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Characterisation of the genetic diversity of the southern cattle tick, *Rhipicephalus microplus*, populations from South Africa

by

Tanzelle Oberholster

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MAGISTER SCIENTIAE (Genetics)

in the

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Dissertation Summary

Rhipicephalus microplus belongs to the Ixodidae, the largest family of ticks, which are of great economic importance due to their negative socio-economic impact on agriculture (BROUQUI 2011; PORTO NETOA *et al.* 2011; SONENSHINE 1991). Acaricides have been the first choice in tick control for cattle farmers, but *R. microplus* rapidly acquires resistance to these chemicals. Replication slippage and recombination drive genetic diversity in tick populations (BAFFI *et al.* 2007; GUERRERO *et al.* 2007; LI *et al.* 2007); generating point mutations and frame shifts within the genes targeted by acaricides, resulting in resistance (BAFFI *et al.* 2007; HERNANDEZ *et al.* 2002; HERNANDEZ *et al.* 2000; JONSSON *et al.* 2010; MORGAN *et al.* 2009). In addition, resistance can quickly accumulate in a population due to the pangamy mating structure of ticks (CHEVILLON *et al.* 2007b; CUTULLÉ *et al.* 2010) and their ability to produce multiple generations within one season (BUDELI *et al.* 2009; LI *et al.* 2007).

Vaccines have become increasingly important to control ticks, as acaricide resistance can be acquired by field tick populations within two years (RODRIGUEZ-VIVAS *et al.* 2011). Although Bm86 has been successful against multiple-acaricide resistant ticks, recent reports indicate that the Bm86 vaccine has become ineffective, possibly due to resistance (PARIZI *et al.* 2009). Also, Bm86 vaccines display great variability in terms of their efficacy against ticks isolated across Argentina (GARCIA-GARCIA *et al.* 2000; PARIZI *et al.* 2009). This is hypothesised to be due to the genetic variability between *R. micoplus* populations.

The majority of phylogenetic studies on ticks have been based on slow evolving sequences, such as *18S* or *28S rRNA*, which provide genus-level resolution. The *COI*, *D3*, *ANT* and *ITS2* genes have the potential to resolve intra-specific and interspecies variation, and may assist with the identification of cryptic speciation within *R. microplus* of South Africa (ANSTEAD *et al.* 2011; BARKER 1998; CAREW *et al.* 2009; MURRELL *et al.* 2000; SONGA *et al.* 2011). Phylogeography is a multidisciplinary field that utilises phylogenetic (molecular evidence of speciation) and population genetic principles (coalescence theory), in combination with additional data (such as geography and population history), to determine the genetic relationships of populations within a species (AVISE 2009) and was one of the main aspects of this study. The phylogenetic and population genetic structure of *R. microplus* will provide valuable information to geneticists, farmers and acaricide/vaccine suppliers about the different *R. microplus* tick populations of South Africa. The information will facilitate more efficient and targeted tick control whether acaricide or vaccine based as opposed to the inefficient approaches generally adopted to tick control.

Keywords: *Rhipicephalus microplus*, genetic diversity, phylogenetics, phylogeography, geographical distribution, acaricide resistance.

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Declaration

I, Tanzelle Oberholster, declare that the dissertation entitled "Characterisation of the genetic diversity of the southern cattle tick, *Rhipicephalus microplus*, populations from South Africa", which I hereby submit for the degree Magister Scientiae Genetics at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution. Where secondary material is used, this has been carefully acknowledged and referenced in accordance with university requirements. I am aware of university policy and implications regarding plagiarism.

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Dissertation outline

Chapter 1: Literature Review

This chapter provides a comprehensive overview of *Rhipicephalus microplus* and the factors that likely contribute to its genetic diversity. The scope of the literature review includes broad topics pertaining to *R. microplus* of which not all are applicable to the research Chapter 2, but for the discussions in Chapter 3. The sections applicable to Chapter 2 include: Population structure, subdivision and gene flow (section 1); Overview of ticks and their importance in parasitology (section 2); Biology and systematics of the southern cattle tick, *Rhipicephalus microplus* (sections 3.1, 3.3-3.6, 3.8-3.11) as well as; Molecular Biology and genetics of the southern cattle tick, *Rhipicephalus microplus* (sections 4.1-4.5, 4.7-4.11). It outlines the study research questions, aims, hypotheses and objectives.

Chapter 2: Genetic evaluation of *Rhipicephalus microplus* with nuclear and mitochondrial markers

This chapter represents the research chapter of this study. It is written as a manuscript according to the guide to the authors of the journal Ticks and Tick-borne Diseases, where figures are provided at the end of the text and a single reference list is provided at the end of the dissertation. Chapter 2 has been combined with information from Chapter 3 and the Appendix into a concept article in fulfilment of the requirements set by the University of Pretoria. Additional information is provided in the Appendix (provided electronically on a CD), which contains its own table of contents, lists of figures and tables as well as references.

Chapter 3: Concluding discussion

This chapter aims to interpret the results of Chapter 2 and evaluate their implications. It serves to discuss additional future studies and to bring resolution to the dissertation.

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Abbreviations

28S rRNA	Large nuclear ribosomal subunit
18S rRNA	Small nuclear ribosomal subunit
5.8S rRNA	Large nuclear ribosomal subunit
5S rRNA	Large nuclear ribosomal subunit
16S rRNA	Large mitochondrial ribosomal subunit
12S rRNA	Small mitochondrial ribosomal subunit
ABI	Applied Biosystems (now Life Technologies)
ANT	Adenine nucleotide translocase or ADP/ATP translocase
BLAST	Basic local alignment search tool
bn	Billion
bp	Base pair
CO	Cytochrome oxidase genes
COI	Cytochrome oxidase c subunit I
COII	Cytochrome oxidase c subunit II
COIII	Cytochrome oxidase c subunit III
D3	Divergent region 3
DNA	Dioxyribonucleic acid
EDTA	Ethanol diamine tetra-acetic acid
EST	Expressed sequence tag
EtOH	Ethanol
gDNA	Genomic DNA
GTR	General time reversible
ITS	Internal transcribed spacer region
MCMC	Markov chain Monte Carlo
$MgCl_2$	Magnesium chloride
ML	Maximum Likelihood
MP	Maximum Parsimony
mt	Mitochondrial
Mya	Million years ago
NaCl	Sodium chloride
NJ	Neighbour-Joining
nt	Nucleotide
nu	Nuclear
PCR	Polymerase Chain Reaction
R. ann	*Rhipicephalus annulatus*
R. app	*Rhipicephalus appendiculatus*
R. aus	*Rhipicephalus australis*
R. dec	*Rhipicephalus decoloratus*
R. gei	*Rhipicephalus geigyi*
R. mic	*Rhipicephalus microplus*
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SSR	Simple sequence repeat
STR	Short tandem repeat
T92	Tamura-3-parameter
TE	Tris EDTA
Tris	Tris (hydroxyl methyl) amino methane

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Chapter 1: Literature Review

1. Population structure, subdivision and gene flow

"A population is a local, geographically defined, group of conspecific organisms sharing a common gene pool" (KING and STANSFIELD 2002) and/or "A group of organisms of the same species occupying a particular space at a particular time" (KREBS 1994; WAPLES and GAGGIOTII 2006). Partial or complete geographical isolation (subdivision) may lead to divergence among populations, whereas gene flow homogenises populations (HALLIBURTON 2004). The interaction between subdivision and gene flow results in population structure, where a species range is described by local breeding groups (KING and STANSFIELD 2002), each with different alleles or allelic frequencies. Additionally population structure is influenced by non-random mating, selection, drift and mutation (HALLIBURTON 2004), but for simplicity, subdivision and gene flow are regarded as the major contributing factors to describe population structure.

Population structure can be thought of as a continuum, ranging from panmixia to highly structured populations (Figure 1). In the case of panmixia, a species tends to function as a single population of constant size and the individuals within it mate randomly (WAPLES and GAGGIOTII 2006). In contrast, gene flow between groups of individuals becomes restricted and populations become genetically distinct over space and/or time (WAPLES and GAGGIOTII 2006). Intermediate levels of structuring are governed by a complex system of interacting evolutionary forces (WAPLES and GAGGIOTII 2006). Examples of panmixia and its transition to subdivision through gene flow are illustrated in Figure 1 (A-D).

Gene flow is the movement of individuals (or gametes) from one population to another and their subsequent breeding (HALLIBURTON 2004). The exchange of genes between populations of the same species is produced by migrants (KING and STANSFIELD 2002); either in one direction only (from population A to population B: A→B) or in both directions (A↔B). There are several models to explain the structure of populations based on the amount and direction of gene flow between the populations, (see Figure 1 E-H).

1.1 The continental-island model

In this model, one-way gene flow occurs from a single large population (the continent or mainland) to single/multiple smaller population(s) (the island(s)) (HALLIBURTON 2004; HEY and MACHADO 2003).The equilibrium allele frequency of the island will be equal to the number of migrants from the mainland (the amount of gene flow) (HALLIBURTON 2004). Alleles would have originated from one or more

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generations in the mainland population's past if the island had received immigrants from the mainland every generation (HALLIBURTON 2004). This model effectively explains the effects of gene flow in captive breeding programs or river re-stockings with hatchery fish (HALLIBURTON 2004). The continental-island model is illustrated by Figure 1E.

1.2 The island model

The island model, describes populations that exchange immigrants, where gene flow occurs in multiple directions between the islands and at random intervals. The number of islands, the number of immigrants and the rate of gene flow results in different structures of the final population (HEY and MACHADO 2003). The island model can be applied to natural island systems (Hawaiian Islands) or 'habitat islands', where organisms are separated by habitat fragmentation (HALLIBURTON 2004). Therefore each island's allele frequency is dependent on the gene flow from another island. All the islands will have different allele frequencies. The mean allele frequency of the islands will approach the average allele frequency among all the islands, if all the other islands are regarded as "the continent" in relation to a single island. The island model is illustrated by Figure 1F.

1.3 Stepping stone models and isolation by distance

This model can have one or two-dimensional gene flow and incorporates space (geography) into the model (HALLIBURTON 2004; HEY and MACHADO 2003). In the one dimensional model, several populations are adjacent to one another and gene flow only occurs in one direction between the adjacent populations, of a discontinuous geographical range (HALLIBURTON 2004). Thus populations closer together (or in the middle) will be more similar than populations further apart (or on opposite sides), illustrated in Figure 1G. Two-dimensional gene flow occurs when populations are distributed over a continuous geographical range, but the likelihood of gene flow between populations decreases as distance increases (HALLIBURTON 2004), illustrated by Figure 1H. The same principle applies as for one-dimensional gene flow; populations closer together will be more genetically similar. This illustrates the principle of isolation by distance (HALLIBURTON 2004). The genetic divergence of populations depends on the distance between them, the number of migrants and the ability of organisms to disperse (e.g. birds will disperse more widely and more effectively than mammals).

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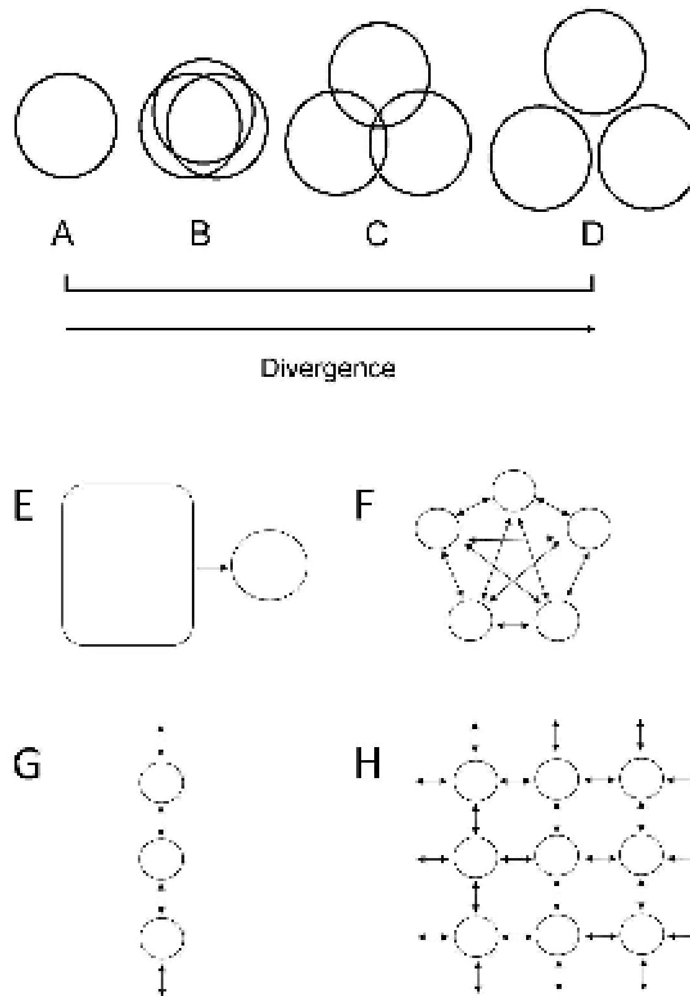

Figure 1: Models to describe population structure based on gene flow. Circles represent groups of subpopulations with varying degrees of connectivity (either geographically or through gene flow). 1A: Panmixia, where subpopulations are homogeneous. **1B:** Substantial connectivity. **1C:** Modest connectivity. **1D:** Complete independence, where subpopulations are separate. **1E:** Continent-island model, unidirectional gene flow from the continent to the island. **1F:** Island model, multi-directional gene flow at random intervals between islands. **1G:** One-dimensional stepping stone model between adjacent populations. **1H:** Two-dimensional stepping stone model between continuously distributed populations arranged in two dimensions. Modified from Hey and Machado (2003), figures A-D, and Halliburton (2004), figures E-H.

The metapopulation model is related to the island models of gene flow. A metapopulation is a "set of local populations which interact via individuals moving among populations". The model acts on different scales of the population, which are continuous and hierarchical, such as local (between individuals) to geographical (over a species range) (HANSKI 1991). Extinction and (re)colonisation events are an integral part of a metapopulation, due to the isolation of populations across the species range (HANSKI 1991; HEY and MACHADO 2003). This suggests that individual organisms do not only move between populations, but individual populations can also move over space and time (HANSKI 1991; HEY and MACHADO 2003). The change in the local population over the geographical range constitutes its population structure and the survival of the species depends on the number of combined local populations of organisms.

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Therefore, gene flow tends to homogenise populations, whereas subdivision (or isolation by distance) leads to genetic differentiation (HALLIBURTON 2004). The models describing population structure makes several assumptions; first they assume that populations are infinitely large (HALLIBURTON 2004), secondly that no other evolutionary forces act on the genes under study (HALLIBURTON 2004) and lastly that populations have reached an equilibrium state with no change in the genetic variation within and between subpopulations (HEY and MACHADO 2003). Therefore natural populations may display slight differences to the modelled population structures, especially if the dispersal of the organisms is dependent on another organism, such as the dependence of parasites on a host, which will influence the geographical spread and gene flow of parasite populations.

Parasites are evolutionary complex organisms, due to their host-dependent lifestyle, which involves an interacting system of multiple factors associated with the host, the parasite itself and the environment (BARRETT *et al.* 2008; CRISCIONE *et al.* 2005; MCCOY 2008). These contribute to parasite survival, diversity and spread, and include the affects of host movements, host availability and the interactions with other parasites on the host (BARRETT *et al.* 2008; CRISCIONE *et al.* 2005; MCCOY 2008). The population structure of the parasites as vectors in turn effect the population structure and spread of parasite associated disease, such as the transmission of tick-borne diseases by ticks. This may result in the increase of disease occurrence and spread, which will have implications for agriculturally important host species. Therefore, in order to elucidate the underlying evolutionary mechanisms of tick populations; knowledge about tick life history, demographics, hosts and associated pathogens are required (BARRETT *et al.* 2008; MCCOY 2008).

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2. Overview of ticks and their importance in parasitology

Ticks are obligate haematophagous parasites, able to feed on mammals, birds, reptiles and amphibians (GUZINSKI *et al.* 2008; SCHUMAKER and BARROS 1994; SONENSHINE 1991). Ticks are ranked second to mosquitoes as vectors for debilitating or lethal zoonotic diseases of animals and humans (SONENSHINE 1991). Therefore, tick control and research is important for the economy, society, medicinal and veterinary industries, due to the livestock losses caused by tick feeding and their associated disease, which have been implicated in zoonosis.

Ticks are broadly classified into; Ixodidae (hard) and Argasidae (soft) ticks (WALKER *et al.* 2003). They are further categorised by the number of hosts that are fed upon during their life cycle. Three and two host ticks have several intermediate hosts upon which they feed before maturing and feeding upon the definitive host (WALKER *et al.* 2003). Within the *Rhipicephalus* genus; three host ticks include *R. appendiculatus* and two host ticks include *R. evertsi evertsi*, both of which parasitise cattle as their final host (OBEREM *et al.* 2006). One host ticks will feed, moult and mature on a single host animal, such as *R. microplus*, the southern cattle tick (OBEREM *et al.* 2006). All three of the abovementioned ticks are vectors of different tick-borne disease pathogens, which infect cattle and will result in the death of cattle if left untreated. Table 1 lists the important ticks and their associated tick-borne diseases, which have an impact on the South African livestock industry.

Table 1: Important livestock-associated ticks found in South Africa, with large geographical distributions, and their associated tick-borne diseases (CENTERS FOR DISEASE CONTROL & PREVENTION ; OBEREM *et al.* 2006; WALKER *et al.* 2003).

Species	Common Name	Diseases or disorder	Symptoms in livestock
*Ornithodoros savygni**	Sand tampan	Sand tampan toxicosis	Cattle paralysis in young calves due to severe biting stress.
Rhipicephalus microplus	Southern cattle tick, Asiatic blue tick	*Babesia bovis* (Asiatic redwater)	Haemolytic anaemia, fever, jaundice, red urine, muscle tremors, convulsions and death. Confused with heartwater.
		Babesia bigemina (African redwater)	Haemolytic anaemia, fever, jaundice and red urine. Milder disease than Asiatic redwater.
		Borrelia theileri (Bovine spirochaetosis)	Lameness, swollen joints, stiffness, laminitis, abortions and fever.
		Anaplasma marginale (Gallsickness)	Anaemia, cardiovascular changes, haematuria, diarrhoea, anorexia and weight loss.
		Tick worry	Heavy tick infestation affects cattle appetite and condition.
Rhipicephalus decoloratus	African blue tick	*Babesia bigemina* (African redwater)	Haemolytic anaemia, fever, jaundice and red urine. Milder disease than Asiatic redwater.
		Anaplasma marginale (Gallsickness)	Anaemia, cardiovascular changes, haematuria, diarrhoea, anorexia and weight loss.
		Tick worry	Heavy tick infestation affects cattle appetite and condition.
Rhipicephalus evertsi evertsi	Red legged tick	Spring lamb paralysis	Paralysis of new born lambs during heavy infestation due to tick toxin.
		Anaplasma marginale (Gallsickness)	Anaemia, cardiovascular changes, haematuria, diarrhoea, anorexia and weight loss.

