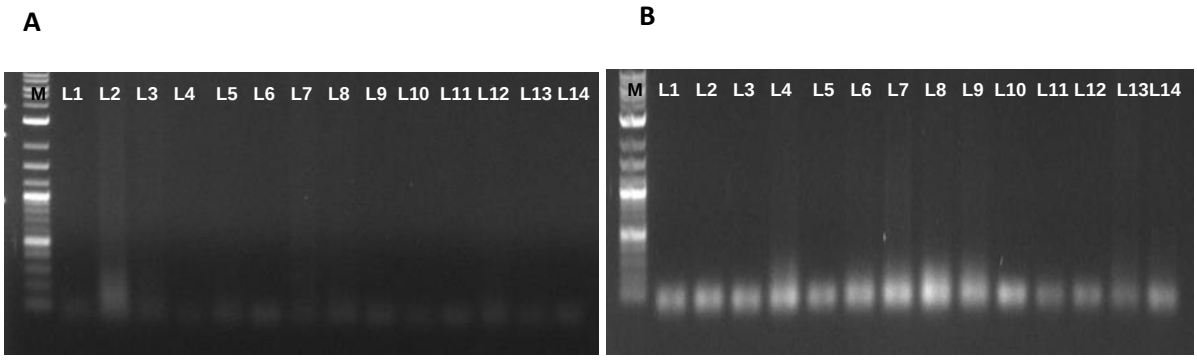

Figure 4.19. Agarose gel showing PCR results for *T. ovis* and *T. lestoquradi*. (A) primers P418S-F and P518S-R were used for *T. ovis*. (B) primers TI-1-1-F and R were used for *T. lestoquardi*. The L1 is a negative control and L2, L 14 are tick DNA from ovine. Lane M is 1kb DNA ladder.

Chapter 5

Discussion, conclusion and recommendations

5.1. Characterization of ticks from Lesotho districts

Ticks are known for being hematophagous ecto-parasites that feed solely on blood (Parola & Raoult, 2001) and advance infections of tick-borne haemoprotozoans on domestic animals (Thompson *et al*., 2008; Motloang *et al*., 2008; Penzhorn, 2011; Mtshali & Mtshali, 2013). Thus it is important to identify and characterize ticks in a particular region, because their impacts, as a result of primary and secondary infections, can extend beyond low production of livestock to waning economic growth (Rajput, 2006; Manjunathachar *et al*. 2014). Furthermore, tick identification has proven to have a significant role in the development of tick control measures (Lv *et al*., 2014). Track records of ticks in various districts of Lesotho is not available, due to the lack of documented scientific data on ticks infesting domestic animals, but there are wide reports on occurrence and distribution of various species of ticks found in the Republic of South Africa, which surrounds Lesotho (Horak *et al*., 2009; Mbati *et al*., 2002; Mtshali, 2012; Spickett *et al*., 2011; Nyangiwe *et al*., 2013). The current study was aimed at identifying ticks infesting domestic animals in Lesotho and detecting haemoparasites which they are possibly harbouring.

A total of 1654 tick specimens from domestic animals in five districts of Lesotho were recovered. Qacha's Neck district had the highest tick abundance of 39%, followed by Butha-Buthe 29% and the least number of ticks were recorded from Leribe 8% and Mafeteng 5%. Out of five districts of Lesotho, a total of four Ixodid tick species and one Argasid tick species were recovered. *Rhipicephalus evertsi evertsi* (52%) was the most abundant tick species collected from various domestic animals, i.e. cattle, sheep, goats, horses and unrecorded hosts, in five districts of Lesotho. The large abundance of *R. e. evertsi* from Lesotho and its commonness throughout all the five district of Lesotho is due to the fact that *R. e. evertsi* is the most widespread species among the genus *Rhipicephalus* found in Africa as reported by Walker *et al*. (2014). Survey conducted by Horak *et al.* (2009), in the south-east Africa, and Spickett *et al*. (2011), in North West Province, also reported that *R. e. evertsi* is widely distributed throughout parts of South Africa. Hence, it is not surprising that *R. e. evertsi* tick species is highly abundant in sampled areas of Lesotho.

