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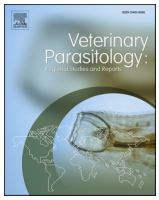

Original Article

Ticks of domestic animals in Lesotho: Morphological and molecular characterization

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ABSTRACT

A total of 3311 tick specimens were randomly collected from domestic animals including cattle, sheep, goats, horses, donkeys, and dogs from Lesotho districts namely, Berea, Butha-Buthe, Leribe, Mafeteng, Maseru, Mohale's Hoek, Mokhotlong, Qacha's Nek, Quthing and Thaba Tseka. Tick species were identified morphologically and verified by amplification and sequencing of the CO1 and 18S rRNA genes. Nine species were identified under different genera namely, *Haemaphysalis elliptica* 0.1% ($n = 2$), *Hyalomma rufipes* 2.6% ($n = 87$), *Hy. truncatum* 1.2% ($n = 41$), *Otobius megnini* 13.6% ($n = 451$), *Rhipicephalus appendiculatus* 0.1% ($n = 3$), *Rhipicephalus decoloratus* 9.3% ($n = 308$), *Rhipicephalus evertsi evertsi* 65.1% ($n = 2156$), *Rhipicephalus glabroscutatum* 1.3% ($n = 43$) and *Rhipicephalus microplus* 6.6% ($n = 220$). There was a significant difference at $p = 6.2E\text{-}06$ ($\chi^2 = 1.923$, df = 7) in the distribution of tick species and their abundance $p = 0.04$ ($\chi^2 = 1.923$, df = 7) from each population. The CO1 and 18S rRNA sequences matched the morphological determinations on the NCBI database and clustered with relevant species on the phylogenetic tree. Genetic analysis of CO1 and 18S rRNA provided very strong support for monophyly of the Rhipicephalinae and Ornithodorinae complexes. Both CO1 and 18S rRNA are useful genetic markers for the specific and generic characterization of tick species in Lesotho and elsewhere. This is the first scientific publication of tick species occurring in Lesotho.

1. Introduction

Ticks are globally recognized as ubiquitous hematophagous ectoparasites of terrestrial vertebrates including amphibians, birds, mammals, and reptiles (Jongejan and Uilenberg, 2004). Ticks are also vectors of a diversity of pathogens (Papa et al., 2017) causing severe communicable diseases in humans and animals (Sirigireddy, 2008; Chitimia et al., 2010), making them second to mosquitos as blood-feeding vectors of both medical and veterinary importance which transmit a large variety of parasitic diseases (Nyangiwe et al., 2013).

In Africa, tick species from ten genera are well recognized in their role of infestation on domestic animals; seven of them being ixodids (hard ticks) with more than 80 species occurring in South Africa (SA) and the three genera are Argasid (soft ticks) (Jongejan and Uilenberg, 2004). Tick infestation and the diseases they transmit during blood meals have resulted in vast economic losses in livestock production and continue to severely plummet the livestock industry mostly in developing, resource-limited countries especially in sub-Saharan Africa (Parola and Raoult, 2001; Estrada-Peña and de la Fuente, 2014). High infestations of ticks consequently result in extensive damage to animal skin which subsequently erodes the commercial value of hides (Asokan and Asokan, 2016).

In humans, tick-borne diseases (TBDs) pose a significant public health risk by transmitting parasitic micro-organisms such as bacteria, protozoa, *Rickettsia*, and viruses; resulting in high morbidity and mortality (Chitanga et al., 2014). Tick-borne diseases reported in southern Africa include the tick-borne relapsing fever (TBRF) caused by *Borrelia duttonii*, babesiosis caused by *Babesia microti*, Crimean-Congo

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hemorrhagic fever (CCHF) caused by Nairovirus (Lew-Tabor and Valle, 2016). Rickettsioses caused by *Rickettsia aeschlimanii*, *Rickettsia conorii,* and *Rickettsia africae*, as well as anaplasmosis is caused by *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Anaplasma centrale* and *Anaplasma ovis* (Mapholi et al., 2014).

Eighty percent of the world's livestock population is at risk of ticks and TBDs (Mangold et al., 1998; Spickett et al., 2011).

Direct approaches for species and population genetic characterization have been demonstrated in recently developed DNA-based techniques for sequence analysis (Mangold et al., 1998). During the implementation of phylogenetic studies several markers can be exploited, these include both coding and non-coding loci (Beati and Keirans, 2001). There are a number of coding genes used in tick phylogenetic analyses and these include mitochondrial rDNA (12S and 16S) and nuclear rDNA (18S and 28S), with 12S rDNA providing more resolution at the genus and species level (Black IV and Roehrdanz, 1998; Dabert et al., 2010; Low et al., 2015; Baron et al., 2018).

The mitochondria cytochrome *c* oxidase subunit 1 (CO1) gene has been successfully used to determine intraspecific genetic variation (Folmer et al., 1994; Song et al., 2011) for phylogenetic inference on morphologically similar species such as *Ixodes cornuatus* and *Ixodes holocyclus* (McLain et al., 1995; Navajas and Fenton, 2000). The CO1 gene is a conserved protein-coding gene in the mitochondrial genome of eukaryotes; which is desirable for evolutionary time depth (Murrell et al., 2001). Non-coding loci used in phylogenetic studies of ticks include the internal transcribed spacers (ITS1 and ITS2) of nuclear rDNA sequences (Walker et al., 2003; Latif, 2013; Kumsa et al., 2016). These nuclear markers have been described for tick identification but, fail to discriminate tick species from the same complex (Madder et al., 2013).

The objective of this study was to pioneer documentation of tick species infesting domestic animals in Lesotho using both morphological identification and genetic analysis of the CO1 and 18S rRNA genes.

2. Materials and methods

2.1. Study area

The Mountain Kingdom of Lesotho (referred to as Lesotho) is a landlocked country surrounded by South Africa. It is known for its green pastures for domestic animals and the only country globally that is entirely more than 1000 m above sea level (https://www.sadc.int/member-states/lesotho/). Lesotho has ten districts (Fig. 1), and collection sites were Berea, Butha-Buthe, Leribe, Mafeteng, Maseru Mohale's Hoek, Mokhotlong, Qacha's Nek, Quthing and Thaba Tseka.

2.2. Specimen collection and identification

Tick specimens were collected during 2017–2019 from naturally infested domestic animals including cattle, dogs, donkeys, goats, horses, and sheep, some tick species were collected from the vegetation where animals grazed. They were collected randomly from different predilection sites depending on the area of abundance and visibility, this included heads, ears, neck, abdominal areas, and perineum using sterile fine-tipped forceps. The collected ticks were stored in individual vials and preserved in 70% ethanol. They were morphologically identified to species level using a stereomicroscope and using taxonomic keys by Walker et al. (2003), Madder et al. (2013) and Latif (2013). Identifications were also verified at the Gertrud Theiler Tick Museum, Agricultural Research Council (ARC)-Onderstepoort Veterinary Research, and voucher numbers for specimens were issued (Supplementary Table S1).