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Hyalomma trancatum	Bont legged tick	Sweating sickness	Tick toxicosis causes inflammation of the skin and mucous membranes (including organs), usually in calves. High temperature, loss of appetite, listlessness, wet dermatitis, leakage of serum from skin and depression.
Hyalomma marginatum rufipes	Bont legged tick	*Nairovirus*, Crimean-Congo haemorrhagic fever (CCHF)	High fever, red eyes, petechiae, jaundice, severe bruising and severe nosebleeds.
		Anaplasma marginale (Gallsickness)	Anaemia, cardiovascular changes, haematuria, diarrhoea, anorexia and weight loss.
		Babesia occultans (Babesiosis)	Haemolytic anaemia, fever, jaundice and red urine.
Rhipicephalus appendiculatus	Brown ear tick	*Theileria parva* strain adapted to cattle, East coast fever (ECF)	High fever, swollen lymph nodes, lung oedema, severe diarrhoea, abortions and death after 15 days.
		January disease	Acute blood loss, anaemia, reduced immunity and chronic fatal anaemia due to tick feeding.
		Theileria parva, buffalo-derived strain, Corridor's disease	Similar to ECF, but more acute with more than 80% cattle mortalities.
		Theileria taurotragi, bovine theileriosis	Benign bovine theileriosis.
		Anaplasma bovis, Bovine ehrlichiosis (Gallsickness)	Anaemia, cardiovascular changes, haematuria, diarrhoea, anorexia and weight loss.
Rhipicephalus simus	Glossy brown tick	*Anaplasma marginale* (Gallsickness)	Anaemia, cardiovascular changes, haematuria, diarrhoea, anorexia and weight loss.
		Paralysis	Tick toxin causes paralysis in calves and lambs.
Amblyomma hebraeum	Bont tick	*Ehrlichia ruminantium* (Heartwater)	Fever, listlessness, high-stepping gait, difficulty breathing, chewing, head pressing, convulsions and death. Accumulation of fluid around the heart, chest cavity and lungs in post-mortem examination.
		Theileria mutans, Bovine theileriosis	Benign bovine theileriosis
		Abscesses	Abscesses form at tick bite sites due to large wounds, blowfly and screw worm infestation, and secondary bacterial infection.
Ixodes rubicundus	Karoo paralysis tick	Karoo paralysis	Salivary toxin causes paralysis, especially in sheep and goats, sometimes cattle.

* The only Argasidae species in the table

Rhipicephalus microplus was the main focus during this study and is a potent carrier of several tick-borne diseases (Table 1). These include *Anaplasma marginale*, which is distributed worldwide, where *R. microplus* is its main vector (ESTRADA-PEÑA *et al.* 2009). The most prominent of these diseases is babesiosis, caused by the protozoan *Babesia bovis* (Asiatic babesiosis or redwater) and *B. bigemina* (African babesiosis) (NYANGIWE and HORAK 2007; WALKER *et al.* 2003). Other tick species such as *R. decoloratus* also carry *Babesia*, but *R. decoloratus* transmits only *B. bigemina*, whereas *R. microplus* transmits both *B. bigemina* and *B. bovis* (WALKER *et al.* 2003). *Babesia bovis* causes the more severe Asiatic babesiosis in cattle (NYANGIWE and HORAK 2007) and is exclusively carried by *R. microplus* (POTGIETER and ELS 1977; TØNNENSEN 2002). Therefore, *R. microplus* is a more economically and

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veterinary important vector of tick-borne disease than *R. decoloratus*. The spread of *Babesia* species to cattle is dependent on tick feeding and mating.

Babesia pathogens are transmitted to cattle when the ticks take a blood meal. *Babesia* replicates within the erythrocytes of the cattle, leading to erythrocyte lysis and eventually death (CHAUVIN *et al.* 2009). Infected female adult ticks transmit the pathogen vertically through the ovaries to their offspring (SIMUUNZA *et al.* 2011). It was also shown that the *Babesia* parasite's genetic diversity is generated by recombination within their tick - and cattle hosts. Older cattle, which were previously infected with *Babesia,* remain reservoirs for genetic recombination events between the *Babesia* present within the cattle and newly transmitted *Babesia* (SIMUUNZA *et al.* 2011). This generates high genetic diversity within the *Babesia* population and if drug-resistant strains arise, it can spread rapidly throughout the *Babesia* population. Babesiosis can also be transmitted to humans who are immune compromised (splenectomised or HIV patients) (CFSPH 2007). *Babesia* lethality and drug-resistance generates economic loss to cattle farmers. Thus, attempts to control the disease through its tick vector by acaricide treatment and tick vaccines are urgently needed.

Babesiosis (*Babesia bovis* & *Babesia bigemina*), theileriosis (*Theileria parva*), anaplasmosis (*Anaplasma marginale*) and heartwater (*Cowdria ruminantium*) are among the most severe tick-borne diseases in South Africa (Table 1). These diseases cause great cattle mortalities and result in large economic losses to the South African economy in excess of billions of rands (OBEREM *et al.* 2006). Current chemical control of ticks is becoming increasingly ineffective due to the misuse or overuse of acaricides (Section 3.9) and has initiated research into more effective tick control methods, such as tick vaccines (Section 3.10). In order to develop effective tick control strategies, knowledge about the biology, systematics, molecular biology and genetics of *R. microplus* is required (POLAND *et al.* 2007).

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3. Biology and systematics of the southern cattle tick, *Rhipicephalus microplus*

3.1 Taxonomy of *R. microplus* relative to other tick families and its species interactions

The monophyletic Parasitiformes is one of two superorders within the subclass Acari, which contains ticks and mites (OLIVER 1989). The hard and soft tick families are part of the Ixodida order of the Parasitiformes (OLIVER 1989; SONENSHINE 1991). Figure 2 represents a brief taxonomic overview of the Ixodida indicating the placement of *Rhipicephalus*.

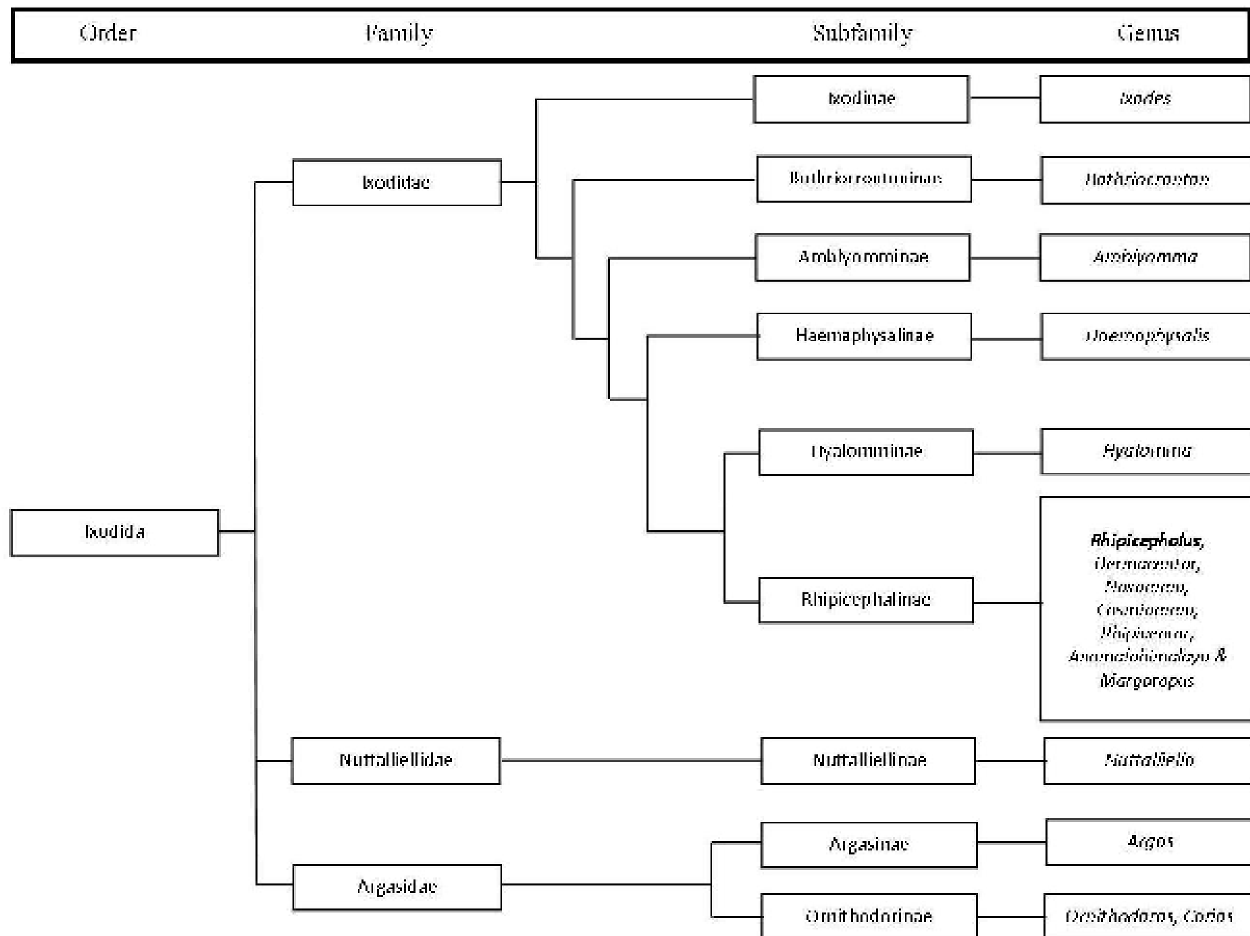

Figure 2: Brief taxonomic overview of the major tick groups within the order Ixodida. Ixodidae represents the hard ticks and Argasidae the soft tick family. Nuttaleillidae contains one tick species, of which little is known. The *Rhipicephalus* genus is contained within Ixodidae. Figure 2 has been constructed from multiple phylogenetic trees and dendrograms of the tick families (BARKER and MURRELL 2002; BARKER and MURRELL 2004; BLACK and PIESMAN 1994; MURRELL *et al.* 2000; MURRELL *et al.* 2001b; OLIVER 1989; SONENSHINE 1991).

The major differences between the Argasidae and Ixodidae are summarised in Table 2. The Argasidae (soft ticks) contains 170 species of ticks with a leathery cuticle and no scutum (SONENSHINE 1991). Ixodidae have more than 650 species of ticks (OLIVER 1989; SONENSHINE 1991), known as 'hard ticks' because of their hard cuticle and sclerotized scutum (SONENSHINE 1991). Ixodidae are an important tick

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family to the medical, veterinary and agricultural sectors due to their large burden on mammals among which the transmission of tick-borne diseases is central (OLIVER 1989; SONENSHINE 1991). *Nuttalliella namaqua*, is the only extant species of the Nuttaleillidae (LATIF *et al.* 2012). It is endemic to South Africa and is proposed to represents the ancestral tick form as it has morphological features of both Ixodidae and Argasidae (LATIF *et al.* 2012).

Table 2: The primary differences between the Argasidae and Ixodidae (SONENSHINE 1991; WALKER *et al.* 2003).

Characteristic	Argasidae (soft ticks)	Ixodidae (hard ticks)
Morphology	Capitulum visible ventrally.	Dorsally visible capitulum (mouth parts).
	Scutum and festoons absent.	Scutum and festoons present.
	Eyes present on lateral surface of supra coxal folds.	Eyes present dorsally on scutum sides.
Amount of time spent feeding	Rapid: Several hours.	Slow: Several days.
Life cycle	Nidicolous or endophilic: Inhabits the nest of the host or remains attached to the host.	Non-nidicolous or exophilic: Ticks seeks the host in the environment.
	Have multiple parasitic phases, where the adult and nymphal stages feed on the same host type. Nymphal stages may be characterised by 2-8 distinct feeding cycles.	Three basic life stages: Eggs hatch to larvae, nymphal and adult stages after feeding and moulting. May target new host in each life stage.
	Multiple blood meals occur between stages.	Single blood meal is taken and moulting occurs between stages.
Host species	Hosts include birds, bats and in some cases, cattle and humans.	Hosts include small mammals (e.g. rodents) and larger mammals, including humans.
Lifespan	Long (exceeding two years)	Short (approximately 1 month, may be longer)
Mating and ovipositing	Multiple ovipositing events occur during the adult life stage and the female lays several batches of eggs during her life time.	Single ovipositing event. Female mates, feeds and lays up to 20 000 eggs. After ovipositing the female dies.

Rhipicephalus includes over 70 species (SONENSHINE 1991) of which *R. decoloratus*, *R. appendiculatus*, *R. evertsi evertsi* and *R. microplus* are the major *Rhipicephalus* ticks found on cattle in South Africa (OLIVER 1989; SONENSHINE 1991; WALKER *et al.* 2003). These four species are of great importance due to their abundance and prominent contribution to the transmission of lethal tick-borne diseases to cattle (Table 1). In addition to these species, *R. annulatus* and *R. geigyi* are found on cattle in the northern parts of Africa (ESTRADA-PENA *et al.* 2006; OLIVER 1989; SONENSHINE 1991; WALKER *et al.* 2003). It is important to taxonomically distinguish between these species, as the *Rhipicephalus* ticks have confounding morphological characteristics.

The taxonomic classification of *R. microplus* has been under dispute amongst scientists for several years. Previously, taxonomists classified the southern cattle tick as *Boophilus microplus* within the *Boophilus* genus, a sister taxon of *Rhipicephalus*, whilst genetic and mating data suggested that *Boophilus* is nested within *Rhipicephalus*. Recently *Rhipicephalus (Boophilus) microplus* was re-classified as *R. microplus*, since the *Boophilus* subgenus had been synonymize into *Rhipicephalus* (HORAK 2009). This was done after phylogenetic data indicated that *Boophilus* - and *Rhipicephalus* are synonymous and thus the *Rhipicephalus* and *Boophilus* genus are paraphyletic (BEATI and KEIRANST 2001; MURRELL and BARKER 2003; MURRELL *et al.* 2000). Doubts still remain as to the sister taxon of *R.*

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microplus. Morphological data suggest that its sister taxa are *R. decoloratus* and *R. annulatus*, which are grouped together within *Rhipicephalus* (BEATI and KEIRANST 2001). In contrast, the phylogenetic data based on the mitochondrial 12S ribosomal DNA group *R. microplus* and *R. annulatus* as sister species and *R. decoloratus* is designated as a sister taxon to the *Rhipicephalus* genus (BEATI and KEIRANST 2001).

Ticks within the *Boophilus* subgenus are regarded as the "cattle ticks", since cattle are their primary host. *Rhipicephalus* (*Boophilus*) *kohlsi* is not found on domesticated animals, but is regarded as a cattle tick (RAHBARI and NABIAN 2007; WALKER *et al.* 2003) and was not included in this study. Phylogenetic data also indicated further subdivision within the species of *Rhipicephalus*. Figure 3 illustrates the current phylogeny of the Rhipicephalinae.

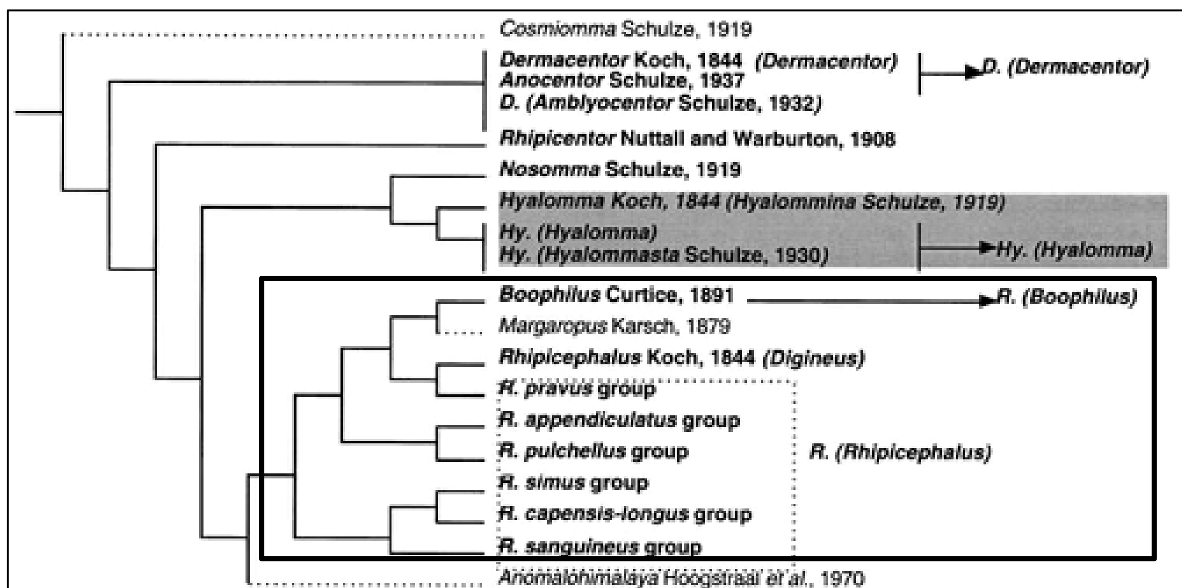

Figure 3: The working hypothesis of the phylogeny of Rhipicephalinae (BARKER and MURRELL 2004). Taxa in plain text at the terminals of broken branches (e.g. Margaropus and Anomalohimalaya) have not been studied with molecular markers. Shading indicates that Hyalomminae may be synonymised with the Rhipicephalinae. The black box indicates the paraphyletic branching within *Rhipicephalus*.

Recently, the Australian cattle tick, *Rhipicepahlus microplus* Canestrini, which included specimens from Australia and New Caledonia, was reinstated as *Rhipicephalus australis* Fuller (ESTRADA-PEÑA *et al.* 2012). The authors based the reinstatement on the morphological dissimilarities between *R. microplus* and *R. australis* in both adult and larval stages. Barker (1998), previously investigated whether Australian *R. microplus* can be considered as a separate species to South African *R. microplus* (BARKER 1998). Although the phylogenetic study with ITS2 sequences did not yield any significant evidence of the speciation, Australian and South African isolates of *R. microplus* did group separately with Brazilian and Kenyan isolates, respectively (BARKER 1998). Furthermore, differences observed in the results of mating experiments between Australian, African and South American *R. microplus* ticks provides evidence for *R. australis* as a separate species. One such study reported that the Australian

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R. microplus is a different species to the Argentinian and African *R. microplus* due to no eggs being produced by Australian female ticks, which were mated with male Argentinian and African ticks (LABRUNA *et al.* 2009). The F1 hybrids produced from mating of male Australian ticks with female Argentinian and African ticks, resulted in a higher egg producing capacity than the mating between male and female Australian ticks, but the hybrids were always sterile (LABRUNA *et al.* 2009), indicating a possible species barrier at the F1 hybrids. The confirmation of *R. australis* as a separate species needs to be further investigated with appropriate genetic markers to conclude this dispute.