Hyalomma rufipes (4%) and *H. truncatum* (2%) were the least abundant tick species in all the five districts. Horak *et al.* (2009) explained that the presence of *H. rufipes* in the Eastern Cape regions, South Africa, is likely to appear in drier regions. Thus this study suggests that the low distribution and occurrence of *H. rufipes* in districts of Lesotho might be due to both extreme dry and wet conditions of the Lesotho. The *H. truncatum* had the lowest occurrence (2%) recovered only from cattle in one district of Lesotho i.e. Qacha's Neck. Despite its low abundance, surveys conducted in southern Africa have shown that *H. truncatum* has a wide distribution. For instance, Golezardy and Horak (2007) collected few tick species of *H. truncatum* from wildlife in three national parks situated in the western regions of South Africa. Close to Lesotho, in the south-east of Bloemfontein, Free State Province, and other two relatively close regions in Limpopo, tick species belonging to *H. truncatum* were collected from warthog population (Matthee *et al*., 2013). This suggests that future tick survey studies in Lesotho should also sample wildlife in order to see whether there will be similar trend of *H. truncatum* host preference as compared to other countries.

The study also recorded uneven occurrences of *R. microplus* from domestic animals in five districts of Lesotho and *O. megnini* from domestic animals in three districts of Lesotho. Horak *et al*. (2014) has produced a third record of *R. microplus* as a non-endemic tick species in the Free State Province. Other studies have shown that *R. microplus* is widely common in other regions of South Africa, i.e. in the North West Province (Spikett *et al*., 2011). Horak *et al*., 2009 has reported relatively isolated, but collateral distribution of *R. microplus* and *R. decoloratus* in two provinces, i.e. Eastern Cape, South Africa and Maputo, Mozambique. In contrast, Nyakiwe *et al*. (2013) reported a possible displacement of the endemic *R. decoloratus* by non-endemic *R. microplus* in the Eastern Cape Province, South Africa. This pattern might account for the sporadic occurrences of *R. microplus* in the five districts of Lesotho as a possible establishment instance, even though this current study was not conducted seasonally as compared to other studies discussed above.

In addition, what was unusual was the recorded three specimens of *R. microplus* from a dog in Qacha's Neck district of Lesotho, because this one host-tick species has previously been recorded mostly from cattle and alternatively from goats in the

southern African region (Horak *et al.,* 2009; Spickett *et al*., 2011), and by a very small chance from other hosts such as eeland, gemsbok and grey rhebok (Horak *et al*., 2015). According to Lu *et al.* (2013), two out of nine tick specimens of *R. microplus* were collected from a dog host in the Hubei Province of China. Therefore, it can be suggested that in the absence of preferred hosts, particularly cattle, the *R. microplus* seeks alternative host such as dogs.

In this study, the soft tick, *O. megnini* has been collected Maseru, Qacha's Neck and Mafeteng districts of Lesotho. Its nymphae have been found to infest cattle calf in two regions of the Eastern Cape Province, South Africa (Horak *et al*., 2009). Spickett *et al*. (2011) recorded four tick specimens of *O. megnini* from three regions of the North West Province, South Africa, but were listed under ticks of lesser importance and low abundance. In the Free State Province, South Africa, there are records of other Argasid ticks, such as non-endemic ticks species of *Ornithodoros savignyi* collected from a paralyzed cow in the western region of Free State Province (Horak *et al*., 2015). Walker *et al*., (2014) states that though *O. megnini* is endemic to North America, its distribution pattern spreads from the semi-desert areas of South Africa to neighbouring countries such as Namibia, Botswana and Zimbabwe. Despite the pathogens not known to be carried by *O. mignini* (Walker *et al*., 2014), its parasitic nature of penetrating the internal parts of the external ear of livestock is thought to cause considerable damages which might lead to otitis (Jongejan and Uilenberg, 2004). Inflammations and secondary bacterial infections of the ear of livestock can also attract fatal myiasis of flies (Walkere *et al*., 2014). It remains to be studied whether there is any transtadial transmission of pathogens from *O. megnini* nymphae to adults and transovarial transmission from adults to eggs, as this could be a good indicator of whether this tick species is a vector of any pathogen in livestock.

It is apparent that existing constrains which lies with the traditional methods of tick identification makes it difficult to rely exclusively on physical traits of ticks in order to deduce a particular species (Anderson *et al*., 2004; Dergousoff & Chilton, 2007; Zhang & Zhang, 2014). This current study has not only relied on the morphological traits and published identification books of tick specimens to draw a conclusion, but has further taken upon a route of including expertise of tick taxonomists and,

molecular techniques to identify and characterize ticks in order to produce validated results on tick species recovered from five districts of Lesotho. The tick specimens collected in Lesotho have been submitted to the Onderstepoort Veterinary Research Tick Museum of the Agriculture Research Council where the identification was confirmed by tick taxonomist and voucher specimen numbers were issued as shown in the results section in Chapter 4.