2.3. DNA isolation, polymerase chain reaction (PCR) and sequencing

The genomic DNA of tick specimens was extracted from the complete body of an individual tick using the Zymo Tissue DNA extraction kit (Zymo Research Corporations, USA) by following the manufacturer's protocol and stored at −20 °C until used. A ~ 710 bp fragment of the

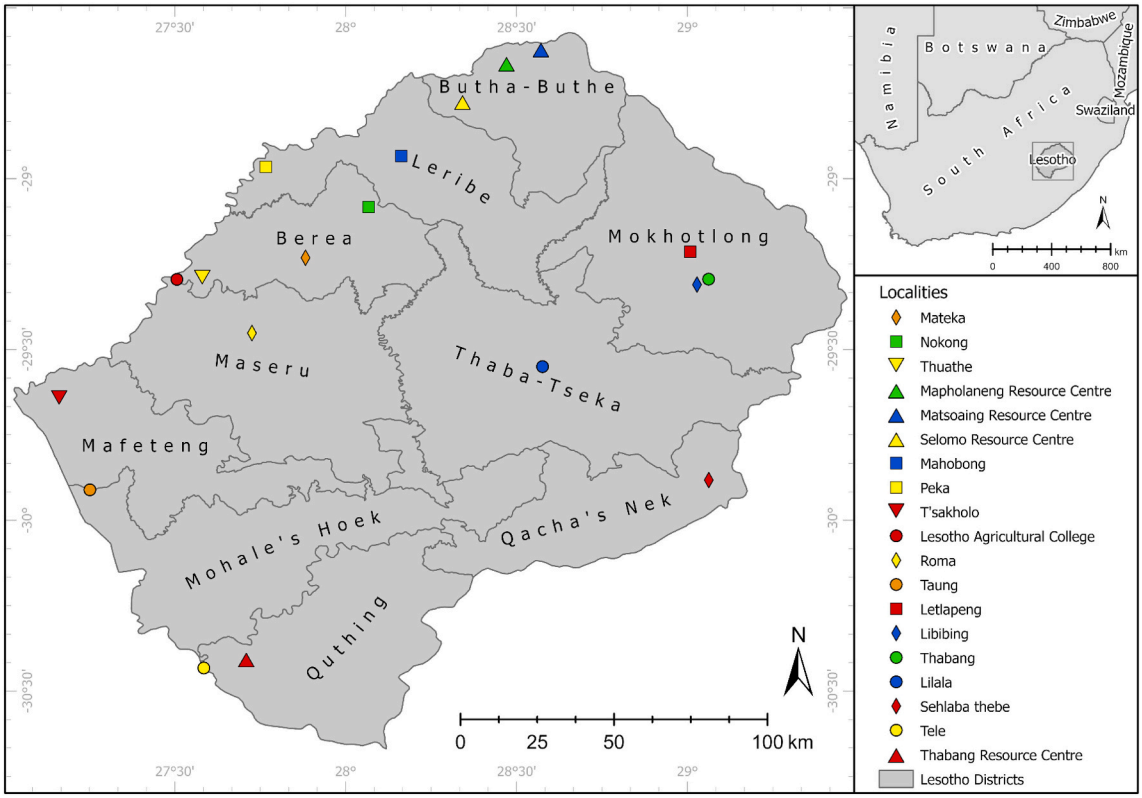

Fig. 1. A map of The Mountain Kingdom of Lesotho, with the ten studied regions encircled.

cytochrome oxidase c subunit 1 (CO1) gene and a ~ 550 bp fragment of the 18S rRNA gene were amplified using the primers and PCR protocol as listed in Table 1. The PCR reactions were performed in a 25 µl volume with 12.5 µl DreamTaq PCR Master Mix (Thermo Scientific, US), 3 µl of each primer (10 µM each primer), 2 µl of genomic DNA template and 4.5 µl deionized water (ddH_2O) was added to final volume. All PCR reactions were performed using BIO-RAD T100 thermocycler (BIO-RAD Laboratories, CA, USA). The PCR products were electrophorized on a 1% agarose gel stained with ethidium bromide and visualized in a UV transilluminator, (Chemidoc system, Neiker, Spain). The positive control was obtained from the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine Japan. The amplified PCR products were sent to Inqaba Biotech (Pretoria, South Africa) for purification and sequencing. Nucleotide sequences were analyzed using MEGA X sequence alignment editor software (Kumar et al., 2018). To confirm sequences obtained from all PCR amplicons the NCBI BLASTn analysis (http://www.ncbi.nlm.nih.gov.blast) was used and a match score of 90–100% similarity was considered as significant.

Consensus sequences were deposited in GenBank under the accession numbers **MK541811, MN037792, MK551198, MK551200 – MK551207, MK551209 – MK551211** and **MN453665 – MN453672** (CO1 sequence from Berea, Butha-Buthe, Leribe, Mafeteng, Maseru, Qacha's Nek and Quthing); **MN015029, MN015030**, **MN320365 - MN320377** (18S rRNA sequences from Berea, Butha-Buthe, Leribe, Mafeteng, Maseru, Qacha's Nek and Quthing).

2.4. Statistical analyses

The data were analyzed statistically for each species collected and identified, the prevalence was presented in proportions and summarized into a table of tick species identified for each district. The analysis of variance ANOVA by ranks was used to determine the significance in the overall distribution of tick species collected in all the districts at a significance of 95% confidence level.

2.5. Phylogenetic analysis

The CO1 (45 and 9) and 18S rRNA (31 and 9) gene nucleotide sequences for hard and soft ticks respectively, were aligned using Clustal W multiple alignments under default parameters in MEGA X software (Kumar et al., 2018) and trimmed manually to remove uneven ends from the aligned sequences. Both Maximum Likelihood (ML) and Bayesian phylogeographic analyses were performed, as consistency between the topologies of two different approaches enhances confidence in the interpretation of patterns. Maximum Likelihood and Bayesian Inference (BI) analyses are considered appropriate for assessing evolutionary relationships. Maximum Likelihood phylogenetic analysis was carried out using MEGA X (Kumar et al., 2018). The phylogenetic analysis incorporated published sequences from several congeneric species (Supplementary Table S2) and *Ixodes ricinus* and *Varroa destructor* was used as an outgroup for soft ticks and hard ticks, respectively. The ML analysis for soft ticks was performed using GTR + G + I model for CO1 and K2 + G model for 18S rRNA. The ML analysis for hard ticks was performed using HKY + G model for and a K2 model for 18S rRNA. Support for each node was assessed by bootstrapping, with heuristic analysis of 10,000 replicates datasets.

Bayesian Inference (BI) phylogenetic analysis was carried out for CO1 and 18S rRNA individually using Mr. Bayes Ver. 3.2.2 (Brown, 2014). The same model parameters used in ML were used for BI analysis, and Markov Chain Monte Carlo (MCMC) searches were executed with a total of four chains of 40,000 generations, with 200 sampled every 1000 generation. The first 25% of the trees were discarded as burn-in. Additionally, sequences available in GenBank (http://www.ncbi.nlm.nih.gov.blast) of *Hyalomma*, *Otobius,* and *Rhipicephalus* genera from other countries were included (Supplementary Table S2).

3. Results

3.1. Tick species collected in Lesotho

A total of 3311 ticks were collected ten districts in Lesotho as shown in Table 2. Morphological identification revealed a total of four different genera from the sampled areas namely: *Haemaphysalis*, *Hyalomma*, *Otobius*, and *Rhipicephalus* with a total of nine different species. The tick species captured were *Ha. elliptica*, *Hy. rufipes*, *Hy. truncatum*, *O. megnini*, *R. appendiculatus*, *Rhipicephalus decoloratus*, *Rhipicephalus evertsi evertsi*, *Rhipicephalus glabroscutatum* and *Rhipicephalus microplus* (Table 3 and Fig. 2). Plates I–XII shows pictures of the different tick species as well as eggs and developing larvae. The dominant species from the ten samples district were *R. e. evertsi* (65.1%) and *O. megnini* (13.6%), whilst *Ha. elliptica* (0.1%) was the least. Qacha's Nek district had the highest species abundance (35.2%) followed by Leribe (22.5%), Maseru (15.6%), Mohale's Hoek (8.2%), Butha-Buthe (6.6%), Berea (5.0%), Mafeteng (2.3%), Mokhotlong (1.8%), Thaba Tseka (0.4%) and Quthing (0.2%) with the least number of ticks captured (Table 3 and Fig. 2). There was a significant difference at $p = 6.2E\text{-}06$ ($\chi^2 = 1.923$, df = 7) in the overall distribution of the tick species and their abundance $p = 0.04$ ($\chi^2 = 1.923$, df = 7) from each population throughout Lesotho districts.

3.1.1. Haemaphysalis elliptica, Koch (1844)

This species was collected only in Qacha's Nek district of Lesotho. Following the description of Madder et al. (2013) and Walker et al. (2003). The specimen has a conspicuous lateral extension to the palp article 2, forming mouthparts with a distinctive conical shape (Plate I). In addition, the coxae 1–3 spurs are medium and coxae 4 of males have only medium-length spurs. The hypostome and palp are short. The scutum is yellow and is covered with numerous small punctuations and the festoon is present. Eyes are absent and there are no additional plates in males.