The correct identification of *Rhipicephalus* (*Boophilus*) species has additional challenges due to the occurrence of hybrids. *R. microplus* has been successfully mated with *R. australis* (LABRUNA *et al.* 2009) and *R. annulatus* (DAVEY *et al.* 1994). *R. australis* has been successfully mated with *R. decoloratus* (SPICKETT and MALAN 1978) (although the article title states '*R. microplus*', these studies were conducted in Australia suggesting *R. australis* rather than *R. microplus*). Hybridisation occurs only in 10% of the offspring under natural conditions. This suggests that although hybridisation can occur, ticks would more often mate with their own species (assortive mating) (NORVAL and SUTHERST 1986). One trend is that *Rhipicephalus* females are able to mate with males from the other species and produce sterile F1 hybrids (LABRUNA *et al.* 2009; SPICKETT and MALAN 1978). Male *R. microplus* x *R. annulatus* hybrids are sterile, whilst female hybrids are fertile and produce viable egg masses for three generations when backcrossed with wild-type parents, but all males produced from these crosses remain sterile (THOMPSON *et al.* 1981). *R. microplus* has a higher reproductive capacity than *R. decoloratus*, producing 500 more eggs (WALKER *et al.* 2003) and more larvae mature (NORVAL and SHORT 1984; WEDDERBURN *et al.* 1991) also males have shorter life cycles and are more abundant (HORAK *et al.* 2009) than those of *R. decoloratus*. If the same principle of *R. microplus* x *R. annulatus* hybrids holds true for *R. microplus* x *R. decoloratus* hybrids; *R. microplus* might be able to not only displace *R. decoloratus*, due to its higher reproductive capability and reduction of the *R. decoloratus* population (due to sterile males) over several tick generations, but may assimilate *R. decoloratus* characteristics into the *R. microplus* population. Larvae with a mosaic of morphological characteristics from both *R. microplus* and *R. decoloratus* have been observed on cattle farms (NYANGIWE *et al.* 2013a). Hybrids would provide a significant explanation for the observed climatic and geographic adaptation of *R. microplus* in South Africa. The mating procedures developed by Thompson and colleagues (1981) would be a suitable method to determine the hybrid dynamics of *R. microplus* and *R. decoloratus*.

The correct classification of the *Boophilus* subgenus remains a challenging prospect, due to their morphologically similar characteristics and the existence of hybrids. Yet, most of the controversial taxonomic classifications of *R. microplus* and the other *Boophilus* cattle ticks have largely been resolved by the combinational use of phylogenetic data, fossil records and the study of tick evolutionary origins.

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3.2 Origins of ticks and the fossil record

Fossils are used to establish a timescale of evolution. This provides dates for which extinct ancestors gave rise to extant organismal lineages. These dates, in millions of years, can be utilised in phylogenetic and cladogenetic studies to calibrate molecular clocks and estimate the relative divergence times of extant taxa, inferred from genetic data provided by molecular markers (DUNLOP 2010).

Within the phylum Arthropoda (organisms with an exoskeleton), the subphylum Chelicerata contains major classes including Arachnida (scorpions, spiders, ticks and mites), Xiphosura (horseshoe crabs) and Pycnogonida (sea spiders) (DUNLOP 2010). Figure 4 shows the evolutionary age and phylogenetic relationships within the Arachnida, in relation to the geological time scale.

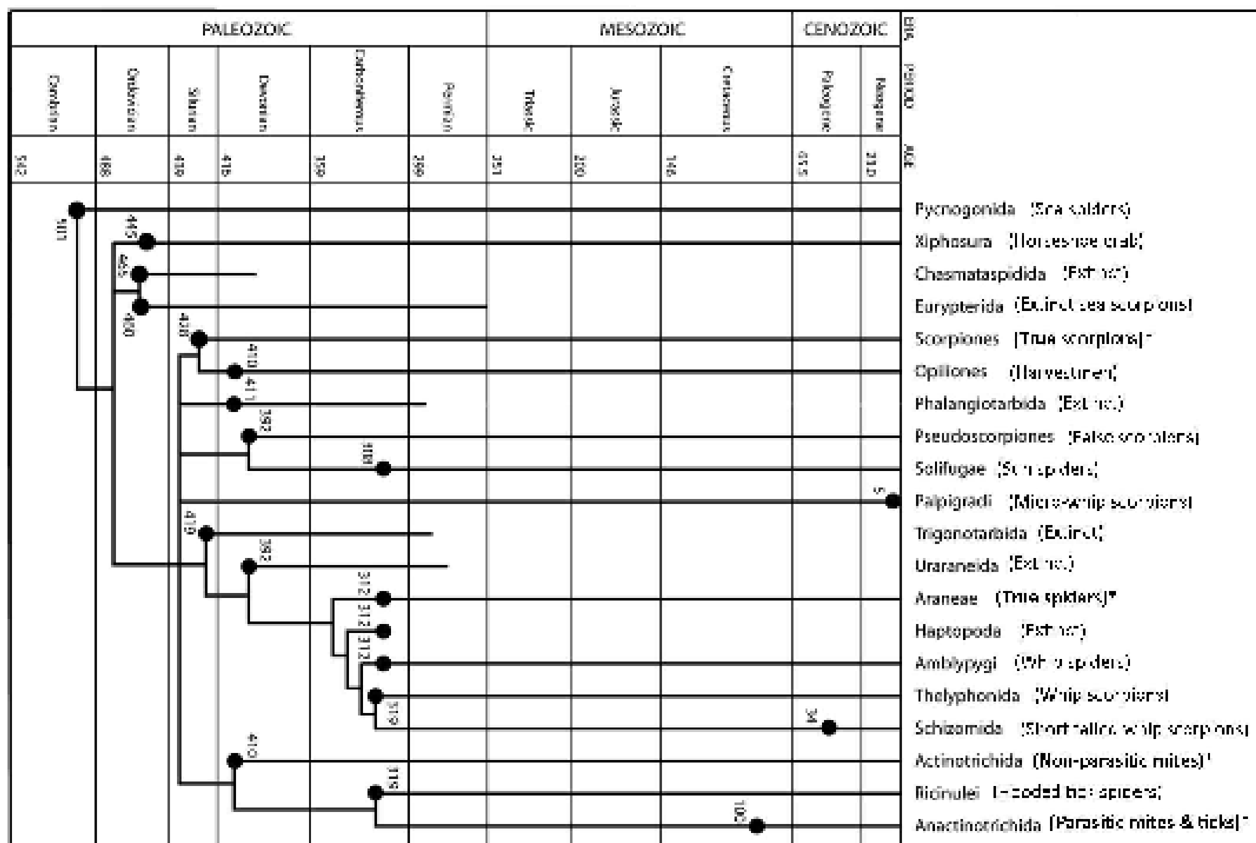

Figure 4: The evolutionary age and phylogeny of arachnids superimposed on the geological time scale, modified from Dunlop (2010). Major extant taxa are indicated in bold and with a (*). Circles indicate recorded fossils.

According to Figure 4, the subclass Acari includes; the Actinotrichida (non-parasitic mites synonymous with the superorder Acariformes), Anactinotrichida (parasitic mites and ticks synonymous with the superorder Parasitiformes), which are separated by the Ricinulei (hooded tick-spiders). It also indicates

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that Actinotrichida (410 Mya) evolved prior to Anactinotrichida (100 Mya), and suggests that ticks first evolved 100 Mya, although older fossils may exits.

Tick and mite bodies consist of cuticle, which does not readily fossilise. Thus most preserved Acari are found in amber (fossilferous resin of conifers and other trees) (DE LA FUENTE 2003). Amber does not alter the morphology of embedded ticks or mites and allows detailed studies of these fossils with transmitted and reflected light microscopy (DE LA FUENTE 2003). Fossils of Actinotrichida are more abundant than Anactinotrichida, yet both are under-represented in the fossil record (DUNLOP 2010). Several fossils are available for the Ixodidae and Argasidae, but have not yet been recorded for Nuttalleilidae (DUNLOP 2010).

There are 14 fossils available for ticks (Table 3), of which 11 are Ixodidae ticks and most represent larvae (DE LA FUENTE 2003). *Ixodes tertiarius* and *Dermacentor* near *reticulatus* represent two non-amber fossils (DE LA FUENTE 2003). Due to the lack of resemblance of *I. tertiarius* to ticks (extant or otherwise) and the loss of the type specimen, *I. tertiarius* has been removed from the tick fossil record (DUNLOP 2011).

Table 3: The available fossils for ticks, including present day host(s) and origins (DE LA FUENTE 2003).

Tick species	Present day host range for the genus[a]	Characteristics	Origin[b]
Carios jerseyi	Birds Mammals	Larva (soft tick) (Argasidae)	New Jersey amber (Cretaceous, 90–94 mya)
Ixodes succineus	Birds Mammals	Female (Ixodidae)	Baltic amber (Tertiary, 35–50 mya)
Ixodes spp.	Birds Mammals	Larva (Ixodidae)	Baltic amber (Tertiary, 35–50 mya)
Hyalomma spp.	Mammals Reptiles Birds	Male (Ixodidae)	Baltic amber (Tertiary, 35–50 mya)
Amblyomma near testudinis	Reptiles Birds Mammals	Male (Ixodidae)	Dominican amber (Tertiary, 15–40 mya)
Amblyomma spp.	Reptiles Birds Mammals	Larva (Ixodidae)	Dominican amber (Tertiary, 15–40 mya)
Amblyomma spp.	Reptiles Birds Mammals	Larva (Ixodidae)	Dominican amber (Tertiary, 15–40 mya)
Amblyomma spp.	Reptiles Birds Mammals	Larva (Ixodidae)	Dominican amber (Tertiary, 15–40 mya)
Not known	Not known	28 first instar larvae (Ixodida)	Dominican amber (Tertiary, 15–40 mya)
Amblyomma spp.	Reptiles Birds Mammals	Adult (Ixodidae)	Dominican amber (Tertiary, 15–40 mya)
Not known	Not known	Adult (Argasidae)	Dominican amber (Tertiary, 15–40 mya)
Ornithodoros antiquus	Amphibians Reptiles Birds Mammals	Female (soft tick) (Argasidae)	Dominican amber (Tertiary, 15–40 mya)
Ixodes tertiarius	Birds Mammals	(Ixodidae)	Tertiary, Oligocene deposits (ca. 30 mya). Wyoming, USA
Dermacentor near reticulatus	Mammals	Male (Ixodidae)	Exterior auditory canal of a woolly rhinoceros, *Tichorhinus antiquitatis* Tertiary, Pliocene (2–5 mya)

[a]The host range information was extracted from Klompen et al. (1996). [b]The age of the amber deposits was obtained from Poinar (1992, pp. 16–63) except for New Jersey amber deposit (Klompen and Grimaldi 2001).

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With regards to the age of the tick fossils and their current host ranges, several hypotheses exist for tick evolutionary origins. The most acceptable hypothesis is that tick evolution is based on host-associations due to co-speciation, and that ticks originated in the late Paleozoic-early Mesozoic, where they fed on slow moving, smooth-skinned reptiles approximately 250 Mya (DE LA FUENTE 2003; HOOGSTAAL 1985). New analyses based on molecular and morphological data suggest that the main influence on tick evolution is biogeography, ecological specificity and host size (DE LA FUENTE 2003). This led to the new hypothesis that Ixodidae ticks have evolved from Argasidae-like ancestors, 120 Mya during the late Cretaceous, after the breakup of Gondwanaland and when Australia became isolated (BLACK and PIESMAN 1994; DE LA FUENTE 2003; KLOMPEN *et al.* 2000). Due to the multiple host ranges of the ancestral ticks of recorded fossils, the original host animal is difficult to determine. The original host could have been reptiles, amphibians, mammals or birds, all of which were present during the Cretaceous period. *Nuttalliella namaqua* is speculated to be a multiple host tick and given the fact that it is regarded as the evolutionary missing-link between the main tick families (LATIF *et al.* 2012), the ancestral tick could have been a generalist and may have fed on multiple hosts.

The Rhipicephalinae ticks have diverse morphology (hood ornamentation, Figure 5A) and host-life cycles (Figure 5B), but these characteristics fail to explain the evolution of ticks; as no logical evolutionary flow can be derived from these characteristics (BARKER and MURRELL 2002; MURRELL *et al.* 2000; MURRELL *et al.* 2001b). However, the historical biogeography, or geographical point of origin (Figure 5C) seem to be one of the main factors that has influenced the evolution of ticks (BARKER and MURRELL 2002; MURRELL *et al.* 2000; MURRELL *et al.* 2001b) and provides the logical evolutionary progression when combined with the historical movements of the earth's land masses (BARKER and MURRELL 2002). The historical zoography of the respective lineages in the Ixodidae family is indicated in Figure 5 and in Table 4.

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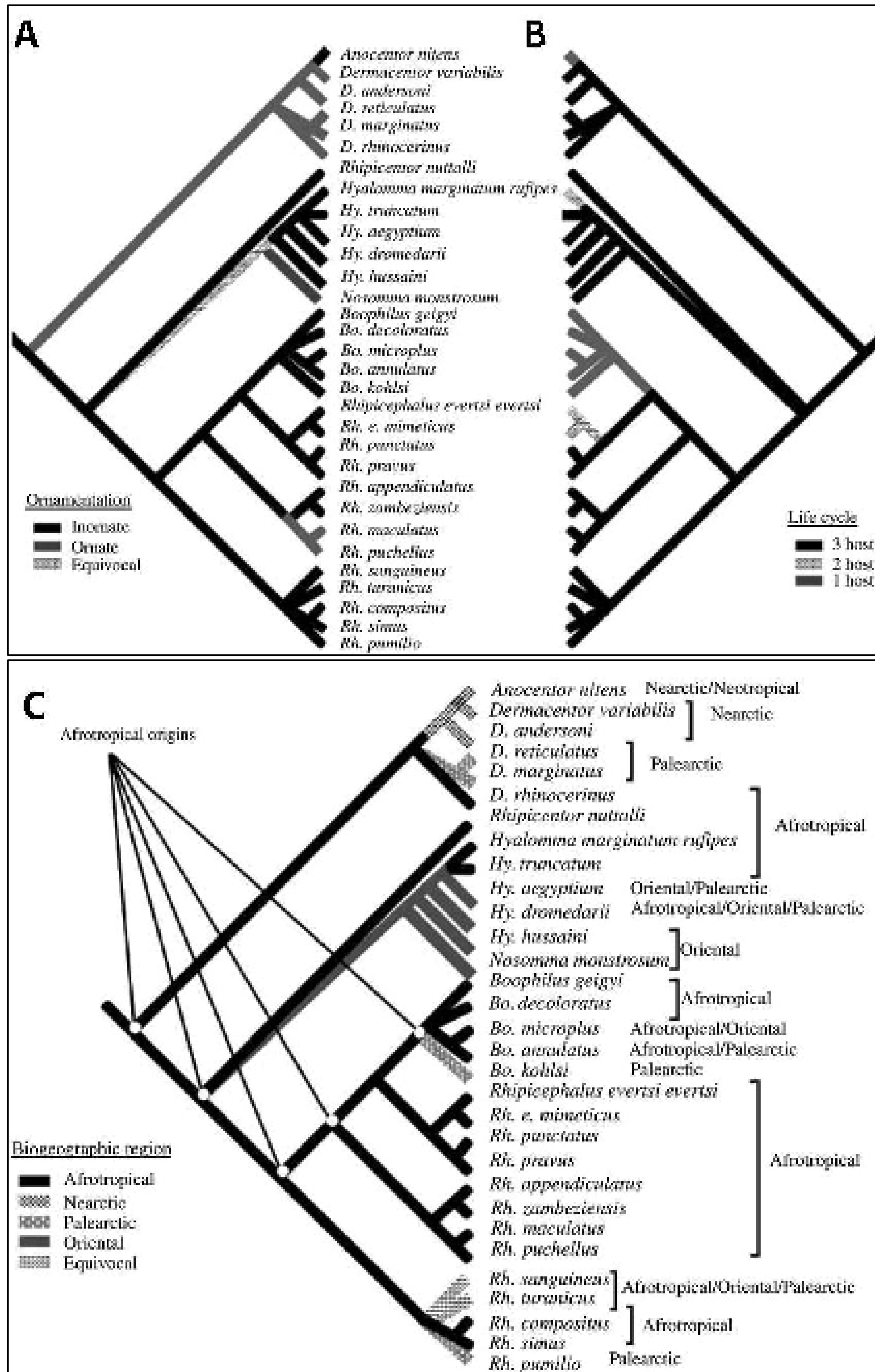

Figure 5: Cladograms representing the genetic, morphological and historical zoogeography of the Rhipicephalinae ticks and sister taxa based on maximum parsimony (BARKER and MURRELL 2002; MURRELL *et al.* 2000; MURRELL *et al.* 2001b). A+B: Cladograms based on genetic markers (12S, COI, ITS2, 18S rRNA), morphological and biological features of different Ixodidae ticks. *Hyalomma dromedarri* has 1, 2 and 3 host-life cycles (BARKER and MURRELL 2002). **C:** Cladogram representing the biogeographical origins of each of the Ixodidae lineages, including the *Rhipicephalus* genus. The introduced *of Rh.* (*Bo.*) *microplus*, *Rh.* (*Bo.*) *annulatus* and *Rh. sanguineus* into biogeographical regions by humans have been excluded. *Boophilus* was previously considered a subgenus of *Rhipicephalus* (BARKER and MURRELL 2002; MURRELL *et al.* 2001b).

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Table 4: Divergence times and origins of ancestral lineages for the Rhipicephalinae and sister subfamilies. The table was constructed from Barker and Murrell (2002).