Incorporation of the COI and ITS2 genes as genetic markers for the current study was vital when conducting analysis pertaining to evolutionary and phylogenetic analysis (Cruickshank 2002; Ronaghi *et al*., 2015). In this study we conducted molecular work only on ticks specimens collected from Butha-Buthe and few from Qacha's Neck, because most of the ticks were preserved in 30% glycerol and 70% mixture which made it difficult to extract DNA from the specimens. Multiple sequence alignment was conducted with 622 bp COI gene for *O. megnini* from this study versus soft tick species from NCBI database. The Argasid tick specimen, from domestic animals of Lesotho, identified as *O. megnini*, had a nucleotide sequence identity of 99% with that of the Madagascar, Africa (KC769589), to support morphological analysis made by this study. Further, sequence analysis of COI for soft ticks, showed that there was a less intraspecific divergence of the *O. meginini* from Lesotho, Madagascar, Africa (KC769589) and South Africa (KJ133592) supported by p-distance value of 0.2 - 0.6% (1nt) as compared to the interspecific divergence of the other soft ticks from NCBI database, supported by p-value of 0 – 3.7% (1 – 133nt). This proved that the *O. megnini* from Lesotho was more genetically closely related to the *O. megnini* from Madagascar (KC769589) and South Africa (KJ133592) when compared to the other soft tick species from various regions. In addition, the phylogenetic position of *O. megnini* based on COI gene provided results which were congruent to the morphological and sequence results, as both ML and NJ trees for COI gene where all the *O. megnini* formed their own separate cluster supported by bootstrap value of 100.

The multiple of sequence alignments were conducted with 622 bp COI gene for *H. rufipes* and *R. e. evertsi* against other hard ticks from NCBI database. The Ixodid ticks from domestic animals of Lesotho identified as *R. e. evertsi* A and *R. e. evertsi* Dhad sequence identity of 96 and 97%, respectively, with *R. e. evertsi* (AF132835)

from Kenya, based on the COI gene to supplement morphological findings of the study. Sequence analysis of the COI gene also showed that the intraspecific divergence of *R. e. evertsi* A and *R. e. evertsi* D from Lesotho, Kenya, Africa (AF132835) and Uganda, Africa (AB934398) with p-distance value of 0 – 3.1% (14nt) was smaller than the interspecific divergence of other *Rhipicephalus* species from the with p-distance value of 0 - 16% (0 - 73nt). Thus this indicates that *R. e. evertsi* A and D from Lesotho have lower genetic diversity from the *R. e. evertsi* of the Kenya, Africa (AF132835) and Uganda, Africa (AB934398) as compared with other *Rhipicephalus* species. Both morphological and sequence analysis were congruent to the positioning of *R. e. evertsi* A and *R. e. evertsi* D from Lesotho and Kenya, Africa (AF132835) and Uganda, Africa (AB934398) which formed their own cluster supported by bootstrap values of 99 for both ML and NJ trees. In addition, the two *R. e. evertsi* A and *R. e. evertsi* D of Lesotho and *R. e. evertsi* from Uganda and Kenya (AB934398 & AF132835 respectively) further formed a monophyletic group which isolated them by bootstraps values of 99 and 70 for both ML and NJ trees. Such isolation, suggested that *R. e. evertsi* A and *R. e. evertsi* D species of Lesotho are more genetically related to each other as compared to those of Kenya (AF132835) and Uganda (AB934398).

In the sixth bottom clade of COI trees, *H. rufipes* from Lesotho and Zimbabwe (AF132823) grouped together with other *Hyalomma* species to confirm their status as identified through morphological analysis and sequence deduction (sequence identity of 98% based on BLASTn), with supporting bootstrap values of 98 for ML and 97 for NJ trees. Sequence analysis of COI further verified the close genetic relatedness between the *H. rufipes* from Lesotho and Zimbabwe (AF132823) with low intraspecific divergence of a p-distance value of 0.2% (1nt) when compared to other *Hylomma* species that had interspecific divergence of 0 - 14.1% (0 - 64nt). The moderate interspecific divergence represented by a p-distance value of 0 - 22.8% (0 - 104nt) between the *Rhipicephalus* and *Hyalomma* species and, the two major monophyletic group of among the species supported by bootstrap values of 67 (ML and NJ trees) and 100 (ML and NJ trees) indicate that *Rhipicephalus* and *Hyalomma* species are two different species with close genetic relation at a genus level.