3.1.2. Hyalomma rufipes, Koch (1844)

This species was collected from Berea, Maseru, Mohale's Hoek, Qacha's Nek, and Quthing in Lesotho. Following the description of Madder et al. (2013) and Walker et al. (2003). The specimen is large and is hairy shiny dark-brown to nearly black in color. The scutum and conscutum are dense and uniformly covered with punctuations obscuring lateral grooves. The conscutum of the male is very characteristic evenly rounded and lacking the grooves. The posterior margin of the scutum is evenly rounded and brown legs are distinctly brightly-banded with ivory-coloured rings (Plate II). The genital aperture of the females is a wide shield with broad posterior margins. The adanal plates have square ends and sub-anal plates are distinct but small and aligned with adanal plates. The spiracle of males and females are surrounded by setae.

3.1.3. Hyalomma truncatum, Koch (1844)

This species was collected from Leribe, Maseru, and Qacha's Nek in Lesotho. Following the description of Madder et al. (2013) and Walker et al. (2003). The specimen has a dark brown conscutum, in males, it is narrow and glossy with few punctuations (Plate III). The central festoon is not well defined enough to form a parma in males. Females have genital aperture a distinctive transversely elongated oval, posteriorly appears flat, **U**-shape wider than long. In males, the lateral lines are distinctly extending to the eyes. The spiracle of males and females are surrounded by setae.

3.1.4. Otobius megnini, Dugès (1844)

This species was collected from Mafeteng, Maseru, Mohale's Hoek, and Qacha's Nek in Lesotho. Following the description of Madder et al. (2013) and Walker et al. (2003), the adults are dark-grey in color and violin-shaped (Plate IV), the nymphs are diamond-shaped and have no eyes. Numerous small pits are present on the integument, the lateral

Table 1
Primer sets, and PCR protocol used for the amplification of the CO1 and 18S RNA genes.

Target	Primer name	Primer sequence (5′ – 3′)	References	PCR protocol
CO1	LCO1490F	GGTCAACAAATCATAAAGATATTGG	Kumar et al. (2018)	98 °C, 5 min; 36 x [94 °C, 30s; 47 °C, 50s; 72 °C, 2 min]; 72 °C, 10 min
	HCO2198R	TAAACTTCAGGGTGACCAAAAAATCA		
18S rRNA	NS1F	GTAGTCATATGCTTGTCTC	Brown (2014)	98 °C, 5 min; 36 x [94 °C, 30s; 47 °C, 50s; 72 °C, 2 min]; 72 °C, 10 min
	NS2R	GGCTGCTGGCACCAGACTTGC		

margin of the body is thick without a definite suture line. The nymphs are covered with short, rigid setae in the form of spines. The mouthparts are rudimentary and situated on the ventral surface of the body.

3.1.5. *Rhipicephalus appendiculatus, Neumann (1901)*

This species was collected only from Maseru in Lesotho. Following the description of Madder et al. (2013) and Walker et al. (2003). The mouthparts are short and the basis capitulum is hexagonally shaped in females. The eyes are flat. The adanal plates are long and in engorged males, a caudal appendage is present (Plate V). In males, the legs increase in size from I to IV. The species is brown. Coxae 1 has an anterior spur that is prominent and visible from the dorsal side. Female ticks have cervical fields with fewer punctations, and genital pore is shallow **V** -shape.

3.1.6. *R. decoloratus, Koch (1844)*

This species was collected from Butha-Buthe, Leribe, Mafeteng, Maseru, Mokhotlong and Qacha's Nek in Lesotho. Following the description of Madder et al. (2013) and Walker et al. (2003). The males have distinct cornua. Adanal spurs are distinct. The mouthparts are short and the dentition on the hypostome is arranged in two columns, each consisting of 3 + 3 rows. A protuberance bearing setae on the inner margin of palp article 1 is present. Coxae 1 has both spurs distinct, while coxae 2 and 3 only have posterolateral spurs distinct. The eyes are small, and females have two distinct cervical grooves dividing the scutum into a central yellow area and two lateral areas that are reddish-brown (Plate VI). The segments of the pale-yellow legs are beady in appearance.

3.1.7. *R. evertsi evertsi, Neumann (1897)*

This species was collected from all ten districts, Berea, Butha-Buthe, Leribe, Mafeteng, Maseru, Mohale's Hoek, Mokhotlong, Qacha's Nek, Quthing and Thaba Tseka in Lesotho. Following the description of Walker et al. (2003) and Madder et al. (2013). The conscutum and scutum of the specimens are densely punctuated very dark-brown in color with a reddish-orange body wall. The eyes are convex and orbited and the legs are reddish-orange (Plate VII). The adanal plates are triangular and large. The circum-spiracular integument is covered with dense prominent setae. Caudal appendages are absent in fed specimens.

3.1.8. *R. glabroscutatum, Du Toit (1941)*

This species was collected from Butha-Buthe, Maseru, and Qacha's Nek in Lesotho. It was identified by the characteristics described by Du Toit (1941). This species is uniformly chestnut brown including the legs, with hemispherical orbited eyes. The scutum is oval-shaped with few punctations scattered over the anterior in males and females, the punctuations are more numerous anteriorly (Plate VIII). Festoons are well marked. Cervical grooves in females are deep anteriorly and curved laterally, almost reaching the scutum margin. In engorged males, a short caudal process is apparent.

3.1.9. *R. microplus, Canestrini (1888)*

This species was collected from Butha-Buthe, Leribe, Maseru, Qacha's Nek, and Thaba Tseka in Lesotho. Following the description of Madder et al. (2013) and Walker et al. (2003). The specimen has 4 + 4 dentition on its hypostome (Plate IX). A protuberance bearing setae on the inner margin of palp article 1 is absent. In males, the cornua are distinct and caudal appendages are narrow in fed specimens. Adults are slightly larger and the scutum is redder in color.

3.2. *Sequence analysis*

The partial CO1 (548–674 nt) fragments of the ticks from Berea, Butha-Buthe, Leribe, Mafeteng, Maseru, Qacha's Nek and Quthing, shared 96–99% identity. Online BLASTn search of *R. e. evertsi* (Lesotho) nucleotide sequences shared 98% similarities with published tick sequences from DRC (MF458972) and Uganda (AB934398); nucleotide sequences for *R. decoloratus* (Lesotho) shared 99% similarities to other *R. decoloratus* ticks collected in Burkina Faso (KY678127), and SA (KY678128). Online BLASTn search for *R. appendiculatus* (Lesotho) shared 98% similarities with other published tick sequences collected in Uganda (KY688464; KY688466), nucleotide sequences of *R. microplus* from this study shared 99% similarities with other tick sequences collected in Brazil (KP226173), Colombia (MF363057), Benin (KY678120) and Cameroon (MG983831), and online BLASTn search of *Hy. rufipes* (Lesotho) showed 97% similarities with the same sequences reported in France (KX000641; KX000643) (Supplementary Table S3). The search also revealed 99% similarities for *O. megnini* (soft ticks) CO1 partial sequence from this study with other published tick sequences collected in Madagascar (KC769589) and SA (KJ133592) (Supplementary Table S3). Partial 18S rRNA fragments (220–465 nt) of the ticks from Berea, Butha-Buthe, Leribe, Mafeteng, Maseru, Qacha's Nek and Quthing were identical to sequences of *Rhipicephalus*, *Hyalomma* and *Otobius* ticks from neighbouring countries (Supplementary Table S2).

3.3. *Phylogeny of Lesotho ticks*

The sequences used for both ML and BI phylogenetic analyses were from members of the genera *Rhipicephalus* and *Hyalomma* as well as *Otobius,* from the Ixodidae and Argasidae families, respectively. *V. destructor* (LN873226, AY620940) and *I. ricinus* (AY945438, GU074707) were used as an outgroup for ixodid (Figs. 3 and 5) and argasid ticks (Figs. 4 and 6), respectively. The partial CO1 ML and BI tree revealed phylogenetic relationships between tick species with higher resolution than that the 18S rRNA tree (Figs. 3–6). All the ticks from the CO1 and 18S rRNA sequences generated from this study clustered with the corresponding congener. In both tree topologies monophyly within the families was well supported with high bootstrap value (100%) and similar topologies were obtained. The ML analysis (Fig. 3) revealed two major clades (I-V) with strong bootstrap support values, namely, Clade I-IV *Rhipicephalus* spp. and Clade V-*Hyalomma* spp., and Fig. 5, revealed only two major clades (Clade I-II). Figs. 4 and 6, produced three clades, namely, I-*Otobius*, II-*Ornithodoros* and III-*Argas*, respectively.