Ancestral lineage	Origin	Comments
Dermacentor–Anocentor	Afrotropical	Dispersion into Eurasia dates to 50 Mya (Eocene) during the migrations of mammals between Africa and Eurasia.
Nosomma–Hyalomma	Oriental region	Ancestor evolved in the early Miocene (19 Mya). Dispersals between Eurasia and Africa likely occurred multiple times.
Boophilus–Rhipicephalus	Afrotropical	Originated and radiated in Africa, when Africa was isolated from the Paleartic and Oriental regions, before land-bridge formations between Africa and Eurasia. After land-bridge formation (14 Mya) further radiation and dispersal occurred into Eurasia and Asia during the Miocene.
Rhipicentor	Afrotropical	Two species evolved and remained in Africa. Possible dispersal or radiation into other countries could have occurred, but resulted in extinction of those lineages.
*Anomalohimalaya**	Paleartic and Oriental regions	Phylogeny not resolved.
*Cosmiomma**	Afrotropical	One species, phylogeny not resolved.
*Margaropus**	Afrotropical	One species, phylogeny not resolved.

*Phylogenetic positions of these genera have not yet been resolved and therefore no inferences can be made about their biogeographic history.

According to Figure 5 and Table 4, Rhipicephalinae evolved more than 14 Mya in Africa, with subsequent radiation into Europe and Asia. This divergence date, in combination with sister subfamily dates of *Hyalomma* and *Amblyomma* (Figure 2), can be used to estimate the divergence dates for individual *Rhipicephalus* tick species, given the appropriate genetic markers.

Although the archetypal host and life cycle cannot be conclusively determined, it is possible that *Rhipicephalus microplus* evolved on the African continent and through specialisation began to exclusively feed on cattle. The transition of the ancestral tick as a multiple-host generalist to present day one-host specialist will be reflected in the life history and biology of *R. microplus.*

3.3 The history of ticks and tick-borne disease and its prevalence in South Africa

The history of tick spread through South Africa is important for genetic studies of *R. microplus*, especially when concerned with the origins of recent tick introduction and distribution. Table 5 provides a summary of tick distribution records and tick-borne diseases in South Africa. The time line of disease outbreaks, tick abundance and cattle movement, especially through cattle imports, provides a historical record of the possible introduction and spread of ticks in South Africa. The impact of historical events on the present day distribution and population structure of ticks and tick-borne disease was discussed below with reference to Table 5.

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Table 5: The dates and events pertaining to tick distribution ranges and the outbreaks of tick-borne disease, during 1893 to 1998 in South Africa. The information was summarised from the Master's dissertation of Manamela (2001), updated and expanded with additional available information.

Date	Events affecting tick and tick-borne disease distributions
Pre 1893	Ticks and tick diseases not a problem to cattle farmers. Cattle populations consisted of mostly indigenous breeds with resistance to native ticks and their diseases.
1893	First cattle dip trails to control ticks in South Africa
1896	Rinderpest epidemic in South Africa caused the loss of 70-80% of cattle in South Africa. Indigenous cattle suffered the most losses. Cattle stocks replenished by importing tick and tick-borne disease susceptible cattle from Europe.
1899	Decline of rinderpest outbreaks (AU-IBAR 2011).
1901	Last recorded outbreaks of rinderpest (AU-IBAR 2011).
1902	First reports of East Coast Fever in KwaZulu-Natal with 95% mortality rate.
1905	Rinderpest eradicated from South Africa (AU-IBAR 2011). **First record of Asiatic redwater (*Babesia bovis)* in South Africa (POTGIETER and ELS 1977; TØNNENSEN 2002).**
1904-1954	East Coast Fever outbreaks throughout South Africa.
1908	Increased cattle dipping at communal dipping areas and immunisation of cattle against East Coast Fever. **First record of *R. microplus* in the Cape Province, South Africa (HOWARD 1908; NYANGIWE *et al.* 2011).**
1920	Cattle trade restrictions and more stringent quarantine and cattle movement restrictions between the provinces of South Africa.
1941	East Coast Fever outbreaks decline with each successive year due to increased tick control methods and slaughtering of infected cattle.
1954	East Coast Fever eradicated from South Africa and regulations on dipping relaxed.
1960-1970	Increase of tick abundance and their associated tick-borne diseases due to poor tick control practices and cattle movement.
1961	Tick populations and outbreaks of tick-borne diseases increase due to the increased resistance of tick populations against acaricides.
1962	**First records of *R. decoloratus* displacements by *R. microplus* in the south-eastern Cape (NYANGIWE *et al.* 2011; THEILER 1962; TØNNENSEN 2002).**
1976	Tick-borne disease outbreaks in various parts of South Africa due to higher than usual rainfall which interfered with regular dipping of cattle. Redwater became especially problematic.
1983	Tick-borne disease outbreaks due to drought and poor tick control management on cattle farms. Drought and water restrictions result in less frequent dipping of cattle.
1984	Animal Disease Act No 35 announced that dipping of cattle will not be compulsory and that farmers are responsible for their own tick control.
1985-1986	**First records of *R. microplus* collected in Swaziland (WEDDERBURN *et al.* 1991).**
1985-1994	Political unrest and violence contributed to increased tick abundance and tick-borne disease outbreaks due to cattle farmers not dipping cattle at communal dipping lots.
1997-1998	Increases in tick populations and tick-borne disease occurrence due to optimal weather conditions (warm temperatures and good rain). Abundant rainfall led to reduced cattle dipping.

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In the late 1800s, the increase of cattle dipping would mark the first stages of the development of acaricide resistance in cattle ticks. Presently, acaricide resistance is problematic to farmers as resistant ticks induce a greater economic loss, due to their continuous survival and transmission of tick-borne disease. During the late 1800s and 1900s, Rinderpest and East Coast Fever devastated the cattle population of South Africa, which led to the import of cattle from Europe (MANAMELA 2001) and suspected import from East Africa (no clear records). Multiple cattle imports occurred during the late 18th and early 19th century to replace the losses caused by Rinderpest and East Coast Fever outbreaks. Therefore, exotic ticks have been introduced to South Africa by importing cattle from abroad. It included *R. microplus* and its spread would be dictated by tick control, cattle trade, cattle movement restrictions, the political and social factors during the 1900s and onwards.

Several social and cultural factors also influenced the dipping of cattle, cattle movement and the occurrence of ticks and their associated diseases. Since 1994, cattle in rural communities were dipped less. The change in the political dispensation in South Africa had rural children attending school, who previously would have taken the cattle to communal dipping lots (MANAMELA 2001). Political resistance and conflict prior to 1994 also caused cattle dipping lots and presumably feeding lots to be abandoned, as inhabitants feared to take their cattle to dipping and feeding lots (MANAMELA 2001). The lack of cattle dipping would have contributed to flaring up of tick populations and tick-borne disease outbreaks, along with the increased incidence of acaricide resistance due to the lack of regular tick control.

The contributing causes for the spread of *R. microplus* and other ticks throughout South Africa is complex and involves many factors, not limited to only biological and environmental factors, such as host distribution and climate. Determining the introduction and subsequent spread of *R. microplus* is seldom apparent due to the multiple imports of cattle from overseas; cattle movement between provinces and even bordering African countries through legal/illegal trade; movement and trade in reservoir hosts; and the exchange of ticks at communal agricultural exhibitions, auctions, dipping and feeding lots.

3.4 Geographic distribution

Rhipicephalus microplus is distributed throughout the world's tropical and subtropical areas, including Australia, Africa, Madagascar, South and Central America and parts of Asia (CUTULLÉ *et al.* 2009; LABRUNA *et al.* 2009; WALKER *et al.* 2003). Many of these areas in Africa were previously dominated by native *R. decoloratus* (DE MATOS *et al.* 2009; LYNEN *et al.* 2008; TONETTI *et al.* 2009; TØNNENSEN *et al.* 2004). *R. microplus* is a non-native tick species introduced to South Africa; the first documented record of *R. microplus* in South Africa was made by Howard and colleagues during 1908 in the Eastern and Western Cape (HOWARD 1908; NYANGIWE *et al.* 2011). This would suggest the *R. microplus*

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introduction coincided with a large number of cattle imports from overseas following the Rinderpest epidemics and East Coast Fever outbreaks in the late 1800s and early 1900s (refer to Table 5).

Since the introduction of *R. microplus* to areas such as the eastern parts of South Africa and Tanzania, it has increased its geographical spread and is reproductively outcompeting the native tick species *R. decoloratus* (NYANGIWE *et al.* 2013a). Studies conducted in Tanzania (LYNEN *et al.* 2008), West Africa (MADDER *et al.* 2012), the Free State (TONETTI *et al.* 2009), the Eastern Cape (NYANGIWE *et al.* 2011; NYANGIWE *et al.* 2013a) and the Limpopo Provinces (TØNNENSEN *et al.* 2004) of South Africa have shown the steady and recent encroachment of *R. microplus* into previously *R. decoloratus* dominated areas where *R. microplus* ticks have not been found in the past (HORAK *et al.* 2009; MADDER *et al.* 2011). In areas where more than one species of *Rhipicephalus* cattle ticks occur, the distribution ranges of the ticks differ as to nearly exclude one another from their local ranges. In Tanzania, *R. microplus* occupies temperate mainland, whereas *R. decoloratus* occupies colder high altitude mountains and plains (LYNEN *et al.* 2008). Similar range exclusion was observed in the parapatric boundaries between *R. annulatus* and *R. microplus* in the United States along the Texas/Mexico border (LOHMEYER *et al.* 2011). This may have implications for tick control and spread, as elimination of one species may allow encroachment and range expansion of the other into the recently ‘unoccupied’ area (LOHMEYER *et al.* 2011). Presently *R. microplus* is mainly distributed along the coast line of South Africa and in parts of Mpumalanga and Gauteng (NYANGIWE *et al.* 2011; OBEREM *et al.* 2006). Figure 6 represents the geographical distribution of *R. microplus* and *R. decoloratus* in 2004 in South Africa, and Figure 7 indicates *R. microplus* and *R. decoloratus* distributions in Africa (based on data from 2004 collected by the Integrated control of ticks and tick-borne diseases, ICTTD, program).

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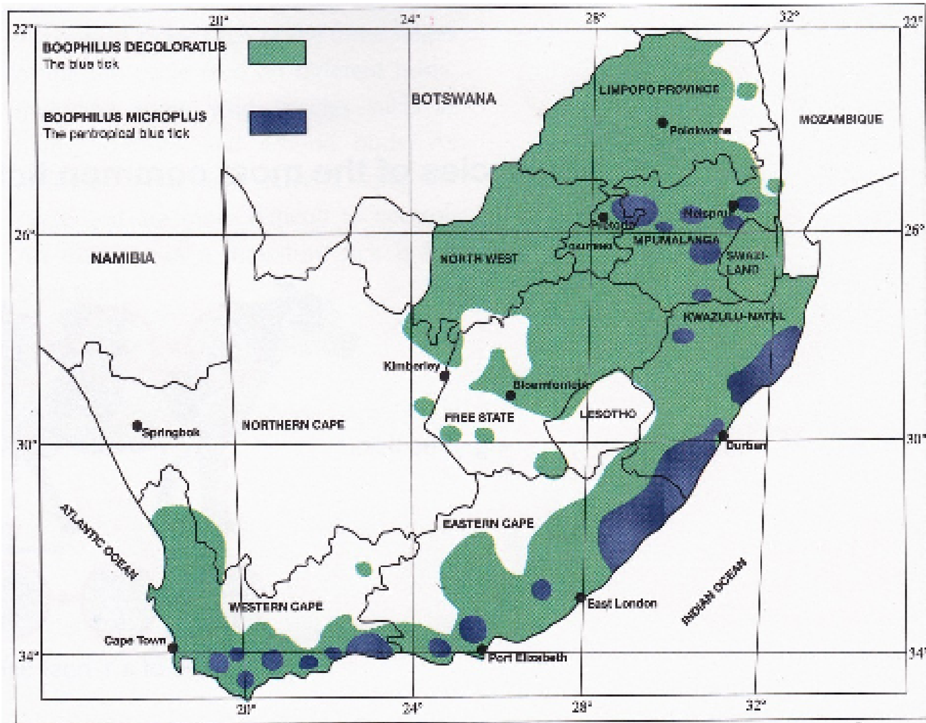

Figure 6: A map of South Africa indicating the geographical distribution of *R. microplus* and *R. decoloratus* during 2004 (OBEREM *et al.* 2006). Blue shading indicates the fragmented distribution of *R. microplus* in temperate regions, whereas *R. decoloratus* distribution has a more continuous distribution indicated in green shading.

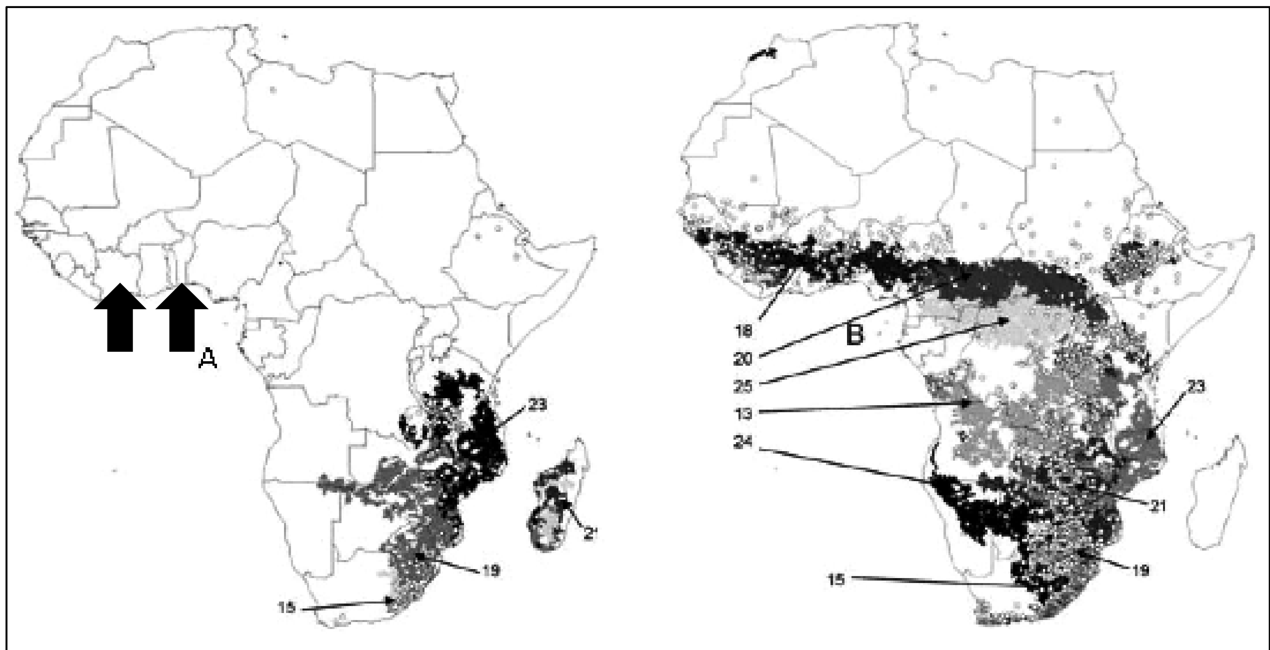

Figure 7: Modified map of the geographical distribution of *R. microplus* (A) and *R. decoloratus* (B) in Africa (ESTRADA-PENA *et al.* 2006). Shaded regions and numbers indicate different environmental categories according to monthly unsupervised NDVI (Normalized Derived Vegetation Index) classifications. Areas where less than 5% of tick records of each species have been collected are not shown (ESTRADA-PENA *et al.* 2006). Large arrows in (A) indicate recent *R. microplus* introductions into West Africa (MADDER and HORAK 2010).

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Historically *R. microplus* distribution was confined to the coastline of South Africa and *R. decoloratus* was more widespread (CUMMING 1999; HOWELL *et al.* 1978). Since 2009 it has been observed that *R. microplus* has increased its range in the Free State (TONETTI *et al.* 2009) and Eastern Cape Provinces (HORAK *et al.* 2009; NYANGIWE *et al.* 2011; NYANGIWE *et al.* 2013a). In 2007 and 2008, *R. microplus* was collected in the Ivory Coast and Benin, likely imported on cattle into West Africa (MADDER and HORAK 2010). It is suspected that *R. microplus* was imported to Benin on Girolando cattle from Brazil (MADDER *et al.* 2007) and has since spread further into the country from the harbour area (DE CLERCQ *et al.* 2012). The distributions of *R. microplus* and *R. decoloratus* suggest that they occupy slightly different climate types, as evident from Figures 6, 7 and 8.

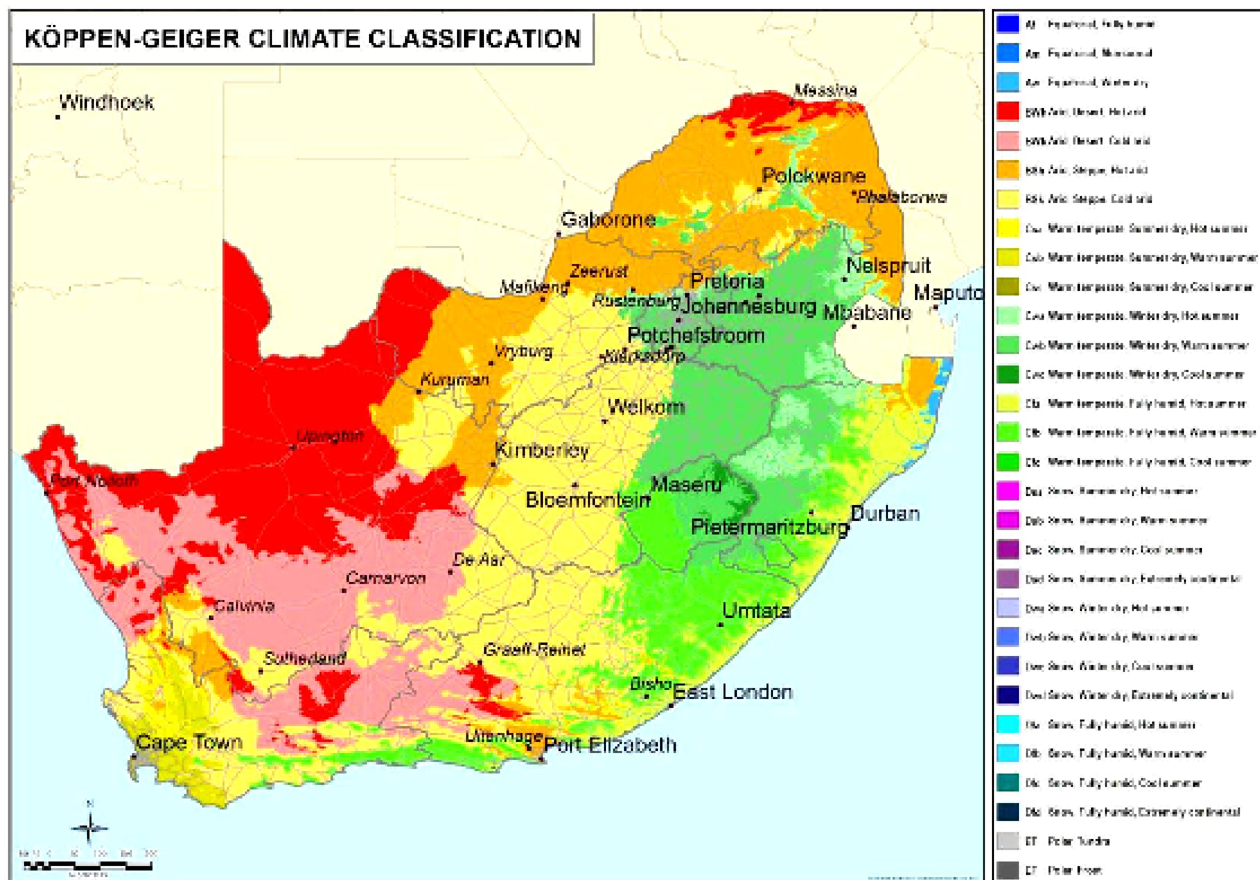

Figure 8: A map based on the South African Weather Services data (1985-2005) on a very fine 1 km x 1 km grid, constructed by the CSIR using the Köppen-Geiger climate classification system (CONRADIE 2012).