The ITS2 multiple sequence alignment was constructed with 475 bp ITS2 gene of *R. microplus, R. e. evertsi* and other hard ticks from NCBI database. Tick specimens from Lesotho identified as *R. microplus* based on morphological features was supported by ITS2 sequence identity of 92% shared with *R. microplus* from China, Asia, (JF758642) and close genetic relation indicated by low intraspecific divergence of 0 - 1.4% (0 – 9nt) with *R. microplus* from China (JF758642, JQ625705 and KC20336) and Nigeria (MF373428; MF373427 and MF373429). Such morphological and ITS2 sequence analysis, was in agreement with the position of all the *R. microplus* from Lesotho and NCBI as they formed an isolated cluster supported by bootstrap values of 100 by both ML and NJ trees to validate their close genetic relation at species level. In contrast, this isolated cluster of *R. microplus* also branches further to form two small branches with bootstrap values of 94and 98 in both ML and NJ trees. This might be due to the fact that *R. microplus* from Lesotho appears to have low genetic diversity from the two *R. microplus* of China (JF758642 and JQ625705) as compared to the other three *R. microplus* of Nigeria (MF373428; MF373427 and MF373429) and China (KC20336).

The *R. e. evertsi* A and *R. e. evertsi* D from Lesotho identified in this study by their morphological features were supported by sequence identity of 96% against *R. e. evertsi* (DQ849266) from Zambia, Africa, 95% against *R. e. evertsi* (U97701) from Kenya, Africa and 96% against *R. e. evertsi* (AF271279) based on ITS2 gene. Their intraspecific divergence showed low genetic diversity supported by p-distance value of 0 – 2.6% (0 - 17nt). Both morphology and ITS2 sequence analysis were in agreement with phylogenetic results as all the *R. e. evertsi* of the study and NCBI database formed a major separate cluster indicating that they are same species and was supported by bootstrap values of 93/98 for ML/NJ trees. In addition, two *R. e. evertsi* A and *R. e. evertsi* D from Lesotho and *R. e. evertsi* of Zambia (DQ849266) and from Kenya (U97701) branched further to form a small cluster with bootstrap values of 99for both ML and NJ trees. This highlighted that the two *R. e. evertsi* A and *R. e. evertsi* D from Lesotho were genetically diverse from *R. e. evertsi* of Zambia (DQ849266) as opposed to *R. e. evertsi* from Kenya (U97701). The monophyletic group formed by *R. microplus* and *R. e. evertsi* with bootstrap values of 100/100 and 93/98 for ML/NJ trees indicated that this two species share the same

genus and was also in congruent to the low interspecific divergence of 0 – 12% (0 – 81nt) based on ITS2 sequence analysis.

5.2. Detection of protozoan parasites from Lesotho ticks

There were no *B. bigemina* infections detected by nPCR from the screened tick samples, whilst 7.9% of tested samples were positive for the presence of *B. bovis* from DNA of ticks collected from cattle. Such tick DNA samples, which were positive for *B. bovis* included, five *R. microplus* from cattle of Ha khabile, Belo, Ha Mantlobo and Matsoaing villages in Butha-Buthe district. The remaining were from eight *R. e. evertsi* from cattle of Ha Khabile village, Seboche village, Ha Mantlobo, Matsoaing village and Resource centre in Butha-Buthe district. The presence of *B. bovis* and absence of *B. bigemina* in ticks collected from cattle of Butha-Buthe district from the current study could be accounted on the basis of vector and causative agent relation, because several studies have supported the fact that *R. microplus* plays a role as the main vector tick for *B. bovis* as compared to other *Rhipicephalus* tick species (Potgieter, 1977; De Vos, 1979; Tonnsen *et al*., 2004; 2006). The out-numbering detection of *B. bovis* in *R. e. evertsi* as compared to that of *R. microplus*, can be explained by the highest abundance of *R. e. evertsi* 52% than *R. microplus* 48% throughout Butha-Buthe district observed in the current study. It must be noted that *R. e. evertsi* can also act as a vector to both *B. bovis* and *B. bigemina* (Potgieter, 1977; De Vos, 1979; Tonnsen *et al*., 2004; 2006). In contrast, in South Africa Mtshali *et al*. (2014) conducted nPCR and reported high incidences of cattle infections by both *B. bigemina* and *B. bovis* in all the nine provinces, whereby four provinces, i.e. Free State, KwaZulu-Natal, Gauteng and Limpopo, had the highest prevalence of *B. bigemina* and were thought to have reached an endemic status. In addition, in Brazil frequencies of *B. bovis* and *B. bigemina* which were similar in adult and young cattle were reported in endemic areas through blood smears, PCR and nested PCR (Oliveira-Sequeira *et al*., 2005).