4. Discussion

In this study, *Ha. elliptica* has been found infesting dogs in Lesotho (Walker et al., 2003) and is distributed in southern countries of Africa and all provinces of SA (Walker et al., 2003; Madder et al., 2013). Foxes, larger cats, jackals and wild dogs are considered as a host for *Ha. elliptica*. In SA, *Ha. elliptica* is known to transmit *B. rossi* to dogs causing canine babesiosis as well as *R. conorii* bacterium causing tick typhus or the Mediterranean spotted fever, resulting in tick bite fever in humans (Horak et al., 2002; Walker et al., 2003). Only two tick specimens were collected from dogs with a 2.7% prevalence from 0.1% of the overall tick

Table 2
Number of sampled tick specimens, localities and developmental stages collected from domestic animals in Lesotho.

	Berea			Butha-Buthe			Leribe			Mafeteng			Maseru			Mohale's Hoek			Mokhotlong			Qacha's Nek			Quthing			Thaba Tseka			Total per host
	M	F	N	M	F	N	M	F	N	M	F	N	M	F	N	M	F	N	M	F	N	M	F	N	M	F	N	M	F	N	N
Cattle	[a]-	–	–	102	105	50	55	111	11	4	18	53	89	141	103	16	45	36	17	18	8	51	101	179	–	–	–	4	5	0	**1322**
Dogs	–	–	–	–	–	–	2	1	0	–	–	–	–	–	–	–	–	–	–	–	–	4	25	41	–	–	–	–	–	–	**73**
Donkeys	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10	1	1	–	–	–	–	–	–	–	–	–	–	–	–	**12**
Goats	43	24	0	10	8	0	80	95	44	–	–	–	–	–	–	4	5	6	3	4	0	45	66	30	–	–	–	3	0	0	**470**
Horses	–	–	–	–	–	–	1	2	0	–	–	–	–	–	–	11	6	13	–	–	–	26	42	20	5	3	0	–	–	–	**129**
Sheep	58	39	0	2	6	4	91	228	16	–	–	–	–	–	–	40	37	42	3	7	0	39	43	88	–	–	–	–	–	–	**743**
Vegetation	–	–	–	4	0	0	3	5	1	–	–	–	58	114	13	–	–	–	–	–	–	74	147	143	–	–	–	–	–	–	**562**
Total per district	164			291			746			75			518			273			60			1164			8			12			3311

[a] - = Not collected, M = Male tick, F = Female tick, N = Nymph.

occurrence. From the overall collection of ticks, this species was the least collected from all sampled districts.

H. truncatum occurred in 4 different domestic animals; dogs were highly infested with 5.5% prevalence, followed by unaccounted hosts at 3.4%, the horses had 1.6% of tick infestation and sheep at 1.2% and the least were cattle at 0.7%. The results obtained are consistent with (Spickett et al., 2011) findings where, 89.9% were from cattle, followed by 8.5% from goats, sheep and unaccounted hosts shared 0.8% tick prevalence. Nonetheless, we were able to collect *Hy. truncatum* and confirm its abundance and prevalence in Lesotho. The *Hy. truncatum* was collected in 3 districts of Lesotho including Leribe, Maseru, and Qacha's Nek, and this tick is known to be a vector of *B. caballi*, a causative agent of equine piroplasmosis. *H. rufipes* was collected from Berea, Maseru, Mohale's Hoek, Quthing and Qacha's Nek district. This tick specimen transmits *B. occultans*, a known causative agent of benign babesiosis in cattle (Walker et al., 2003).

In this study, *Hy. rufipes* collected from unaccounted hosts had the highest tick infestation of 10.4%, followed by horses (4.7%), cattle (1.9%), sheep (0.4%), and goats were the least at 0.2% prevalence. Specimens collected were very low in numbers with 2.6% overall occurrence from sampled localities and some localities had no *Hy. rufipes* counted for. *H. rufipes* was also collected on horses. This finding corresponds with the study by (Horak et al., 2017), where 10.5% tick infestation was found in horses.

O. megnini was collected in all domestic animals of Lesotho, including cattle, sheep, and goats. According to Walker et al. (2003), domestic animals are known as the primary host for the spinose tick. In the current study, *O. megnini* was the most commonly collected species from Mafeteng, Maseru, Mohale's Hoek, and Qacha's Nek districts with 13.6% overall occurrence. Qacha's Nek district had a higher population of *O. megnini* with 64.1% abundance. Ticks were collected from dogs (32.9%), unaccounted host and cattle shared (18.3%), sheep (10.8%), horses (8.5%), whilst remaining 0.6% was from goats. Dogs had the highest percentage of tick infestation. Data obtained in this study agrees with the report by Walker et al. (2003) that domestic animals including cattle, horses, and dogs are the preferred hosts of *O. megnini*.

R. appendiculatus does not occur in open grasslands without bush as this tick prefers tall interspersed with trees, in the eastern regions of SA, *R. appendiculatus* is restricted to higher-rainfall areas and is scant in the north-east regions(Walker et al., 2003; Horak et al., 2009; Spickett et al., 2011). In this study, 0.1% of the overall occurrence of *R. appendiculatus* was only in the Maseru district. The species abundance was at 100%. Tick infestation was prevalent at 4.7% from the vegetation, no specimens were collected from domestic animals (Table 4). According to Spickett et al. (2011), study, 11.2% was accounted for *R. appendiculatus* in North-West, South Africa; 86.9% was from cattle, 7.4% from goats, and 5.0% from sheep. These findings are similar to observations by Horak et al. (2015), where *R. appendiculatus* abundance has a high prevalence in northern Free State, South Africa, with some specimens collected from vegetation similar to the current study. *R. appendiculatus* is considered as an endemic species in SA and as a three-host tick, adult activities commence only during the summer months (December–March) and prefers large domestic and wild ruminants as hosts (Horak et al., 2009; Horak et al., 2017). This tick is a vector of *Theileria parva* and a buffalo-derived *T. parva* a causative organism of East Coast fever and Corridor disease, respectively in cattle (Horak et al., 2017).

R. decoloratus was the most commonly collected species from Butha-Buthe, Leribe, Mafeteng, Maseru, Mokhotlong, and Qacha's Nek (Table 4) with 9.3% overall occurrence, Maseru district had more population of *R. decoloratus* with 61.7% species abundance. Tick infestation from cattle was at 20.9% and 5.8% was from unaccounted host followed by 3.1% from vegetation whilst the remaining 0.1% was from sheep. Cattle had the highest percentage of tick infestation. These findings agree with Spickett et al. (2011) study, where they had 98.1% prevalence of tick infestation on cattle and only 1.1% were of unaccounted hosts; 15.6% prevalence was observed on horses from Horak et al.

Table 3
Tick species identified from ten districts in Lesotho and the total number of tick specimens collected in each district.