Figure 8 indicates the major climate types in South Africa according to the Köppen-Geiger climate classification. When compared to the distribution map of *R. microplus* and *R. decoloratus*, it is clear that *R. microplus'* range is more restricted to temperate areas (C climate types, yellow and green). Whereas *R. decoloratus* can occupy more arid and colder climate types (B climate types, red and orange) as well as hot and humid climate types. Temperature and humidity seem to be limiting factors to the spread of *R. microplus*, where *R. microplus* is less tolerant to cold and dry conditions (SHORT *et al.* 1989). This suggests that *R. microplus* may not encroach into areas with non-optimal conditions,

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even in the absence of *R. decoloratus* or other competing tick species. Therefore, both environmental and biological (availability of host) factors influence the distribution of *R. microplus* across South Africa and will influence the range expansion of *R. microplus* into new areas.

Other animals, such as small mammals and birds, may contribute to the geographical spread of *R. microplus* ticks. These animals do not represent host species, but can re-locate adult and immature ticks to new areas. Small mammals, such as rodents (HORAK *et al.* 2005) and birds such as the helmeted guinea fowl, larks, bulbuls and robins (VAN NIEKERK *et al.* 2006) have been found to carry ticks of the *Rhipicephalus* genus. Although this has not been described for *R. microplus*, small mammals and birds may transfer ticks between cattle and wildlife, even over long distances.

Climate change influences the distribution of *R. microplus* into areas that were previously unpopulated by creating optimal conditions for *R. microplus* to thrive, as indicated by a study of *R. microplus* distribution in the Americas (ESTRADA-PEÑA *et al.* 2005). These distribution shifts will increase the prevalence of both the *R. microplus* ticks and their tick-borne diseases into previously unaffected areas (ELSA LÉGER *et al.* 2013). The geographical range shifts in *R. microplus* will also impact on the host shifts of the tick towards other animals such as livestock that co-habituate with cattle and wildlife. Therefore, constant surveillance and possible control measures of *R. microplus* movement is required to monitor the spread of the tick and its tick-borne diseases into other areas of southern Africa.

3.5 Habitat of ixodid ticks

Although ticks are dependent on their host for nutrition, many other biological and environmental factors contribute to their survival. Therefore, ticks need to be adapted to both their host (immune system and parasitic lifestyle) and to their environment (climate and acaricide resistance) (OLIVER 1989; WALKER *et al.* 2003). Non-parasitic life stages (such as the egg and engorged females) are subjected to environmental factors (such as weather conditions and predators) (WALKER *et al.* 2003). Parasitic life stages are subjected mostly to host-related factors, such as the immune system of the cattle (WALKER *et al.* 2003). Therefore, host availability and climatic conditions dictate the geographical distribution and abundance of ticks. This is an essential factor to consider during tick sampling.

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3.6 Tick hosts with specific reference to tick control

Rhipicephalus microplus is a one-host tick that feeds primarily upon cattle from the *Bos* subgenus, such as *B. taurus* and *B. indicus* (BELLGARD *et al.* 2012). *Bos indicus* cattle are also known as 'humped' cattle, due to their characteristic humped backs. Whereas *Bos taurus* cattle are known as 'hump less', due to the lack of the characteristic *B. indicus* hump (DEPARTMENT OF ANIMAL SCIENCE). Table 6 provides a list of the cattle breeds relevant to this study and their subspecies.

Table 6: The cattle breeds present on cattle farms in South Africa and their subspecies allocations (2009; ANIMAL GENETICS TRAINING RESOURCE (AGTR) ; DEPARTMENT OF ANIMAL SCIENCE ; INDIGENOUSBREEDS.CO.ZA ; PIPER *et al.* 2009).

Cattle Breed[a]	**Subspecies (*Bos taurus* or *Bos indicus* or Cross)**
Bonsmara[b]	Cross
Holstein-Friesian	*Bos taurus*
Nguni[b]	Cross
Brahman	*Bos indicus*
Jersey	*Bos taurus*
Beefmaster	Cross
Drakensberger[b]	Cross
Sussex	*Bos taurus*
Afrikaner[b]	*Bos indicus*
Angus	*Bos taurus*
Hereford	*Bos taurus*
Simbrah	Cross
Simmental	Cross
Brangus	Cross
Ayshire	*Bos taurus*
Boran	*Bos indicus*
Hugenoot[b]	Cross
Tuli[b]	*Bos taurus*
Guernsey	*Bos taurus*

[a] Full names are *Bos taurus taurus* (predecessor of *Bos taurus primigenius primigenius*), *Bos taurus indicus* (predecessor of *Bos taurus primigenius namaindicus*) and *Bos taurus africanus* (predecessor of *Bos taurus primigenius africanus* with *B. taurus* and *B. indicus* genetics introduced through migrations) (VUURE 2005). The abbreviations used are the most common nomenclature for the subspecies at present.
[b] Indigenous Sanga cattle breeds from South Africa

Indigenous Sanga (South African) and *B. indicus* (Indian) cattle are more resistant to ticks and tick-borne diseases than the cattle imported from Europe (*B. taurus*) (HUANG *et al.* 2012). Although *R. microplus* is adapted to parasitising cattle, it is able to parasitise other bovid host animals, such as sheep (OBEREM *et al.* 2006; WALKER *et al.* 2003) and equines (LOHMEYER *et al.* 2011; PASCOAL *et al.* 2013; POUND *et al.* 2010). Recent studies conducted in South Africa have found that *R. microplus,* if provided with other hosts, will feed on goats (DE MATOS *et al.* 2009; NYANGIWE and HORAK 2007), wildlife such as gemsbok (TONETTI *et al.* 2009) and eland (personal communication from Prof Ivan Horak). These alternative hosts are in close contact with domestic cattle, especially in South Africa, where they can act as reservoir hosts to the ticks and their tick-borne diseases, such as babesiosis and theileriosis (DE MATOS *et al.* 2009; HORAK *et al.* 2009; NYANGIWE and HORAK 2007; TONETTI *et al.* 2009). This suggests that these animals should also be included in tick control and treatment programmes to successfully control tick populations on cattle farms.

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3.7 Development and feeding cycles of the southern cattle tick

Ixodid ticks are obligate parasites with four stages within their life cycle: (1) eggs, (2) larvae, (3) nymphs and (4) adults (SONENSHINE 1991; WALKER *et al.* 2003). All *R. microplus* parasitic life stages (larvae, nymphs and adults) feed upon one cattle host and it is therefore considered to be monotrophic (WALKER *et al.* 2003). It completes its larval, nymphal and adult stages on one host (CFSPH 2007) and the eggs are laid and hatched in the surrounding environment (SONENSHINE 1991; WALKER *et al.* 2003). The mode of feeding is influenced by each life stage and the environment.

Feeding cycles coincide with the active life cycles on the host (OLIVER 1989). *R. microplus* females have a circadian feeding cycle, interspersed with periods of ingestion, salivation and resting (OLIVER 1989). Photoperiod, temperature, moisture, host and mate abundance influence all stages of the tick, especially feeding and mating (OLIVER 1989). Diapause occurs in all ixodid ticks and is synchronised with biological and environmental conditions in the circadian feeding cycle of *R. microplus* (OLIVER 1989). Diapause is behavioural, such as the delay of engorgement of already attached ticks, or morphogenetic (developmental), which results in the delay of embryogenesis, delay of moulting of immature ticks or ovipositing by engorged females (OLIVER 1989; SONENSHINE 1991). Behavioural diapause occurs during larval stages and allows larvae to over-winter until optimal conditions arise. Morphogenetic diapause induces inactivity in engorged females to delay ovipositing until optimal conditions arise in summer (SONENSHINE 1991).

Rhipicephalus microplus mating occurs on the host during feeding (OLIVER 1989; SONENSHINE 1991; WALKER *et al.* 2003). Engorged females drop from the host and oviposit one large egg mass (3000-5000 eggs) in the natural environment (OLIVER 1989; SONENSHINE 1991; WALKER *et al.* 2003). *R. microplus* can produce 3-4 tick generations per year (OLIVER 1989), increasing the tick population in the environment within a short amount of time. Figure 9 represents the seasonal dynamics of *R. microplus* and *R. decoloratus* tick populations on cattle farms in South Africa.

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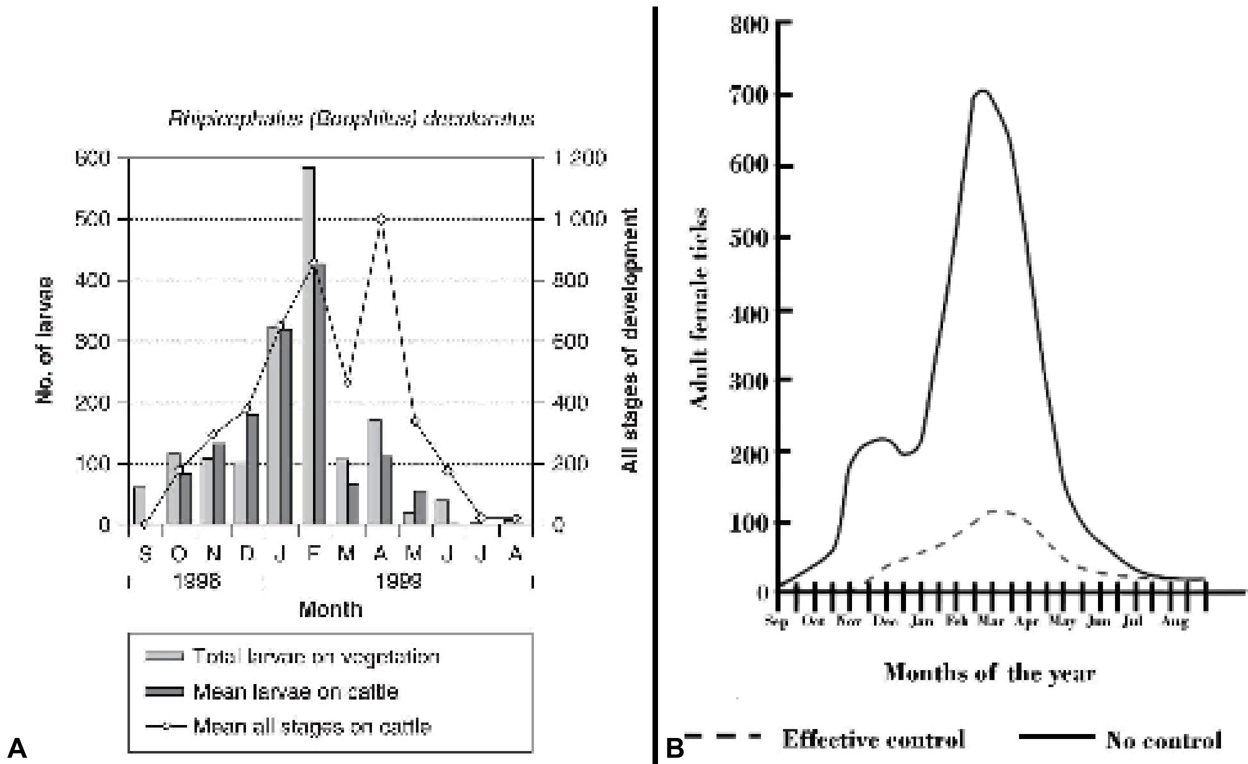

Figure 9: The seasonal change in tick numbers of *R. decoloratus* and *R. microplus* on cattle farms in South Africa. 9A: The seasonal occurrence of *R. decoloratus* on cattle and vegetation during September 1998 to August 1999 in the eastern region of the Free State Province (PHALATSI *et al.* 2004). **9B:** General seasonal dynamics of Blue ticks, *R. microplus* and *R. decoloratus,* on cattle farms in South Africa. During October to December the first generation of larvae can be observed on cattle. During February to April ticks have produced 3-4 generations and are present in large numbers when autumn arrives. Modified from Oberem (2006).

R. microplus and *R. decoloratus* have similar seasonal dynamics and occur in high numbers during the summer months (high temperatures and rainfall) in South Africa. The occurrence of climate change may cause a shift in the seasonality of the ticks as well as an increase in their abundance during optimal conditions. Thus, both biological and environmental factors play a role in tick life cycles and, ultimately, their survival each generation. When optimal conditions arise, these are marked by tick population flares and the increased incidence of tick-borne disease on cattle farms. These large tick populations are able to avoid inbreeding and maintain high genetic diversity through their complex mating strategies.

3.8 Reproduction and mating strategies

The reproductive strategies and mating structure within *R. microplus* populations are varied. This generates a complex mating system and ensures high genetic diversity within tick populations. *R. microplus* ticks are diploid organisms with an XX/XO mating system (OLIVER 1989; SONENSHINE 1991). Females are XX with 22 chromosomes (20 autosomes and two sex chromosomes) and males are XO with 21 chromosomes (20 autosomes and one sex chromosome) (OLIVER 1989; SONENSHINE 1991). Recent fluorescent *in-situ* hybridization (FISH) studies indicate that all the chromosomes are

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holocentric (centromere lacking) and that the X-chromosome is larger than the autosomes (HILL *et al.* 2009). Previously the autosomes were described as acrocentric and the X-chromosome metacentric (SONENSHINE 1991). FISH also indicated large amounts of heterochromatin and repetitive sequences within the chromosomes (HILL *et al.* 2009).

The haplodiploid mating status (XX/XO sex chromosomes) of *R. microplus* follows Haldane's rule of hybrid sterility, where "When in the offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous (heterogametic) sex" (JOHNSON and LACHANCE 2012; SCHILTHUIZEN *et al.* 2011). This may have implications for the divergence and speciation in other heterogametic organisms, such as those with XX/XY and ZZ/ZW systems (CRESPI and NOSIL 2013; JOHNSON and LACHANCE 2012), but may not have an effect on hemizygous (XX/XO) organisms as there may be an absence of strong selection on the haplodiploid males (SCHILTHUIZEN *et al.* 2011). Therefore sexual dimorphisms may not affect the genetic diversity of *R. microplus*, but this remains unclear and requires further investigation.

Rhipicephalus microplus male and females are dimorphic in their adult stages (SONENSHINE 1991). This bisexual species produce progeny in a 1:1 sex ratio (OLIVER 1989). Progeny are mostly produced by fertilised females, but in the absence of male ticks, female *R. microplus* ticks are capable of parthenogenesis (CUTULLÉ *et al.* 2010; OLIVER 1989; SONENSHINE 1991). Thelytoky, a type of parthenogenesis in ticks, produces only female progeny (SONENSHINE 1991), these larvae are often weak and unable to feed and die soon after hatching (OLIVER 1989). Parthenogenesis is not common for *R. microplus*, but is an important reproductive strategy for *Haemaphysalis* spp. (CUTULLÉ *et al.* 2010; SONENSHINE 1991). Although the progeny produced by parthenogenesis still retain their diploid status (CUTULLÉ *et al.* 2010; SONENSHINE 1991), sexual reproduction remains the primary method for generating high genetic diversity among the offspring.

The maintenance of high genetic diversity in *R. microplus* population will likely be similar to that of *R. australis*. Their pangamy mating structure (random association of male and female genotypes) (CHEVILLON *et al.* 2007b) provides offspring with multiple parents (males mate with several females) (CUTULLÉ *et al.* 2010). This reduces the probability of sib-mating (CHEVILLON *et al.* 2007b) and ensures that little inbreeding occurs within the breeding population (KOFFI *et al.* 2006a). High genetic diversity and the production of multiple generations within one year allow *R. microplus* populations to evolve and adapt quickly. The high turnover of ticks each season will also influence the occurrence of tick-borne disease on cattle farms, the acquisition and spread of acaricide resistance in tick populations.

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3.9 The accumulation of acaricide resistance in *R. microplus*

Rhipicephalus microplus control measures include the use of single, multiple and rotational acaricides to limit tick numbers and spread (BAFFI *et al.* 2007; GUERRERO *et al.* 2007; LI *et al.* 2007). Other control programmes include resistance breeding in cattle (GASPARIN *et al.* 2007) and tick vaccines to stem tick infestations and to control their associated tick-borne diseases (CFSPH 2007). The main acaricides used against ticks include amitraz, pyrethroids and acetylcholine esterase inhibitors. *R. microplus* gains resistance to multiple acaricides within a few generations (BUDELI *et al.* 2009; LI *et al.* 2007), due to their high genetic diversity and the pangamy mating structure within tick populations (CHEVILLON *et al.* 2007a; CHEVILLON *et al.* 2007b; CUTULLÉ *et al.* 2010). The high genetic diversity within tick populations is attributable to the slippage of the replication machinery. Slippage generates point mutations and frame shifts within the sequences targeted by an acaricide (CHEN *et al.* 2007; HERNANDEZ *et al.* 2002; HOPE *et al.* 2010; TSAGKARAKOU *et al.* 2009). This leads to altered gene expression between susceptible and resistant tick strains and/or results in the failure of acaricides to recognise their targeted sequences (BAFFI *et al.* 2007; GUERRERO *et al.* 2007; LI *et al.* 2007).