PCR detection of *B. caballi* and *T. equi* from four ticks of horses were all negative, and this could be attributed to the few tick samples used in the current study. This finding cannot represent the entire status of Lesotho districts. However, close to Lesotho, in the north-eastern Free State Province, South Africa, the presence of *B. caballi* and *T. equi* from horses was reported by Motloang *et al*. (2008). Further

detailed epidemiological studies of equine piroplasmosis from both ticks and host could shed light on the presence of *B. caballi* and *T. equi*.

Babesia ovis was positively detected by PCR in three *R. e. evertsi* DNA samples (one from goat and two from sheep of Qalo village) out of 22 tick DNA samples. Though the proportion of infected sheep ticks compared to that of goat ticks was very small, they showed to be relatively unequal in this study. *Rjeibi et al*. (2014) detected more incidences of high prevalence of *B. ovis* in sheep as opposed to goats of North-West Tunisia. None of *B. motasi*, *Theileria ovis* and *T. lestoquardi* infections were detected by PCR from goat ticks and from sheep ticks from Lesotho in this study. However, catch all primers for *Theileria-Babesia* were positive on four DNA sample from ticks represented by *R. e. evertsi* from goats of Qalo village, *R. decoloratus* from goat of Manamela village, and the last two were *R. e. evertsi* from sheep of Qalo and Ha Majara villages. The positive results of catch all PCR means there is presence of some ampicolexan haemoparasite which could either be *Babesia, Hepatozoon* or *Theileria*, the actual species remain to be identified. The reason why tick-borne haemoparasites could not be detected with species specific PCR assays for *Theileria-Babesia* species can be alternatively explained by suspecting that infections could be from other unscreened domestic animal protozoa such as *Theileria sergenti/buffeli/orientalis* group (Thekisoe *et al*., 2007). These are protozoan parasites which always infect domestic animals, but are regarded as of less economic impact or non pathogenic or causing benign infections (Cicek *et al*., 2009; Uilenberg *et al*., 1981). Moreover, collection of ticks only took place in five districts of Lesotho whilst most of the molecular work was performed on samples from one district, i.e. Butha-Buthe, due to poor quality of DNA of ticks from other districts.

5.3. Conclusion

In this study we managed to use the traditional morphological methods to identify five tick species, namely *O. megnini, H. rufipes, H. truncatum, R. microplus* and *R. e. evertsi*, collected from domestic animals of Lesotho. Even though all the species of ticks identified from domestic animals of Lesotho, could not be covered by molecular techniques, the COI gene of *O. megnini, R. e. evertsi* and *H. rufipes* and the ITS2 of *R. microplus* and *R. e. evertsi* were characterized and used to conduct phylogenetic

analyses. The hypothesis of the study was nullified because despite a good number of tick vectors documented in various districts, this study did not detect high number of tick-borne haemoparasites. It is suspected that some of the PCR assays are not sensitive enough for detection of the tick-borne haemoparasites. Despite the ability of ticks to transmit pathogens to domestic animals, they are well known to cause skin damage, skin irritations resulting in weight losses as animals spend less time feeding, anaemia due to blood loss during high infestations. This study has provided information on the present tick species in Lesotho. This has created a baseline for detailed epidemiological studies of ticks and tick-borne diseases in Lesotho.

5.4. Recommendations

- Further tick collections must be conducted over all seasons in the remaining districts of Lesotho, in order to get the full picture of tick diversity and distribution in the whole country.
- Other DNA based assays such as RLB should be conducted to screen for haemoparasite infections in ticks, thus enabling simultaneous detection and higher sensitivity.
- Haemoparasite screening of blood collected from domestic animals should be conducted in order to determine the occurrence of tick-borne haemoparasite infections.

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