Tick species	Number of ticks per district										Total no. of ticks
	Berea (%)[a]	Butha-Buthe (%)[a]	Leribe (%)[a]	Mafeteng (%)[a]	Maseru (%)[a]	Mohale's hoek (%)[a]	Mokhotlong (%)[a]	Qacha's Nek (%)[a]	Quthing (%)[a]	Thaba Tseka (%)[a]	
Haemaphysalis elliptica	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	**2**
Hyalomma rufipes	3 (3.4)	0 (0)	0 (0)	0 (0)	8 (9.2)	7 (8.0)	0 (0)	66 (75.9)	3 (3.4)	0 (0)	**87**
Hyalomma truncatum	0 (0)	0 (0)	13 (31.7)	0 (0)	6 (14.6)	0 (0)	0 (0)	22 (53.7)	0 (0)	0 (0)	**41**
Otobius megnini	0 (0)	0 (0)	0 (0)	38 (8.4)	48 (10.6)	76 (16.9)	0 (0)	289 (64.1)	0 (0)	0 (0)	**451**
Rhipicephalus appendiculatus	0 (0)	0 (0)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**3**
Rhipicephalus decoloratus	0 (0)	4 (1.3)	43 (14.0)	24 (7.8)	190 (61.7)	0 (0)	33 (10.7)	14 (4.5)	0 (0)	0 (0)	**308**
Rhipicephalus e. evertsi	161 (7.5)	168 (7.8)	647 (30.0)	13 (0.6)	244 (11.3)	190 (9.0)	27 (1.3)	694 (32.2)	5 (0.2)	7 (0.3)	**2156**
Rhipicephalus glabroscutatus	0 (0)	8 (18.6)	0 (0)	0 (0)	3 (7.0)	0 (0)	0 (0)	32 (74.4)	0 (0)	0 (0)	**43**
Rhipicephalus microplus	0 (0)	111 (52.7)	43 (19.5)	0 (0)	16 (7.3)	0 (0)	0 (0)	45 (20.5)	0 (0)	5 (2.3)	**220**
Total no. per district	**164 (5.0)**	**291 (6.6)**	**746 (22.5)**	**75 (2.3)**	**518 (15.6)**	**273 (8.2)**	**60 (1.8)**	**1164 (35.2)**	**8 (0.2)**	**12 (0.4)**	**3311**

a = Indicates the abundance of the tick species found per sampled district from Lesotho in percentage.

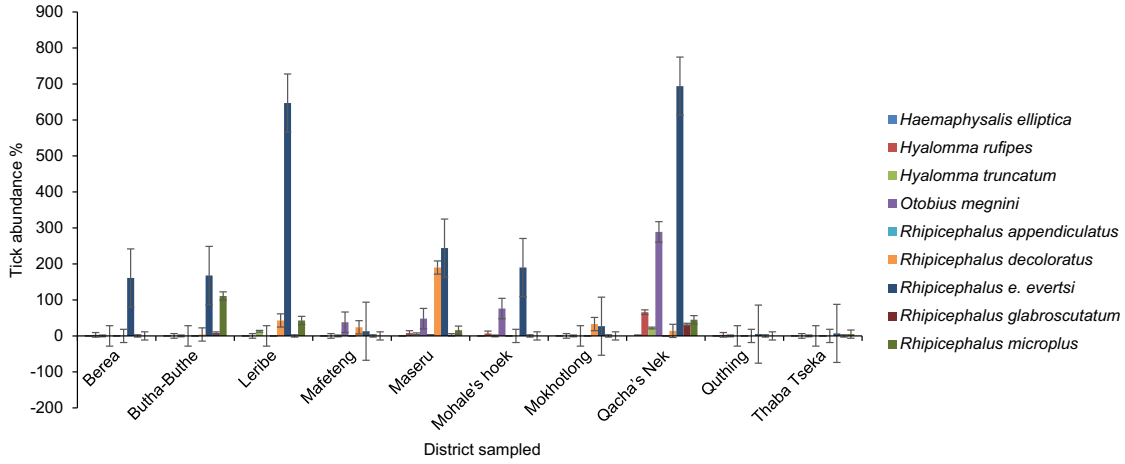

Fig. 2. Prevalence of different tick species from ten sampled districts in Lesotho.

(2017) study. However, no horses were infested with *R. decoloratus* our study. It was found to be common tick infesting cattle and the distribution of *R. decoloratus* confirms its host preference for large ungulates, its prevalence suggests that it is well established in Lesotho.

In this study, 6.6% of the overall occurrence of *R. microplus* was from Butha-Buthe, Leribe, Maseru, Qacha's Nek, and Thaba Tseka districts. Butha-Buthe had a significantly high species abundance at 52.7% (Tables 3 and 4). Tick infestation was more prevalent on cattle with 14.4%, 8.2% in dogs, and 3.4% in goats. The remaining 1.4% of *R. microplus* specimens were not assigned to a host by the collectors, probably collected from cattle, as this species is absent in areas where no cattle occur (Spickett et al., 2011). The seasonality for both *R. microplus* and *R. decoloratus* is similar, with high peak numbers occurring during the autumn months of April and May.

R. microplus and *R. decoloratus* are one-host ticks transmitting *Baconia bigemina* (African babesiosis) (Walker et al., 2003; Horak et al., 2017). *R. microplus* also transmits *Bartonella bovis*, the causative agent of bovine babesiosis (Spickett et al., 2011; Horak et al., 2017). *Babesia bovis* is virulent and can cause severe Asiatic red water and mortality to cattle transported into new areas already infected with the pathogen especially susceptible resident animals (Horak et al., 2015).

In this study *R. e. evertsi* was the most abundant species collected in all ten districts sampled with Qacha's Nek having the highest abundance at 32.2%. The overall occurrence of *R. e. evertsi* was at 65.1% from all species collected. Tick infestation was more prevalent on donkeys with 100%, followed by ticks collected from goats at 95.7%, vegetation (92.2%), sheep (87.5%), horse (85.3%) and 60.6% were from unaccounted host whilst 43% was from cattle and the least infestation was observed on dogs at 6.8% prevalence. *Rhipicephalus e. evertsi* distribution was common throughout the districts that were covered (Table 4), these findings of the current study align well with Horak et al. (2017) findings, in the author's survey *R. e. evertsi* was common in all nine provinces surveyed in SA. *Rhipicephalus e. evertsi* transmits *B. caballi* and *T. equi* to horsed and are causative agents of equine piroplasmosis. It also transmits other pathogens such as *T. separata*, a causative agent of ovine theileriosis, *A. marginale*, a causative agent of anaplasmosis cattle (Jansen and Neitz, 1956; Spickett et al., 2011; Madder et al., 2013).

R. glabroscutatum is a two-host tick commonly known as the smooth brown tick and a parasite of small stock including cattle, sheep and goats, and other wild ungulates. All developmental stages of this tick prefer ungulates as hosts (Golezardy and Horak, 2007). Preferential attachment sites are lower between legs and around hooves (Walker

Plate I: *Haemaphysalis elliptica*

Plate II: *Hyalomma rufipes*

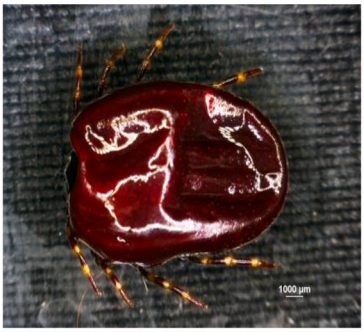

Plate III: *Hyalomma truncatum*

Plate IV: *Otobius megnini*

Plate V: *Rhipicephalus appendiculatus*

Plate VI: *Rhipicephalus decoloratus*

Plate VII: *Rhipicephalus e. evertsi*

Plate VIII: *Rhipicephalus glabroscutatum*

Plate IX: *Rhipicephalus microplus*

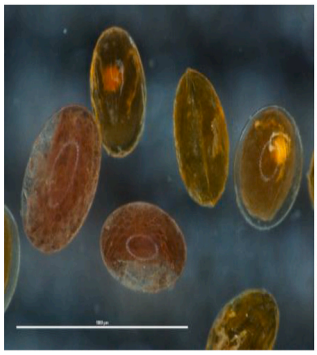

Plate X: Eggs

Plate XI: Eggs developing into larva

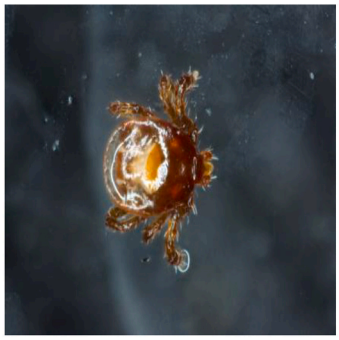

Plate XII: Larvae

Plates I–XII. Collected tick species, eggs and developmental stage of larvae morphologically identified from the ten districts in Lesotho.