Attempts to extend the use of acaricides through rotational programmes using multiple acaricides have proven to be unsuccessful (RODRIGUEZ-VIVAS *et al.* 2011). Resistance genes spread via gene flow to other farms and/or provinces, given that the cattle carrying the resistant ticks move between the areas. The spread of resistance genes between provinces is limited in areas where cattle movement is restricted and cattle are treated with acaricides before their movement into new areas (BUDELI *et al.* 2009; CFSPH 2007; CUTULLÉ *et al.* 2009). This suggests that the main level of resistance acquisition occurs at the farm level rather than the spread of resistance genes amongst cattle, specifically in Australia and America, where cattle movement is highly restricted and monitored (CFSPH 2007; CUTULLÉ *et al.* 2009). The genetic variation generated at farm level remains the main foundation of acaricide resistance generation in South Africa, yet cattle movement is not properly restricted or closely monitored. Thus, the spread of the acaricide resistance from resistant tick populations to susceptible tick populations will continue to be assisted by cattle movement between farms, especially at communal cattle feeding and dipping lots.

Recently multiple countries have reported acaricide resistance of *R. microplus* populations, especially third world countries, such as Brazil (MENDESA *et al.* 2011), Mexico (RODRIGUEZ-VIVAS *et al.* 2011) and India (VATSYA and YADAV 2011) and Latin America (HENRIOUD 2011). Table 7 summarises the acaricide resistance of tick populations in South Africa to single and multiple acaricide classes.

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Table 7: Acaricide resistance for the years 1998-2001 and 2008 (PESTICIDE RESISTANCE FACILITY 2010). With permission of Ms Ellie van Dalen.

	1998-2001	2008
Resistance to one acaricide class	36.1%	43.5%
Resistance to two acaricide classes	19.5%	37.9%
Resistance to three acaricide classes	0	2.25%
Overall increase of acaricide resistance	**29.1%**	

Increased acaricide resistance in tick populations has led to the development of various molecular methods to determine acaricide resistance in ticks (BUDELI *et al.* 2009; LI *et al.* 2007; PORTO NETOA *et al.* 2011). These act as timely warning systems for acaricide resistance accumulation in tick populations. The rapid occurrence of acaricide resistance has driven the search for alternative methods for tick control that does not include the use of acaricides (HENRIOUD 2011; WALKER 2011). One such method for acaricide resistance detection in populations includes the sequencing of specific genes with known mutations that result in acaricide resistance and scoring the status of ticks collected from provinces within a country against the benchmark (CHEN *et al.* 2007; HERNANDEZ *et al.* 2002; HOPE *et al.* 2010; TSAGKARAKOU *et al.* 2009). These molecular methods to determine acaricide resistance will provide constant surveillance of acaricide resistance at a molecular level in tick populations and will promote early actions in tick control to prevent further resistance accumulation and subsequent economic loss. Alternative tick control methods are being developed, which include tick vaccine strategies.

3.10 Variable vaccine efficacy and resistance in *R. microplus*

Tick vaccines have been produced against *R. microplus* based on the Bm86 gut antigen proteins of the tick (DE LA FUENTE *et al.* 2007; PARIZI *et al.* 2009). Cattle are immunised with this inactivated Bm86 protein as a tick control strategy in the field. Vaccines offer several advantages over acaricides: first vaccines are cost effective for the farmer in comparison to acaricides; secondly they do not contaminate the environment, milk or meat products as with acaricides; and thirdly the use of vaccines has also been shown to reduce the transmission of ticks and tick-borne diseases (DE LA FUENTE *et al.* 2007). It is therefore no surprise that vaccines are increasingly favoured as tick control by cattle farmers.

The Bm86 vaccines act mainly against the female ticks and tick population expansions. This is achieved by reducing the number of engorged female ticks on cattle, reducing the weight of ticks and reducing the reproductive capacity of female ticks (DE LA FUENTE *et al.* 2007). Tick vaccines are a more promising tick control strategy than acaricides, although, many limitations still exist. Immunisation of cattle with the tick gut proteins only provides partial immunity to bovines (PARIZI *et al.* 2009). The Bm86 vaccine's effectiveness varies from 51-91% depending on the characteristics of each tick population and the breed of cattle immunised (PARIZI *et al.* 2009). The variation of the tick vaccine results may be

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due to the sequence difference in the Bm86 sequences of the different tick populations (PARIZI *et al.* 2009). The Argentinian (South American) *R. microplus* has shown great sequence diversity within its Bm86 gene, which was not observed in the Cuban strains or in *R. australis* (PARIZI *et al.* 2009); this might explain the resistance observed in the Argentinian strain to Bm86-based vaccines. Reports have indicated that Bm86-based vaccines are becoming ineffective, due to ticks gaining possible resistance against the vaccines (GARCIA-GARCIA *et al.* 2000; PARIZI *et al.* 2009).

Effective vaccine development requires understanding of, and information relating to the differences in tick species and tick populations at farm, regional (provincial) and country level. Targeted vaccines developed with reference to variant-specific information should be superior in addressing the negative economic impacts associated with tick infestations.

3.11 Economic impact of *R. microplus* on cattle farming in South Africa

Ticks have an impact on the agricultural and economic sectors of countries. *R. microplus* is economically very significant and it is continuously spreading in both its geographical distribution and in terms of its host range (DE MATOS *et al.* 2009; LYNEN *et al.* 2008; TONETTI *et al.* 2009; TØNNENSEN *et al.* 2004). The loss of cattle condition and the damage to hides due to scar tissue formation around tick bite sites, both as a result of tick feeding, is a major revenue stream problem for cattle famers (WALKER *et al.* 2003). Cattle farmers also lose cattle due to the lethal array of diseases carried by *R. microplus*, such as *Babesia* (redwater), *Anaplasma* (gall sickness) and *Borrelia* (spirochaetosis) (WALKER *et al.* 2003). Cattle deaths constitute a direct loss of capital and is not limited to just a lower income stream. The increased costs associated with cattle farming (cattle rearing, tick control and tick-borne disease control) and the increased number of imports of cattle products into South Africa (18.8% for red meat (DAFF 2011a) and 17.2% for milk (DAFF 2012) have great implications for food security in South Africa. It also impacts on the job safety for 500 000 people employed by the beef industry (DAFF 2010), as well as the other 2 125 000 people dependent on the livestock industry for their livelihoods (DAFF 2010).

Cattle are an important source of food and income in South Africa (VAN DJIK 2008). Approximately 69% of South African agricultural land is used for cattle production (DAFF 2011a). There are 14.1 million cattle in South Africa, of these 60% were owned by commercial farmers and 40% were owned by emerging and communal farmers (DAFF 2011a). The total gross value of agricultural production for 2011/12 was estimated at R158 557 million (DAFF 2011a). Animal products contributed 47.7% (R75 632 million) to the total gross value of the agricultural production and slaughtering of cattle and calves contributed 15.9% (R25 210 million) (DAFF 2011a). The total gross income from milk production for 2011/12 was estimated to be R9 224 million, which contributed 0.5% to global milk production (DAFF 2012). Therefore the total gross income of beef and milk production alone for 2011/12 was R34

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434 million. The largest recorded loss to the agricultural industry was from the use of ectoparasiticides by farmers, this includes ectoparasite control for animal and crop production. During 2011 the expenditure on dips and sprays by farmers to control ectoparasites increased by 19.0% from the previous year (DAFF 2011a; DAFF 2011/2012). The total cost of dips and sprays was approximately R7 500 million, which was 8.4% of the total expenditure on intermediate goods and services during 2011/12 (DAFF 2011a; DAFF 2011/2012).

Globally the economic impact of tick-infestation and tick-related losses is estimated to exceed 10 billion US dollars per year, but probably has not reached 100 billion US dollars per year (PARIZI *et al.* 2009). In South Africa, record of the economic impact of ticks is lacking. Therefore, calculations were performed with reference to the total production costs of milk, cattle and calves slaughtered and the loss in cattle weight due to *R. microplus* feeding (Table 8 and 9). Tick impact on milk production could not be isolated but research data was available for cattle weight loss, which allowed for inference of economic loss in beef production. All calculations are supplied in Appendix A.

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Table 8: Total estimated cost of cattle production, defined as the sum of the total production of beef and milk for the time period of 2005-2012 (DAFF 2011a; DAFF 2011b; DAFF 2011/12).

Year	2005/2006	2006/2007	2007/2008	2008/2009	2009/2010	2010/2011	2011/2012
Total (Rands in billion)	13.8	17.1	21.3	21.5	23.2	24.1	27.7

Table 9A: The estimated economic impact of ticks on the cattle industry in South Africa. The number of ticks, weight retardation, initial and final cattle weights data was obtained from Little (1963) and the 2011/2012 beef production (DAFF 2011a; DAFF 2011b; DAFF 2011/12).

Average number of ticks per animal over period	Expected Retardation ("ER") (lb/year)	Starting weight ("SW")	End weight ("EW"), Uninfested group ("UF") EW = (UF - ER)	Retardation % ("R%") R% = ER/UF	Estimated value of economic loss in beef production assuming average number of ticks per period (Rands in billion)
40	56	344.5	478	10.49%	1.85
50	82	344.5	452	15.36%	2.70
60	108	344.5	426	20.22%	3.56
70	134	344.5	400	25.09%	4.42
80	160	344.5	374	29.96%	5.27

Table 9B: Categories required for the estimated economic impact of quantified tick infestation levels (LITTLE 1963).

Control group gained 55lb more than infested group for the period of experiment		
Category	**Amount**	**Unit**
Average stating weight of infested group = (350+339)/2	344.5	lb
Average end weight of infested group = (498+460)/2	479	lb
Average end weight of uninfested end group ("UF")	534	lb
Value of beef production 2011/2012	17.60	rand billion

From Table 9, the cost of tick burden per year was estimated to be between R 1.85bn (40 ticks per animal) and R 5.27bn (80 ticks per animal). This estimate coincides with another calculation based on the red meat prices and capital losses caused by ticks obtained from previous estimates (BIGALKE 1980), which amounted to R1.3bn – R3.1bn annually (calculations supplied in Appendix A). It must be noted that both these estimates do not include the total cost of ticks to the economy, only the effect on beef production. The total cost would be the sum of cattle losses; the costs of conditioning of cattle expressed in longer production cycles and quality defects due to tick feeding, and the expenses incurred in combating tick infestations and the treatment of sick animals. Thus these values are underestimates of the true cost of ticks to the economy. Due to this large socio-economic impact of ticks and tick-borne diseases, research focusing on the development of new acaricides and vaccines are constantly underway to battle the ever-resistant *R. microplus*. In order to achieve effective tick control, knowledge about the genetic diversity and molecular biology of *R. microplus* is required.

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4. Molecular biology and genetics of the southern cattle tick, *Rhipicephalus microplus*

4.1 Nuclear and mitochondrial genomes

The *R. microplus* genome has not been fully assembled, due to the highly repetitive nature of the genome (BELLGARD *et al.* 2012; GUERRERO *et al.* 2010). Currently 1.8 Gbp of unique genome sequence is available from nine sequence databases on the CattleTickBase website (BELLGARD *et al.* 2012). These databases contain sequences mainly sequenced by Cot sequencing, which removes repetitive DNA to construct a partial genome sequence (GUERRERO *et al.* 2010; MOOLHUIJZEN *et al.* 2011). The *R. microplus* genome is estimated to be 7.1 Gbp in length, which is approximately 2.5 times larger than the human genome (BELLGARD *et al.* 2012). It contains up to 70% repetitive DNA (BELLGARD *et al.* 2012), which mainly consists of moderately repetitive DNA (38%) in short interspersed patterns (60-85%). These are found throughout the genome and includes transposable elements and members of multigene famillies (ULLMANN *et al.* 2005). The *R. microplus* genome has a high AT-richness (54-57%) (BELLGARD *et al.* 2012; GUERRERO *et al.* 2010; MOOLHUIJZEN *et al.* 2011; ULLMANN *et al.* 2005). Additional information of the *R. microplus* genome is limited. Due to this lack of gene structure information, the gene structure of *Ixodes scapularis* (IscaW1), a well-studied ixodid tick species that can be used for comparative purposes is provided in Table 10. Table 10 also includes the mosquito, *Culex pipens* (cpip12), and the fruit fly, *Drosophila melanogaster* (fb5.5), for comparison of ticks to other parasites and model organisms.

Table 10: Gene structure of *Ixodes scapularis* (tick), *Culex pipens* (mosquito) and *Drosophila melanogaster* (fruit fly). Reconstructed from Arthropod gene structure statistics 2009 compiled by D. Gilbert available from *Daphnia* Water Flea Genome Database (http://wfleabase.org/genome-summaries/gene-structure/).

	Tick	Mosquito	Fruitfly
Genome size (Mbp)*	1,760 (753)	580 (400)	180 (120)
No. of genes	20,500	18,900	13,700
Gene density	0.023	0.055	0.168
Gene length	12,500	4,800	4,000
CDS Size	1,070	1,400	1,650
No. of exons/genes	5.7	3.5	4.0
Exon size	190	420	410
Intron size **Mean** **Intron>Exon**	1,730/90 2,400 87%	64/1,900 1,400 36%	63/750 660 27%
UTR size	540	240	800
Alternate transcripts	15%	-	36%
Intergenic size	26, 700	18, 200	5, 400

*The value in brackets, is the total gene-containing sequences (excluding heterochromatin and scaffolds without genes). Gene density is calculated as the sum of coding exon bases/total gene-containing genome bases. Gene length includes introns and UTRs. Exon size is given in mean base pairs. All statistics have a standard deviation close to the mean, whereas the intergenic size has a larger variance.

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With reference to Table 10, it is clear that tick genomes are larger than that of mosquitoes (another haematophagous parasite) and fruit flies (a model invertabrate). This trend is also observed for the number of genes, gene length and intron size. *I. scapularis* has large intergenic regions, which may correlate with the high abundance of repeat sequences found in tick genomes. Information regarding the mitochondrial genome is available for *R. microplus* and it has similar characteristics to the nuclear genome. Figure 10 indicates the relative order of genes in the *R. microplus* mitochondrial genome.

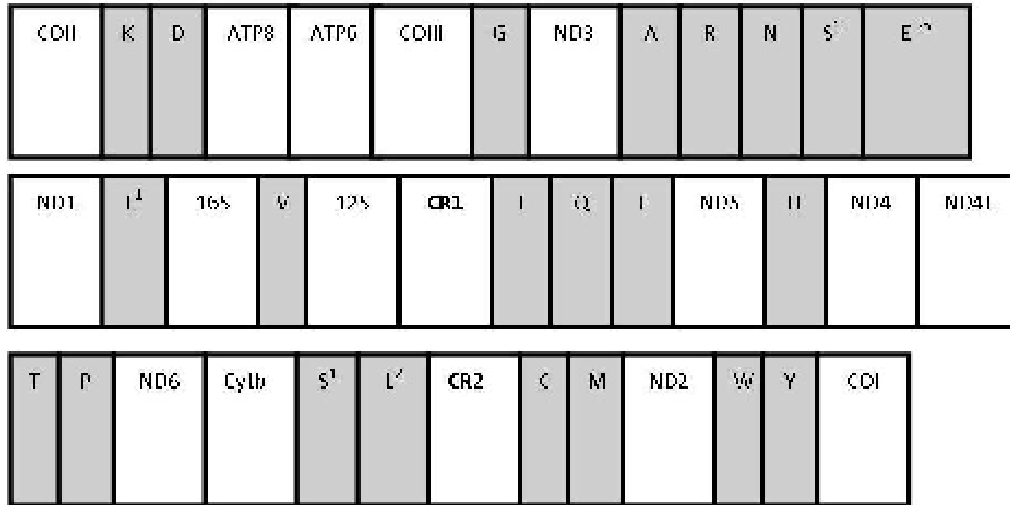

Figure 10: The linear organisation of the *R. microplus* mitochondrial genome. Gene abbreviations: ND = NADH dehydrogenase subunit, CO = cytochrome *c* oxidase subunit, A6 = ATP synthase 6, A8 = ATP synthase 8, Cytb = cytochrome *b* apoenzyme, 12S = small-subunit ribosomal RNA, 16S = large-subunit ribosomal RNA, CR = control regions indicated in bold. The tRNA genes are shaded and labelled with the one-character abbreviation of the amino acid their transcripts transfer. Those tRNA genes labelled with two-character abbreviations are as follows: L^1 = gene for tRNA Leu(UUR), L^2 = gene for tRNA Leu(CUN), S^1 = gene for tRNA Ser(UCN), S^2 = gene for tRNA Ser(AGN), E^{1-5} putative functional tRNA Glu gene, E2–E5 = putative tRNA Glu pseudogenes. Modified from Campbell and Barker (1999).

Approximately, half of the *R. microplus* mitochondrial genome (7 565bp) has been sequenced (CAMPBELL and BARKER 1999). It has a low GC content and a novel fivefold tandem repeat of the $tRNA^{Glu}$ (Figure 10) (CAMPBELL and BARKER 1999). The mitochondrial genome of *R. microplus* has rearrangements of three genes when compared to the genomes of *Ixodes hexagonus*, another hard tick, and *Limulus polyphemus*, the horseshoe crab (CAMPBELL and BARKER 1999). No numts (nuclear copies of mitochondrial DNA) have been recorded for *R. microplus* and no evidence for numts were found in other *Ixodes* species (SHAO *et al.* 2005). Gene duplication, replication slippage and rearrangement or illegitimate recombination events (SHAO *et al.* 2005) are important drivers of the evolution of the mitochondrial genome (CAMPBELL and BARKER 1999).

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4.2 Phylogenetic evaluation of *R. microplus*

Phylogenetic studies investigate the evolutionary relationship amongst genes and/or species (LEMEY *et al.* 2009). This gives an indication as to how closely gene/species are related to one another (LEMEY *et al.* 2009). Phylogenies classify organisms by reconstructing the evolutionary speciation of organisms (ABERCROMBIE *et al.* 2004; KING and STANSFIELD 2002). Phylogenetic research involves considering the amount of similarity between genes/organisms, with the assumption that genes used in the study are homologous (have a common ancestor) (LEMEY *et al.* 2009). Homologous sequences must be sufficiently similar to one another for phylogenetic comparisons. If two sequences have become highly variable, they will not carry enough information about the evolutionary relationship between them (LEMEY *et al.* 2009) and will hence not be suitable for phylogenetic analyses. Homoplasy may obscure phylogenetic relationships due to the evolution of similar structures or sequences without common ancestry (LEMEY *et al.* 2009).

Evolutionary forces introduce differences between sequences over time, such as insertions, deletions and substitutions (LEMEY *et al.* 2009). Therefore, any two sequences derived from a common ancestor that evolve independently of each other will eventually diverge (LEMEY *et al.* 2009). Measures of genetic divergence includes genetic distance and nucleotide substitution models (LEMEY *et al.* 2009). Natural selection and the rate of mutation differ among coding and non-coding sequences. Therefore, each has their own advantages and disadvantages when used for analysis of taxa at family, genus and species levels. Various sequences are used for phylogenetic analysis of ticks and will be discussed below.