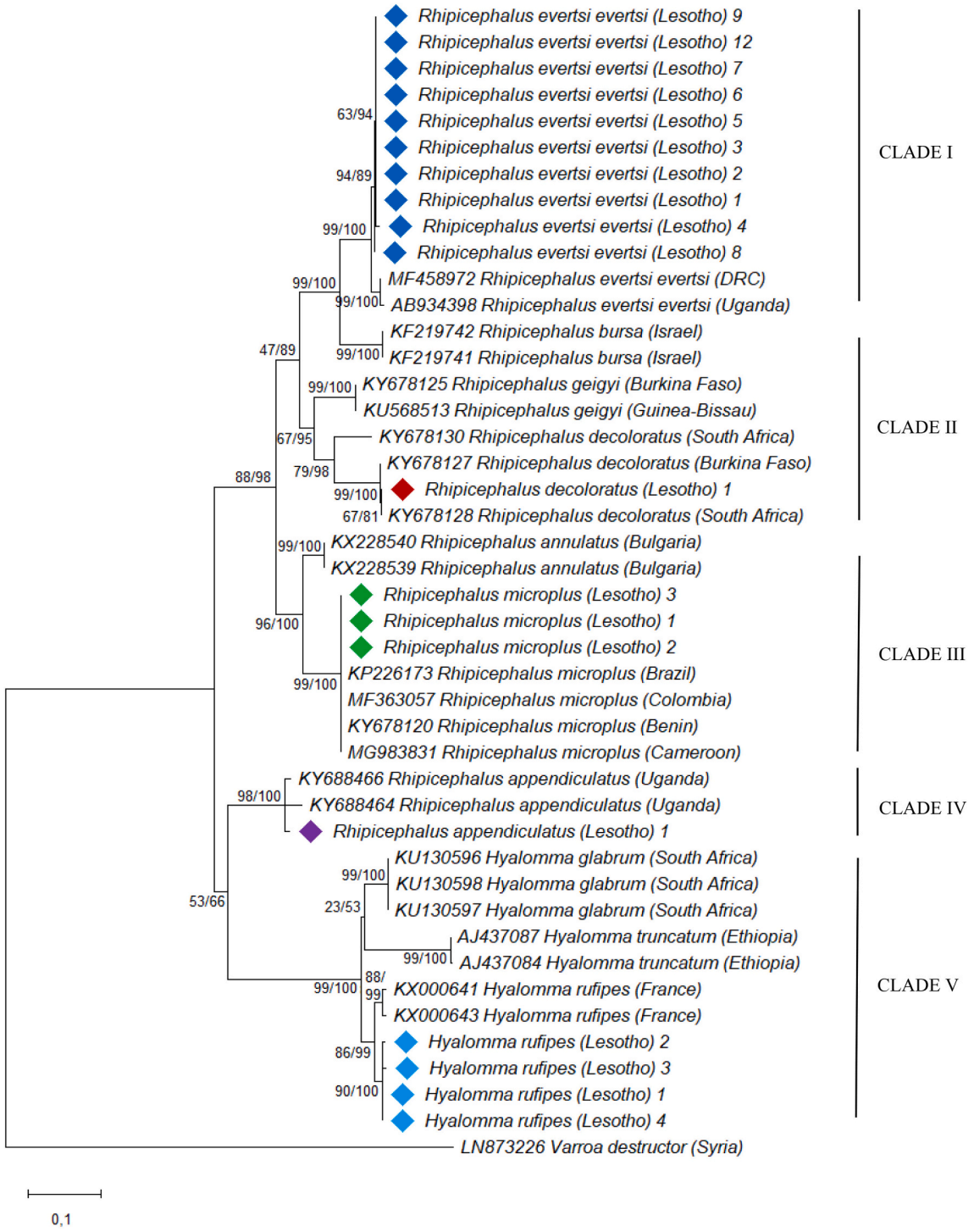

Fig. 3. Maximum likelihood tree inferred from CO1 sequences from the genera *Hyalomma* and *Rhipicephalus*. Sequence data generated in the present study are marked. Support values (Bootstrapping and Bayesian inference test values) are indicated at each node. Scale bar indicates the number of nucleotide substitutions per position.

et al., 2003). Adult ticks are associated with the occurrence of foot and lameness in goats(Golezardy, 2006). In this study, *R. glabroscutatum* was collected in three districts namely Butha-Buthe, Maseru, and Qacha's Nek with 1.3% overall occurrence whereby Qacha's Nek had the highest tick abundance at 74.4%, followed by Butha-Buthe at 18.6% and the least was Maseru at 7%. The highest infestation was observed on dogs with 43.8% and cattle had the least infestation prevalence of 0.8%. The results confirm that *R. glabroscutatum* is a common parasite of domestic animals. Rechav and Knight (1981) collect *R. glabroscutatum* in SA from cattle and goats in lower numbers, this was also observed in this study. *R. glabroscutatum* was not collected from goats in this study. Interestingly, the tick was detected from dogs at higher numbers compared to other hosts. Thus far, there is no documentation on possible pathogens transmitted by *R. glabroscutatum*. No recent studies done in SA have been able to collect this species. Therefore, more robust studies need to be done in understanding the occurrence and distribution and adaptation of this tick species. Also, to improve the morphological illustrations in identifying this tick.

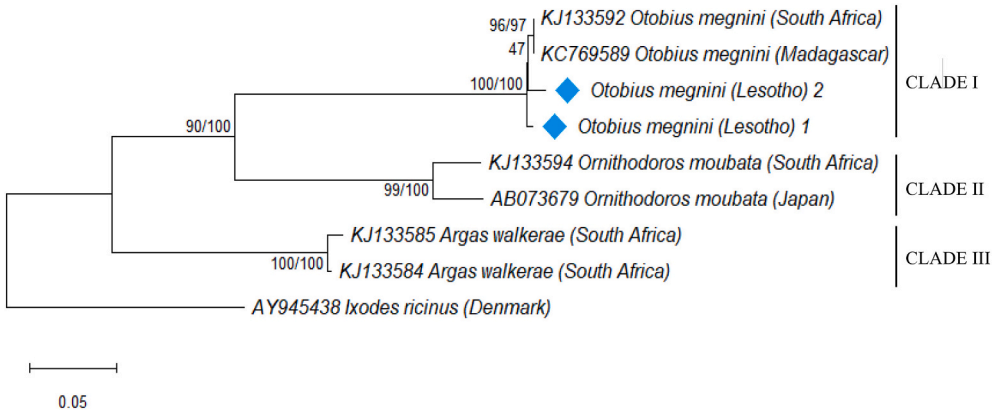

Fig. 4. Maximum likelihood tree inferred from CO1 sequences from the genera *Otobius, Ornithodoros,* and *Argas* species. Sequence data generated in the present study are marked. Support values (Bootstrapping and Bayesian inference test values) are indicated at each node of the branch.