4.2.1 Coding regions

Genes reside in coding regions and two classes of genes are recognised. First, structural genes code for functional proteins and modifications of the product may occur to generate additional/altered functional proteins (KING and STANSFIELD 2002). Secondly, there are genes that are transcribed into transcripts (tRNA, rRNA and snRNA) that are involved in protein production (KING and STANSFIELD 2002). Coding regions have high functional constraint and thus high selection pressure. Synonymous substitutions are tolerated in coding regions as this does not change the final protein product (KREITMAN and COMERON 1999). Coding sequences do not easily gain mutations that will influence the final protein’s function, as this may be detrimental to the organism. Therefore, coding sequences remain conserved in organisms and their evolution is slower than non-coding regions. This allows the use of coding sequences to determine the rate of conserved sequence evolution and it is restricted to the phylogenetic resolution of family or genus level (NAVAJAS and FENTON 2000). Species evolution is

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not always directly related to gene evolution and requires the use of both coding and non-coding sequences with different evolutionary rates (LEMEY *et al.* 2009).

a) Mitochondrial genes

Mitochondrial genes occur in large numbers in the each cell, but as mitochondrial sequences are maternally inherited, they create an evolutionary bottleneck where large portions of the population carries the same sequence (CRUICKSHANK 2002). The mitochondrial genome of *R. microplus* has a high mutational rate and its genes are therefore highly variable (CAMPBELL and BARKER 1999). The strict maternal inheritance of mitochondrial genes are useful for phylogenetic studies (CRUICKSHANK 2002) as it traces the maternal inheritance of genes within the population. The cytochrome c oxidase (*CO*) and ribosomal rRNA (*16S* and *12S*) mitochondrial genes will be discussed, each with their own advantages and disadvantages for their use as phylogenetic markers. With reference to Figure 10 in section 4.1, the *16S* and *12S rRNA* coding genes are located next to one another, whereas cytochrome c oxidase subunit 3 (*COIII*) is separated from cytochrome c oxidase subunit 1 (*COI*) and 2 (*COII*) by ATP synthase genes (CAMPBELL and BARKER 1999).

***16S rRNA* & *12S rRNA* coding genes**

The *rRNA* genes code for subunits of the ribosome, which are involved in the translation of proteins. The *16S rRNA* gene transcript forms part of the large ribosomal RNA subunit and *12S* transcript forms part of the small ribosomal RNA subunit. These genes are not separated by internal transcribed spacers (CRUICKSHANK 2002) as it is in the nuclear genome. The *16S rRNA* gene is mainly used for family and genus level studies for the comparisons of hard and soft ticks (BLACK and PIESMAN 1994). The *12S rRNA* gene has been used for genus and species level phylogeny, where ticks from the *Rhipicephalus* genus were compared with one another (BEATI and KEIRANST 2001). The *12S rRNA* gene has been suggested to resolve more recent speciation events within the *Rhipicephalus* genus (MURRELL *et al.* 2000) and may be suitable for deeper phylogenetic studies. These sequences may be highly variable, due to the high mutation rate and rearrangement events that occur in the mitochondrial genome, which can be advantages to a phylogenetic study if variability can be detected amongst individual *R. microplus* ticks. Or, it can be disadvantageous, where duplications and gene rearrangements may hinder sequence alignments. Norris and colleagues (1999) suggested that *16S* and *12S rRNA* genes are not suitable for higher-level phylogenetic studies as they have low resolution due to the high AT-richness and the high mutational rate of the tick mitochondrial genome (NORRIS *et al.* 1999). They also mentioned that the *18S* nuclear *rRNA* gene is more suited to phylogenetic studies, especially at family level, as its resolution is superior to *16S* and *12S rRNA* genes (NORRIS *et al.* 1999). The authors stated that the *12S* and *16S* may be used in intraspecific phylogenetic studies or the study of closely related taxa (NORRIS *et al.* 1999). Murrell and colleagues (2000) have successfully used the

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12S rRNA and *COI* genes in combination to improve the interspecific phylogeny of the *Rhipicephalus* genus (21 species of *Rhipicephalus*) (MURRELL *et al.* 2000). Therefore, the usefulness of *12S* and *16S rRNA* genes in resolving interspecific and intraspecific relationships may be improved by combining the sequence phylogenies with other mitochondrial genes.

Cytochrome c oxidase genes

Cytochrome c oxidase (*CO*) genes play a role during energy generation within the mitochondrion (KING and STANSFIELD 2002). There are three cytochrome c oxidase subunit genes, cytochrome oxidase subunit 1 (*COI*), 2 (*COII*) and 3 (*COIII*). Cytochrome c oxidase has similar intra-specific resolution capabilities as ITS2, but they evolve slightly faster (CRUICKSHANK 2002) and have been found to be highly polymorphic, despite the high AT-richness of the mitochondrial genome (CRUICKSHANK 2002). *COI* has been successfully used to investigate intraspecific variation in mites (DABERT *et al.* 2010; KAWAZOE *et al.* 2008; ROY *et al.* 2009; SCHÄFFER *et al.* 2010; SCHÖNHOFER and MARTENS 2010) as well as family, genus and species level phylogenies of ticks from the Rhipicephalinae and Hyalominae (MURRELL *et al.* 2001a; MURRELL *et al.* 2001b). Cytochrome oxidase c subunit 1 was also used for the phylogenetic analysis of morphologically similar species of *Ixodes holocyclus* and *Ixodes cornuatus* (SONGA *et al.* 2011). *COII* has not yet been investigated for the use in tick phylogenetic studies, but *COIII* has been successfully used to investigate intraspecific relationships within *Ixodes pacificus* (KAIN *et al.* 1999). Therefore, *COI* remains the most extensively investigated *CO* mitochondrial gene for phylogenetic studies in ticks and has the capacity to resolve interspecific and intraspecific phylogenies.

b) Nuclear ribosomal genes

Nuclear ribosomal genes include the *rRNA* coding genes of the large subunit (*28S, 5.38S* and *5S*) and the small subunit (*18S*) (MURRELL *et al.* 2001a). These molecules form part of the ribosomes, which are involved in the translation of proteins. Internal transcribed spacers (*ITS*) separate the nuclear ribosomal gene cluster. *ITS1* separates the *18S* and *5.8S rRNA* genes, whereas the *ITS2* separates the *5.8S* and *28S rRNA* genes (MURRELL *et al.* 2001a). The *rRNA* genes that flank the *ITS* sequences are highly conserved (MURRELL *et al.* 2001a) and are primarily used for higher phylogenetic studies at family and subfamily levels in ticks. Ribosomal genes *18S* and *28S* are discussed below and the *ITS* sequences are discussed under section 4.5.2a (Non-coding regions).

***18S rRNA* & *28S rRNA* genes**

The *18S rRNA* gene has been used for phylogenetic studies of European hard tick species and the authors suggest that it is not suitable for deeper phylogenies, as it displayed little genetic variation between closely related species (MANGOLD *et al.* 1998). Although the *18S* is not used for intraspecific

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phylogenetic studies, it has greater resolution than *12S* and *16S rRNA* genes for family based studies (NORRIS *et al.* 1999). The *28S rRNA* gene may be used in deep level phylogenies (CRUICKSHANK 2002) and the D3 region of the large ribosomal subunit has been used in phylogenetic studies, as discussed below.

Large ribosomal subunit *D3* region

Divergent region *D3* is AT-rich and lies within a conserved core sequence of *28S rRNA* coding gene (MCLAIN 2001). It is divergent and has proved to be informative in intraspecific phylogenetic studies of six *Ixodes* species (*I. affinis, I. pacificus, I. persulcatus, I. ricinus, I. scapularis* and *I. woodi*) from different geographic localities (MCLAIN 2001). Analysis of the *D3* region for six *Ixodes* species *(I. kingi, I. angustus, I. ricinus, I. scapularis, I. sculptus)*, four *Dermacentor* species (*D. andersoni, D. albipictus, D. variabilis* and *D. occidentalis*) and a *Rhipicephalus sanguineus* tick did not yield high levels of intraspecific variation, but significant interspecific variation (ANSTEAD *et al.* 2011). The authors suggested that this region may not be suitable for all *Ixodes* species, but they did observe sequence variation for the *R. sanguineus* species when they compared it to the published sequence (ANSTEAD *et al.* 2011). It has been suggested that the *D3* region can be used as a species marker in mites (MARK MARAUN *et al.* 2004) and may be applicable to ticks. Therefore the *D3* region may be a good candidate for intraspecific study of *R. microplus*, as some *Ixodes* species are known to have low genetic diversity (MCLAIN *et al.* 2001; NOUREDDINE *et al.* 2011). The genetic diveristy results of the *D3* region can also be compared with the *ITS2* sequence within the same gene region. This can determine the difference in evolutionary rate between the conserved (*D3*) and variable (*ITS2*) regions of the same gene (*28S rRNA*).

c) Nuclear protein-coding genes

Nuclear protein coding genes are challenging to use in phylogenetic studies, due to their single copy number in genomes, which makes them difficult to amplify (CRUICKSHANK 2002). These genes are under functional constraints and therefore have variable substitution and evolutionary rates (CRUICKSHANK 2002; NAVAJAS and FENTON 2000). Adenine Nucleotide Translocase (*ANT*) was used to detect cryptic species within the mite species, *Aceria tosichella* (CAREW *et al.* 2009; NAVAJAS and NAVIA 2010). It displayed limited sequence variation between individuals of the same species (CAREW *et al.* 2009), but may be an informative sequence for intraspecific phylogenetic studies in *R. microplus*. It will therefore be evaluated for the first time in ticks in this study.

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4.2.2 Non-coding regions

Non-coding regions do not code for functional transcripts (ABERCROMBIE *et al.* 2004; KING and STANSFIELD 2002). These include mitochondrial control elements, introns of coding regions and the internal transcribed spacer region (*ITS*) of the nuclear ribosomal genes (CRUICKSHANK 2002; NAVAJAS and FENTON 2000; NAVAJAS and NAVIA 2010). Since, these sequences are non-coding (not always non-functional) they evolve quickly as they are subjected to little selection pressure (CRUICKSHANK 2002). This makes them useful for species and intraspecific phylogenetic studies. Those with low selection pressure are often heterozygous in organisms (CRUICKSHANK 2002) making their analyses difficult. The *ITS* sequences of the nuclear *rRNA* genes will be discussed with reference to section 4.2.1 (Coding regions).

a) Internal transcribed spacer 1 (*ITS1*)

The ITS1 has similar properties to *ITS2*, but is located between the *18S* and *5.8S rRNA* genes (NAVAJAS and FENTON 2000). It is not commonly used in tick phylogenetic studies, but has been used in interspecific and intraspecific phylogenetic studies on mites (CRUICKSHANK 2002; NAVAJAS and FENTON 2000; NAVAJAS and NAVIA 2010). Chitimia and colleagues (2009) used a PCR-RFLP of the ITS1 sequence to differentiate between several ticks species, which included *Dermacentor marginatus, Haemaphysalis punctata, Haemaphysalis parva, Ixodes ricinus* and *Dermanyssus gallinae* (CHITIMIA *et al.* 2009). Due to the rapid evolution of the *ITS* sequences, universal primers (that are located in conserved regions) may fail to amplify the region and these sequences can only be used for intraspecific variation studies (CRUICKSHANK 2002).

b) Internal transcribed spacer 2 (*ITS2*)

The *ITS2* region exists between the *5.8S* and *28S rRNA* genes and is removed after transcription and is non-functional (CRUICKSHANK 2002). The sequence is under very little selection pressure and accumulate substitutions easily (BARKER 1998; CRUICKSHANK 2002), which makes the *ITS2* sequence useful for intraspecific phylogenetic studies. The *ITS2* sequence has been studied extensively and sequence information is available. Studies on *Ixodes* species (*I. scapularis* and *I. holocyclus*), *Amblyomma* (*A. vikirri*), *Aponomma fimbriatum*, *Haemaphysalis humerosa* and *Dermacentor variabilis* ticks indicated that the *ITS2* sequence is highly variable (HLINKA *et al.* 2002). *Ixodes granulatus* species from different geographical localities were distinguished from one another with an intraspecific phylogeny of the *ITS2* sequence (CHAO *et al.* 2011). Therefore, the *ITS2* sequence may be especially useful for phylogenetic studies between closely related species and possibly within species, such as for *R. microplus* from different localities of South Africa.

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Concluding remarks

There are many genes available for phylogenetic studies at inter- and intraspecific levels, each with their own advantages and disadvantages. Some phylogenetic studies of genes, such as the *12S* and *16S* mitochondrial rRNA genes and the *18S* and *28S* nuclear rRNA genes, have yielded various levels of success for both high and deep level phylogenies. Successful studies were highly dependent on the evolutionary rate of the tick species analysed, whereas the intraspecific phylogenetic resolution of the *ITS* sequences is well established. Table 11 summarises the sequences typically used for phylogenetic studies in ticks.

Table 11: A summary of the sequences used for phylogenetic studies in ticks, with their cellular locations and resolution levels.

Sequence	Cellular location	Phylogenetic resolution level
***16S rRNA* gene**	Mitochondrial coding gene	Family, genus, species*
***12S rRNA* gene**	Mitochondrial coding gene	Family, genus, species*
***COI* gene**	Mitochondrial coding gene	Species (intra- and interspecific)
***18S rRNA* gene**	Nuclear coding gene	Family, genus
***28S rRNA* gene**	Nuclear coding gene	Family, genus, species (species resolution in relation to D3 region)
***D3* region**	Nuclear coding gene	Family, genus, species*
***ANT* gene**[†]	Nuclear coding gene	Genus, species
ITS1	Nuclear, non-coding	Species (intra- and interspecific)
ITS2	Nuclear, non-coding	Species (intra- and interspecific)

* Genes that may resolve species phylogenies when combined with other sequences.
[†] New gene evaluated for ticks

The *COI*, *D3*, *ANT* and *ITS2* sequences were used during the present study. These sequences may elucidate intraspecific relationships of *R. microplus* populations from different localities in South Africa.

4.3 Allele networks

Standard phylogenetic relationships are represented as bifurcating leaf-labelled tree (HUSON and BRYANT 2006; HUSON and SCORNAVACCA 2011; LEMEY *et al.* 2009). This assumes that the evolutionary processes underlying the relationship are bifurcating. It may not necessarily be true for a given phylogenetic relationship, especially for relationships influenced by recombination, hybridization, re-assortment and gene transfer (HUSON and BRYANT 2006; HUSON and SCORNAVACCA 2011; LEMEY *et al.* 2009). Therefore, split networks are used to determine whether a bifurcating or multifurcating gene tree or gene genealogy is required to represent the evolutionary relationship between taxa. This provides additional phylogenetic methods to accurately present incompatible evolutionary scenarios in phylogeny and phylogeography (HUSON and BRYANT 2006; HUSON and SCORNAVACCA 2011).

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4.4 An overview of phylogeography relevant to this study

Phylogeography is a multidisciplinary field within biogeography (geographical distribution of plant and/or animals). It is concerned with "the principles and processes governing the geographic distribution of genealogical lineages, especially those within and among closely related species (inter- and intraspecific levels)" (AVISE 1994; AVISE 2009). It deals with historical and phylogenetic components of the spatial distribution of gene lineages as it considers both time and space as axes, which can be mapped onto gene genealogies (AVISE 2000). Figure 11 illustrates the components and disciplines that constitute a phylogeographic study.

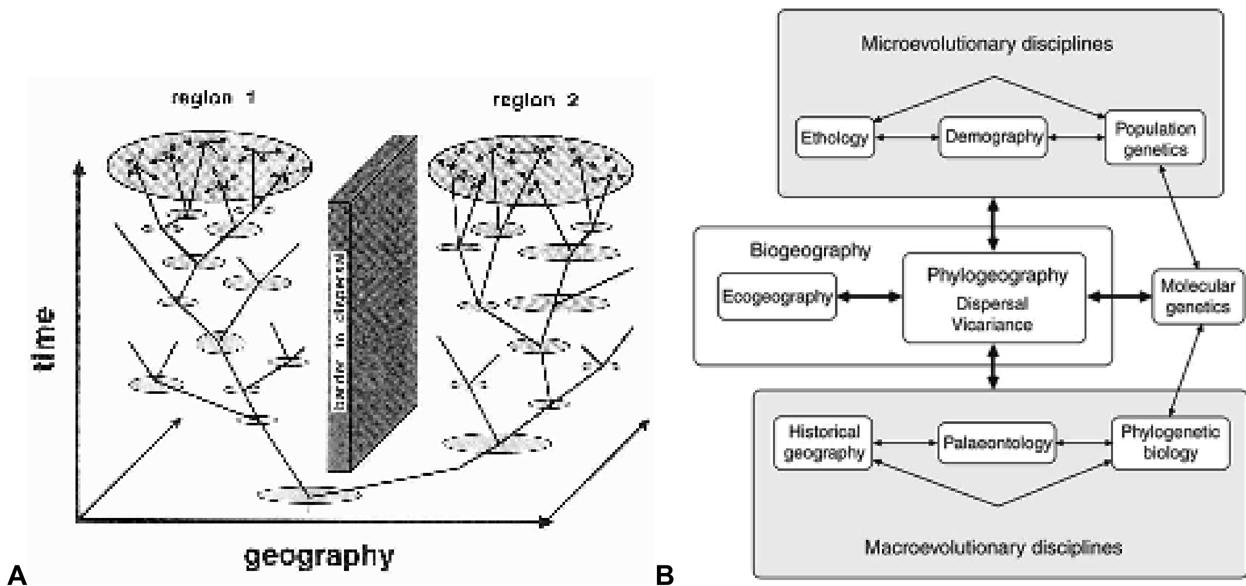

Figure 11: Disciplines that contribute to phylogeography. **11A: "**Phylogeography considers both time and space during the study of gene genealogies. This illustrates the hypothetical gene genealogy for a species displaying restricted gene flow within each of two physically separated regional populations. Shaded ovals represent geographic ranges of particular lineages, and arrows represent vectors in the extant population that denotes special magnitudes of contemporary dispersal of individuals from their natal state." from (AVISE 2000). **11B:** "Multiple disciplines from both macro- and micro-evolutionary fields are included in phylogeographical studies." from Avise (2009).

Phylogeography utilises coalescence and genealogical concordance frameworks to determine the separation of populations (AVISE 2000). The coalescent theory describes the times at which extant lineages trace back to their most recent common ancestor as a function of population size (AVISE 2009; LEMEY *et al.* 2009). This is accomplished by considering the inheritance of selectively neutral genes backwards in time (such as mitochondrial genes and unlinked nuclear genes) (AVISE 2000; CRANDALL and TEMPLETON 1993). For example, mitochondrial DNA is especially useful in phylogeographic studies as it has extensive intraspecific variation, no recombination and is maternally inherited (AVISE 1994). Therefore, the maternal lineage can be traced back through time following the female inheritance pattern and will coalesce at a common ancestor. New developments are underway to extend the

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coalescent theory to multiple loci (BRYANT *et al.* 2011; TAKAHASI 2009), loci under selection and loci that are in epistasis (TAKAHASI 2009). Figure 12 illustrates the concept of the coalescent theory.