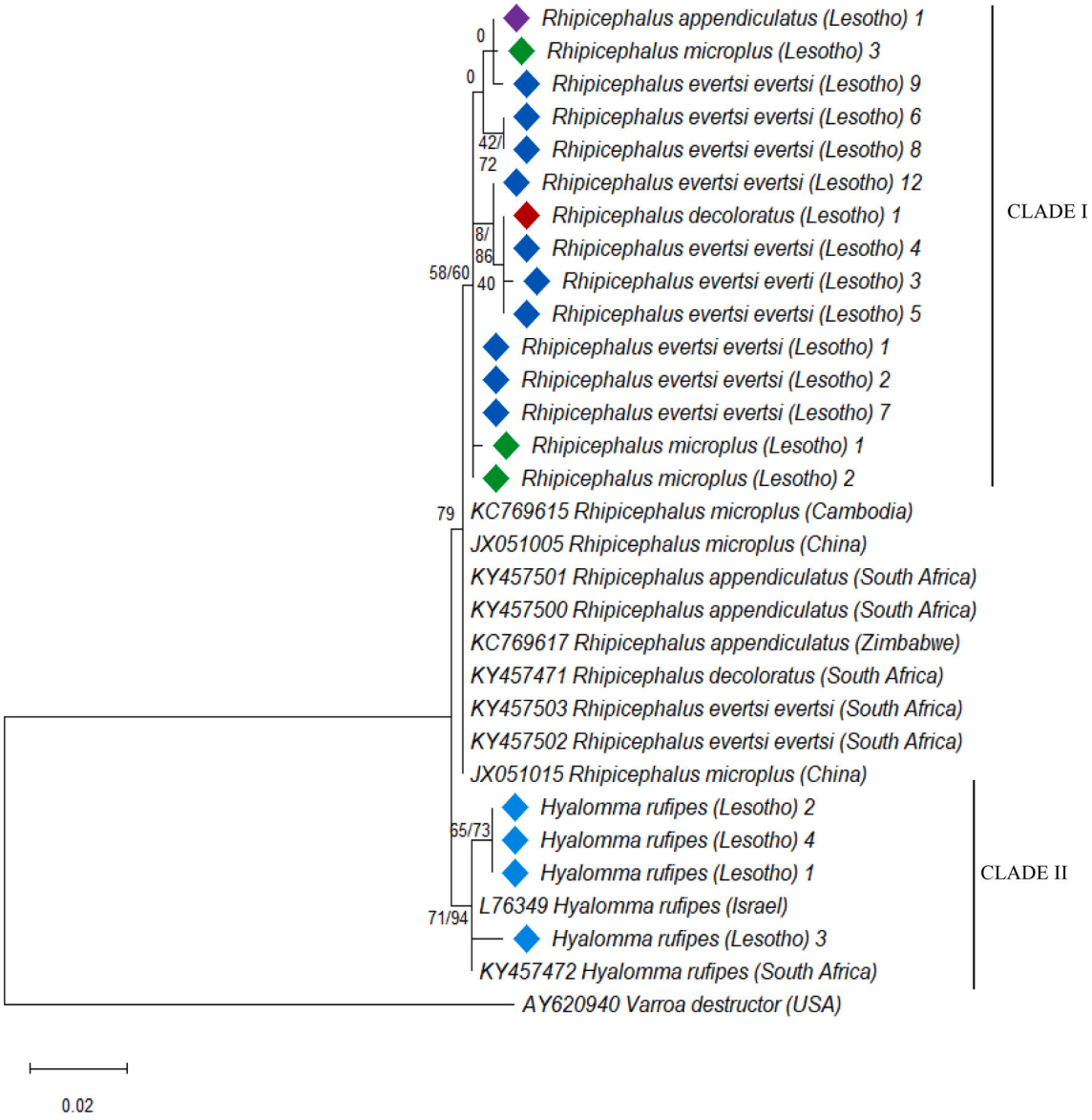

Fig. 5. Maximum likelihood tree inferred from 18S rRNA sequences from the genera Hyalomma and Rhipicephalus species. Sequence data generated in the present study are marked. Support values (Bootstrapping and Bayesian inference test values) were indicated at each node. The tree was rooted using *Varroa destructor* as an outgroup.

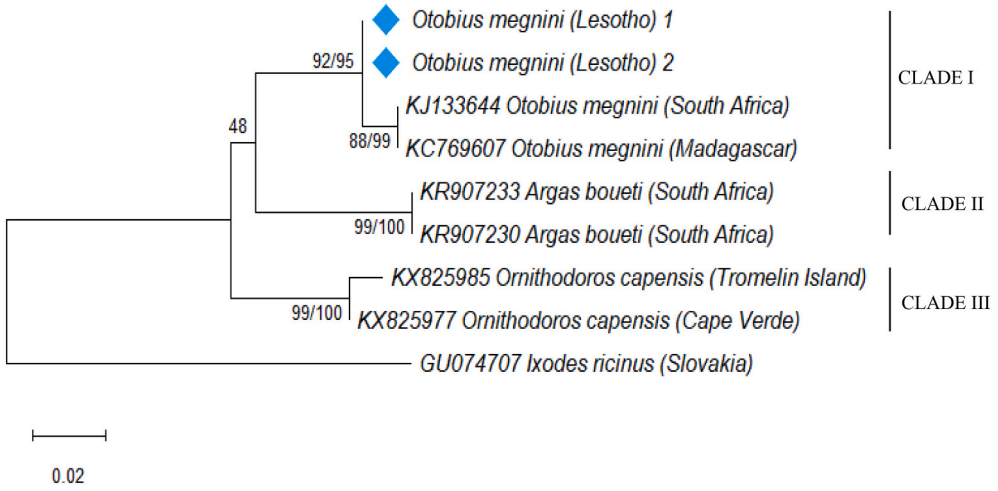

Fig. 6. Maximum likelihood tree inferred from 18S rRNA sequences from the genera *Otobius*, *Argas* and *Ornithodoros* species. Sequence data generated in the present study are marked. Support values (Bootstrapping and Bayesian inference test values) were indicated at each node. The tree was again rooted with *Ixodes ricinus* as an outgroup.

Tick identification is vital for epidemiological studies, establishing distribution maps for tick species and characterization of tick fauna. Seasonal patterns determine the trophic preference and tick species response to pathogens transmission to vertebrates (Kumsa et al., 2016). To understand the tick diversity and activity patterns, life cycle and developmental stages reveal insight into the ecology of tick species (Golezardy et al., 2016). Additionally, climates and vegetation are factors that determine the geographic distribution range of the ixodid and argasid ticks and possibly predict the diversity of the ticks within regions. Diverse tick population density can indicate the capacity of species to survive limited environmental conditions in various seasons of the year, as tick species respond remarkably to temperatures and humidity (Golezardy et al., 2016).

Notably, in the current study some ixodid ticks were collected in lower numbers than others, while some species occurring in SA [Free State (Horak et al., 2015), Eastern Cape (Horak et al., 2017) and KwaZulu-Natal (Horak et al., 2017) provinces that shared border with Lesotho] were not collected throughout this study survey; suggesting that the microclimatic conditions play a crucial role in the dispersal of tick species. Signifying that genetic diversity strains within the same species resulting in variation of a climatic zone (Baron et al., 2018), leading to a distinct population as observed in species collected in this study to those collected previously in SA. Such findings can infer to the inconsistency of vaccines used across different geographic areas.

The phylogeny of ticks observed in this study supports the monophyly in both Ornithodorinae and Rhipicephalinae subfamilies. Similar observations have been reported previously where morphological analysis derived from the external characters as well as molecular techniques targeting mitochondrial and nuclear genes supported the monophyly in Argasidae and Ixodidae families (Csordas et al., 2016; Li et al., 2018; Rivera-Páez et al., 2018; Roy et al., 2018; Yawa et al., 2019). Specimens identified in this study are considered to be the most economically important ticks infesting livestock from a global perspective and the control of these tick species continues to be severely hampered by the occurrence of acaricides resistance. According to Roy et al. (2018), reliability and differentiation of the *R. microplus* species are complex therefore it is crucial and essential to monitor the spread of these ticks and the resistant populations.

Sequenced PCR amplicons of both CO1 and 18S rRNA genes of *O. megnini* (Lesotho) accordingly matched with other *O. megnini* sequences available in the GenBank database with 99% identity which confirmed that the morphological identification was correct. Tree topologies of ML and BI analysis constructed with both markers showed three major clades that support monophyly with the *Otobius* genus. The overall p-distance matrix indicated strong resolution power (0.16%) and standard error (0.01%) for the CO1 marker and (0.07%) and standard error (0.00%) for the 18S rRNA marker. The majority of the population pair shows low levels of genetic difference found among the *O. megnini* tick species used in both markers for the ML tree of this study. The findings of this study indicate the advantage of using the CO1 gene as it can resolve evolutionary relationships (Low et al., 2015) of *O. megnini* with other soft tick species. In comparison to other reference sequences, the reading frame of *O. megnini* revealed a more conserved region among different genera. Variable regions were observed more at the second-position nucleotide which is the intermediate level resolution for the family and genus (Folmer et al., 1994). From this study, it is clear that the genus level is highly retained.

The 18S rRNA marker's phylogenetic relationship between the hard tick samples, along with the additional GenBank Accession entries showed a lack of resolution for the *Rhipicephalus* genus complex as suggested in previous studies (Burger et al., 2012; Burger et al., 2013). There was no clear separation of the species complex into its respective clades, the phylogenetic analysis showed that the clades were related to a specific genus and not species. From the *Rhipicephalus* genus, the reading frame in comparison to referenced sequences revealed variations of nucleotides in all position, showing less conserved regions and the first and third-position, suggesting that the 18S rRNA marker is not highly informative in resolving species taxonomy (Low et al., 2015). The use of 16/18S rRNA markers as a tool in evolution inferring to other tick species is less certain (Roux et al., 1997; Papa et al., 2017). According to Li et al. (2018), the CO1 marker gives sufficient resolution to define closely related and cryptic species, while ribosomal markers are useful in resolving taxonomy. Recent studies on the 18S rRNA strongly support the monophyly of most tick genera, with exception to closely related genera such as *Rhipicephalus* and *Hyalomma*. Nonetheless, 18S rRNA alone does not have sufficient phylogenetic information resolving relationships among Metastriata complex, this marker is mostly significant in only resolve relationships among higher taxa (Burger et al., 2014). The 18S rRNA gene shows conserved regions at the genera level, in this study the ML analysis provided significant topology resolution at the genera level (Lv et al., 2014).

Rhipicephalus species formed a distinctive branch and *Hyalomma* species formed a branch on their own. In most phylogenetic analyses of tick species and other closely related species when using the gene, there are normally uncertainties with the position of most species due to weak support observed from an internal relationship (Burger et al., 2014).

Table 4
Domestic animals that were infested by the tick species collected from ten districts in Lesotho.

Lesotho districts	Studied animals	Tick species identified								
		H. elliptica	*H. rufipes*	*H. truncatum*	*O. megnini*	*R. appendiculatus*	*Rhipicephalus decoloratus*	*Rhipicephalus microplus*	*R. e. evertsi*	*Rhipicephalus glabroscutatum*
Berea (*N* = 164)	Goats	*-	–	–	–	–	–	–	67	–
	Sheep	–	3	–	–	–	–	–	94	–
Butha-Buthe (*N* = 291)	Cattle	–	–	–	–	–	3	95	151	8
	Goats	–	–	–	–	–	–	16	2	–
	Sheep	–	–	–	–	–	–	–	12	–
	Unknown host	–	–	–	–	–	1	–	3	–
Leribe (*N* = 746)	Cattle	–	–	4	–	–	42	40	91	–
	Dogs	–	–	–	–	–	–	3	–	–
	Goats	–	–	–	–	–	–	–	219	–
	Horses	–	–	–	–	–	–	–	3	–
	Sheep	–	–	9	–	–	1	–	325	–
	Unknown host	–	–	–	–	–	–	–	9	–
Mafeteng (*N* = 75)	Cattle	–	–	–	38	–	24	–	13	–
Maseru (*N* = 518)	Cattle	–	2	1	35	–	174	14	104	3
	Vegetation	–	–	–	–	3	2	–	59	–
	Unknown host	–	6	5	13	–	14	2	81	–
Mohale's hoek (*N* = 273)	Cattle	–	4	–	35	–	–	–	58	–
	Donkeys	–	–	–	–	–	–	–	12	–
	Goats	–	–	–	2	–	–	–	13	–
	Horses	–	3	–	10	–	–	–	17	–
	Sheep	–	–	–	29	–	–	–	90	–
Mokhotlong (*N* = 60)	Cattle	–	–	–	–	–	33	–	10	–
	Goats	–	–	–	–	–	–	–	7	–
	Sheep	–	–	–	–	–	–	–	10	–
Qacha's Nek (*N* = 1164)	Cattle	–	19	4	134	–	–	37	137	–
	Dogs	2	–	4	24	–	–	3	5	32
	Goats	–	1	–	1	–	–	–	139	–
	Horses	–	–	2	1	–	–	–	85	–
	Sheep	–	–	–	51	–	–	–	119	–
	Unknown host	–	46	12	78	–	14	5	209	–
Quthing (*N* = 8)	Horses	–	3	–	–	–	–	–	5	–
Thaba Tseka (*N* = 12)	Cattle	–	–	–	–	–	–	5	4	–
	Goats	–	–	–	–	–	–	–	3	–

* = not collected.

Furthermore, *Hy. rufipes* and *Rhipicephalus* spp. exhibited only 99% sequence similarities with other members of the genera and the ML and BI methods revealed 58% and 71%, respectively, nodal bootstrap values among *Rhipicephalus* and *Hyalomma* clades. The overall mean distance revealed a strong resolution power of 0.02% and a standard error (0.01%) for the CO1 marker and 0.02% p-distance matrix and standard error (0.01%) for the 18S rRNA marker. The *Rhipicephalus* spp. clustered well with other genetically related tick species of the same genus in ML tree. The lack of resolution to species level when using this marker is considered as a limitation in the molecular approach for tick species identification, this has been observed in the ribosomal marker (12S rRNA) (Kumsa et al., 2016).

However, for the CO1 marker, there was a monophyly relationship among hard tick species and the topology was well resolved. The topology of the tree inferred by different methods (ML and BI) with different building strategies and distance models was similar, with a small difference in bootstrap values. The phylogenetic tree revealed that the *Rhipicephalus* species clustered together and the *Hyalomma* species was a sister to the cluster consisting of *R. appendiculatus*. Similar findings have been observed in molecular techniques targeting mitochondrial (CO1) and ribosomal (16S rRNA) genes also supported monophyly in hard tick species (Low et al., 2015; Chitimia-Dobler et al., 2016; Rivera-Páez et al., 2018).

Phylogenetic analysis was performed for the mitochondrial CO1 gene using hard tick samples from Lesotho, as well as GenBank Accession entries reported in other studies. See Supplementary Table S2. This was done to decipher the clade allocation of ticks from Lesotho, as well as the relationship between *Rhipicephalus* and *Hyalomma* species. The results revealed that from the Metastriata complex of ticks grouped into five distinctive clades, along with ticks from Uganda, DRC, SA, Burkina–Faso, France, Cameroon, Colombia, Madagascar, and Brazil. It was observed that *R. e. evertsi* (Lesotho) formed its own sister clade and inferred to be closely related to *R. e. evertsi* from DRC, while *R. decoloratus* (Lesotho) inferred to be related to *R. decoloratus* from SA. *R. microplus* (Lesotho) was closely related to species from Brazil and lastly, *R. appendiculatus* (Lesotho) was closely related to the specimen from Uganda. In the *Hyalomma* genus, *Hy. rufipes* was more closely related to species from France. The overall mean distance matrix indicated a strong resolution power (0.13%) and standard error (0.01%).

The cytochrome *c* oxidase 1 (CO1) gene is mostly preferred barcoding marker for animals, while the 18S rRNA gene is too conserved for tick diagnostics at the species level but, useful in identifying tick species at higher taxonomic level. See Supplementary Fig. S1(Lv et al., 2014). Based on the author's schematic diagram it is evident that both markers used in this study are significant for the identification of tick species. Interestingly, for the soft tick species, the 18S rRNA gene strongly supports the transfer of the sub-genus *Ornithodoros* to the genus *Argas* and strong support of *O. megnini* placement as a sister group to *Ornithodoros* (Burger et al., 2014). These findings were similar for this study and there was no weak support observed in the phylogeny of soft ticks with the

placement of *O. megnini* using 18S rRNA, as previously observed by other studies (Burger et al., 2014).

The phylogenetic analysis of hard ticks showed that *R. appendiculatus*, *R. decoloratus*, *R. e. evertsi*, *R. microplus,* and *Hy. rufipes* species from the study clustered well with other *Rhipicephalus* and *Hyalomma* species from other countries. From the CO1 marker, most sequences showed variation at the third–position nucleotide, and regions were conserved throughout the species. These third-region substitutions are saturated at a higher level and still retain information (Folmer et al., 1994). This suggests that phylogenetic resolutions from the CO1 gene can be up to the species level. The phylogenetic relationship between the studied ticks was observed with other populations from other countries. As for the molecular approach, these markers have invariably unravelled the distinct genetic assemblies of Lesotho ticks for both Ornithodorinae and Metastriata complexes.

Credit authorship contribution statement

SICM conducted laboratory experiments and made the first draft of the manuscript. OTZ and OMMT conceptualised, supervised, and revised the manuscript. LB, MM, MJRM, GRM collected tick samples, and LB, MM, MSM revised the manuscript. DB identified the ticks and revised the manuscript.

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Ethical statement

Animal studies have been approved by the appropriate ethics committee of the University of KwaZulu- Natal (Reference: AREC/040/016M) therefore, they have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank animal owners for their cooperation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2022.100691.

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