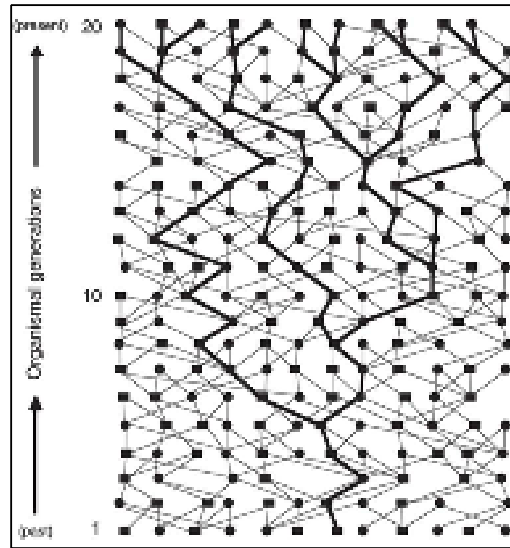

Figure 12: "**Hypothetical gene or haplotype tree (bold lines) of an organism's pedigree over 20 generations.** The most recent common ancestor of the extant population has been traced back in time to the ancestral state. Squares are males, circles are females and lines connect parents to their offspring." The figure was taken from Avise (2009).

Along with the coalescent theory, genealogical concordance is important among different levels of phylogeographical analysis from single gene trees to species trees. Genealogical concordance is a method that recognises separate groups or species in populations when molecular, biogeographic and systematic data coincide (AVISE 2000; GRADY and QUATTRO 2001; MALLET 1995). Therefore, every character of each gene, each gene of a species and additional biogeographic (such as geographical locations) information should group species within a population similarly in order for those species to be considered as separate. Figure 13 illustrates the agreement of phylogenetic pattern required at each level of genealogical concordance to separate distinct phylogenetic groups in populations.

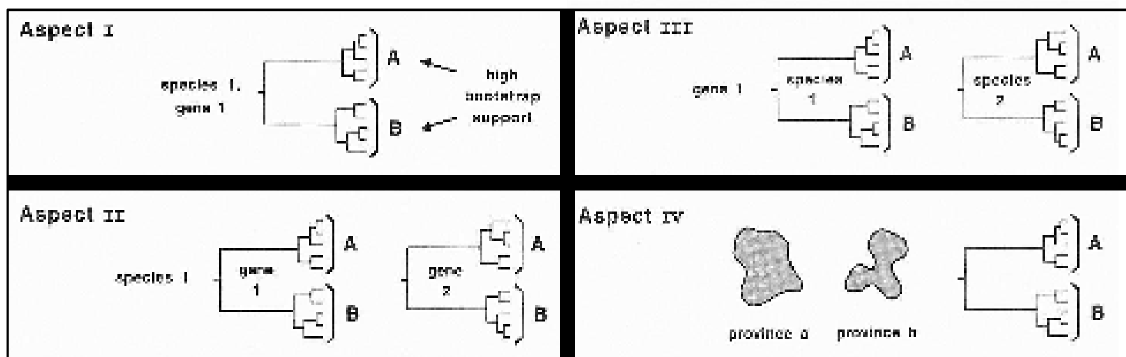

Figure 13: "**The four aspects of genealogical concordance. Aspect I:** Concordance across sequence characters within a gene. **Aspect II:** Concordance in significant genealogical partitions across multiple genes within a species. **Aspect III:** Concordance in the geography of gene-tree partitions across multiple co-distributed species. **Aspect IV:** Concordance of gene-tree partitions with special boundaries between traditionally recognised biogeographic provinces." The figure was taken from Avise (2000).

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Accurate and reliable phylogeographic inferences are highly dependent on similar patterns for every gene tree and for the species as a whole (combinations of multiple gene trees). Coalescence allows determination of the most recent common ancestor of the species currently under study. Therefore, phylogeography relies on the concept of gene trees and that of the coalescent theory to determine the evolutionary forces that shaped extant species.

Phylogeography is a multidisciplinary field of study that utilises gene trees to infer the historical and contemporary forces that have produced the current genealogical architecture of populations and closely related species (AVISE 2009). It differs from phylogenetics in that it is not only reliant on gene genealogies, but includes population history and demographics (AVISE 2009). It differs from population genetics as it also includes gene genealogies and DNA data along with allele frequencies (AVISE 2009). For a complete genetic diversity study of *R. microplus,* each of these disciplines (phylogenetics, population genetics and phylogeography) should be included in the analysis and evaluation of data.

4.5 A brief overview of population genetics relevant to this study

Population genetics is the study of the genetic composition of biological populations (KING and STANSFIELD 2002). It is concerned with the origin, amount and distribution of genetic variation in biological populations and how genetic variation affects the population through space and time (TEMPLETON 2006). Population genetics study the frequencies of alleles, genes, genotypes and phenotypes. Different evolutionary changes and factors influence the genetic variation and allele frequency of populations such as genetic drift, natural selection and mutation (TEMPLETON 2006). This micro-evolutionary change at DNA level ultimately leads to the adaptation of organisms to certain environments. Genetic variation is monitored through space and time by studying molecular markers within species or populations (TEMPLETON 2006). These markers can be coding regions (functional genes) or non-coding regions (microsatellites). Microsatellites are hyper-variable making them ideal for the studying variation in natural populations (BARKER 2002; BHARGAVA and FUENTES 2010; ELLEGREN 2004; GOLDSTEIN and POLLOCK 1997).

4.6 Microsatellites and their contribution to this study

Microsatellites are repetitive DNA sequences of 2-6 base pairs in tandem arrays (NAVAJAS and FENTON 2000). These are also termed; simple sequence repeats (SSR) and short tandem repeats (STR) due to its ubiquitous repetitive nature (BHARGAVA and FUENTES 2010). Microsatellites are further subdivided into firstly perfect (where the repeat sequence is not interrupted), secondly imperfect (where the repeat sequence is interrupted), and finally uninterrupted microsatellites (where small sequences lie interspersed within the main repeat sequence or where more than one repeat motif make up the repeat

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region) (BHARGAVA and FUENTES 2010). Table 12 indicates the classification of microsatellites in relation to their architecture.

Table 12: The classification of microsatellites according to their repeat structure (BUSHIAZZO and GEMMELL 2006).

Class	Number of repeat motifs	Examples[a]
Pure	1	$-(CA)_{12}-$
Interrupted pure	1	$-(CA)_8-(CT)-(CA)_3-$
Compound	2	$-(CA)_9-(GAA)_5-$
Interrupted compound	2	$-(CA)_9-(CAA)-(GAA)_4-$
Complex	>2	$-(CA)_4-(T)_7-CAAT-(CTT)_8-$
Interrupted complex	>2	$-(CA)_2-CT-CA-(T)_7-(CTT)_8-$

[a] These hypothetical sequences are given for clarity. Real examples can be found in the references listed by Bushuazzo and Gemmell (2006).

Microsatellites are excellent candidates to use as polymorphic DNA markers for population genetics and phylogeographic analysis for high resolution genetic mapping (GOLDSTEIN and POLLOCK 1997) and to determine the amount of gene flow that exists between populations (ELLEGREN 2004). This is mainly due to the fact that microsatellites have the following characteristics. Firstly, they are easy to use, identify and score (BHARGAVA and FUENTES 2010). Secondly, they are highly variable in organisms that display little genetic variability (BARKER 2002; BHARGAVA and FUENTES 2010; GOLDSTEIN and POLLOCK 1997). Thirdly, their well-defined repeat structure and unique priming sites allow for high reproducibility (BHARGAVA and FUENTES 2010; GOLDSTEIN and POLLOCK 1997). Lastly, many organisms have microsatellites within their genomes (BARKER 2002; BHARGAVA and FUENTES 2010) and microsatellites offer a low cost tool for use in genetic studies (BHARGAVA and FUENTES 2010).

There are many factors that influence the evolution of microsatellite markers. Microsatellites can be found throughout the genome, and depending on whether they are within coding or non-coding regions, affects their mutational rates (BUSHIAZZO and GEMMELL 2006). Microsatellites within coding sequences will have lower mutational rates, when compared to non-coding regions as selection will influence the addition of repeats in the microsatellite, where trinucleotide repeats are more favourable (ELLEGREN 2004). Microsatellites evolve through the increase in the repeat unit length (facilitated mainly through replication-slippage) but involves an intricate play between recombination, replication slippage and indel slippage (BHARGAVA and FUENTES 2010). Generally microsatellites with larger numbers of repeats will mutate more often and result in higher evolutionary rates of change, leading to more polymorphisms (BHARGAVA and FUENTES 2010). This indicates that different microsatellite repetition types will have different mutation rates, such that tetranucleotide repeats will have a lower mutation rate than those of dinucleotide repeats (BHARGAVA and FUENTES 2010). Interruptions introduced by indels (insertions are more common than deletions) decreases the variability and evolutionary rate, resulting in imperfect microsatellites mutating at a lower rate than perfect repeats (BHARGAVA and

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FUENTES 2010). Too many interruptions in the repeat region leads to instability and the eventual death of a microsatellite (BUSHIAZZO and GEMMELL 2006). Microsatellites also have different mutational rates within organisms of different age and sex (BUSHIAZZO and GEMMELL 2006; ELLEGREN 2004). This is linked to the number of mitotic divisions and germ-line mutations, which tend to be less in females than in males, and will be more prevalent in older organisms (BUSHIAZZO and GEMMELL 2006; ELLEGREN 2004). All of these factors contribute to the final genetic variation at microsatellite loci characterised by high heterogeneity and the occurrence of multiple alleles (ELLEGREN 2004), which are the hallmarks used for genetic diversity studies. The factors influencing microsatellite mutation is summarised in Figure 14.

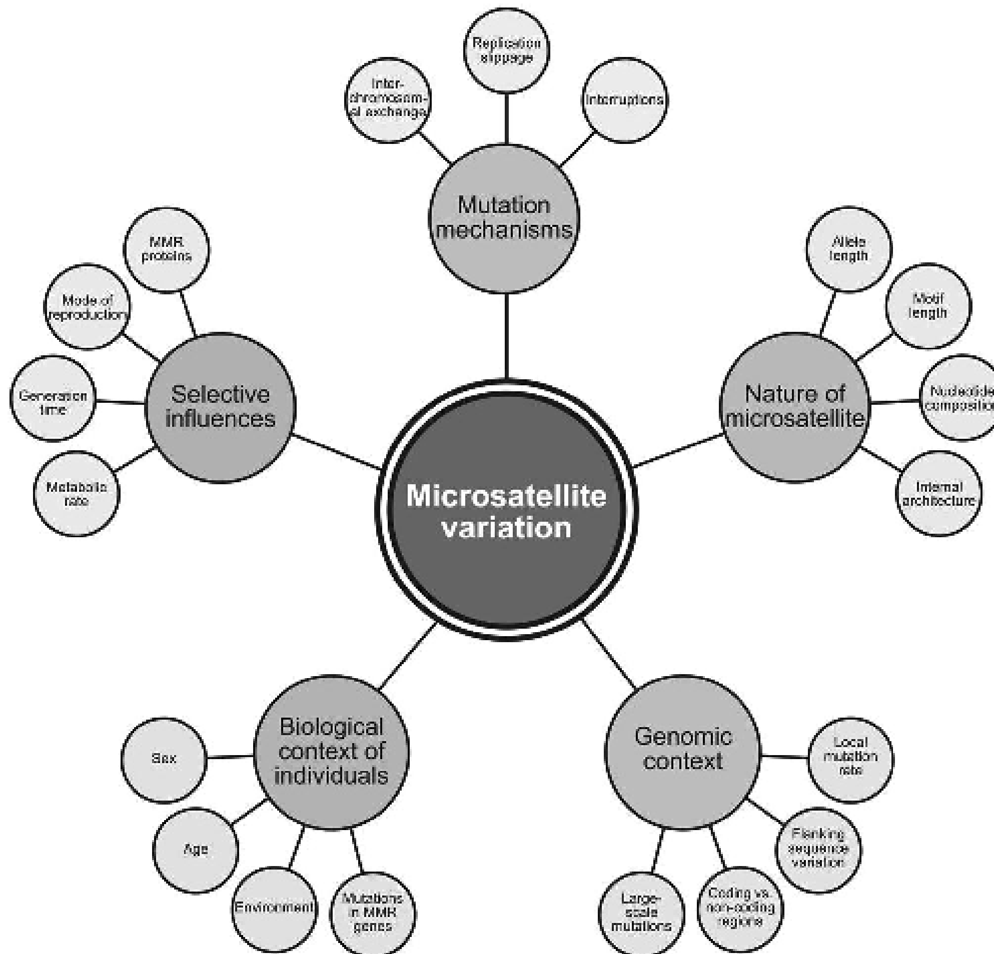

Figure 14: The interconnected factors that influence the mutation of microsatellites, each with variable affects (BUSHIAZZO and GEMMELL 2006).

To date, microsatellites have been used as markers in tick population genetic studies (BARKER 2002; CHAPUIS and ESTOUP 2007; GRIFFITHS *et al.* 2010; GUZINSKI *et al.* 2008; KOFFI *et al.* 2006b) and can offer a promising tool to determine the population structure of *R. microplus* ticks in South Africa. Their highly variable nature and experimental reproducibility allow researchers to distinguish between genetic variants within a species (CASTILLO *et al.* 2008; GADALETA *et al.* 2011; MCCAULEY *et al.* 2009; QUELOZ *et al.* 2010; TAKEZAKI and NEI 2008). Microsatellites would be valuable markers for the study of intra-

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specific variation in the *R. microplus* ticks from South Africa as they have been successfully used in previous genetic studies of ticks from Australia, South America and Zimbabwe (CHIGAGURE *et al.* 2000; CUTULLÉ *et al.* 2009; CUTULLÉ *et al.* 2010).

Eight candidate polymorphic microsatellite loci were identified for *R. microplus* where the number of repeats and their interruptions may allow the identification of subdivision or cryptic speciation within the *R. microplus* species from different geographical locations, including Zimbabwe (CHIGAGURE *et al.* 2000). However, due to the flanking regions of these microsatellites being variable among individuals (OBERHOLSTER 2011), these loci were not regarded as suitable for inclusion in the present study (PDF reference file provided on Appendix CD). These flanking regions will influence the mutational rate of the microsatellite and can lead to incorrect results for the assessment of genetic diversity of tick populations (ELLEGREN 2004). Another nine microsatellites where developed by Koffi and colleagues (KOFFI *et al.* 2006b), following the observation of null alleles when amplifying the microsatellite sequences described by Chigagure and colleagues (2000). This might be due to the fact that Koffi and colleagues (2006b) were using microsatellites from different species, namely *R. australis* vs. *R. microplus*. They developed the latter nine microsatellites for population genetic evaluation of *R. australis* populations by means of GeneScan technology. These microsatellites were used to determine the genetic diversity in a young *R. australis* population in New Caledonia (KOFFI *et al.* 2006b) and indicated sympatric speciation of *R. australis* populations that fed on different host species within the same geographical range in New Caledonia (DE MEEÛS *et al.* 2010). Only two other microsatellite markers for *R. microplus* exist, these are linked to sex-determining chromosomes, where data for BmM1 is available and only the repeat architecture is available for BmM2 (DE LA FUENTE *et al.* 2000).

The availability of microsatellite markers for both *R. microplus* and *R. australis* provides a valuable genetic resource that could be applied to South African tick populations to provide valuable information on the genetic diversity of *R. microplus* ticks. High resolution information on genetic diversity should significantly improve tick vaccine design and implementation to target specific *R. microplus* populations based on their genetic diversity.

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4.7 Technical considerations

4.7.1 Tick identification

The identification of ticks collected from South Africa to genus level can be done visually and without difficulty using the characteristics known for each tick genus is South Africa, namely *Amblyomma*, *Hyalomma* and *Rhipicephalus* (MADDER and HORAK 2010; OBEREM *et al.* 2006; WALKER *et al.* 2003). However, distinguishing between *Rhipicephalus* species, such as *R. decoloratus* and *R. microplus* is challenging, due to the obscure characteristics used to differentiate between these two tick species.

Females constitute the bulk of tick samples, as they are easy to collect due to their large size. Ticks are identified using a light microscope and by their hypostome column dentition: 4x4 for *R. microplus* and 3x3 for *R. decoloratus*. Figure 15 (A and B), indicates the different hypostome dentition between *R. microplus* and *R. decoloratus*.

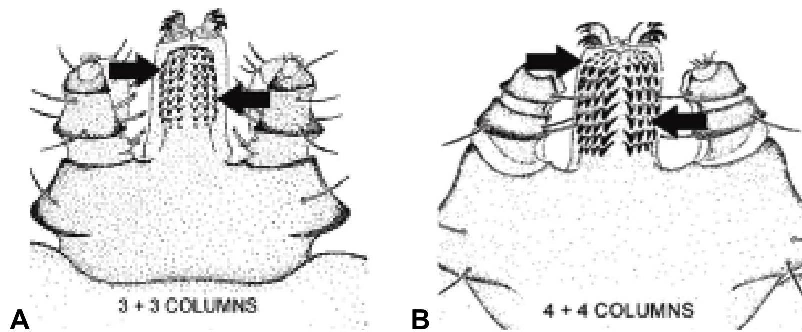

Figure 15: Hypostome column dentition between *R. microplus* and *R. decoloratus* female ticks. 15A: *R. decoloratus* female with 3x3 column dentition. **15B:** *R. microplus* female with 4x4 column dentition. The figures were modified from Walker *et. al.* (2003).

Hypostome dentition of 3.5x3.5 and 4.5x4.5 (three to four additional teeth between the inner row teeth) have been observed for *R. decoloratus* and *R. microplus*, respectively (LEMPEREUR *et al.* 2010). Male spurs of the adanal plates are highly variable, which is the primary characteristic used to differentiate, between *R. microplus* and *R. decoloratus* males (LEMPEREUR *et al.* 2010). Variation in hypostome dentition and adanal spurs can lead to erroneous identification of male and female ticks. This necessitates the use of additional tools for tick identification, such as PCR-RFLPs of the ITS2 sequence of the ticks, developed by Lempereur and colleagues (LEMPEREUR *et al.* 2010), which can distinguish between *R. microplus* and *R. decoloratus* at a molecular level.

4.7.2 Tick sampling

Correct sample collection is an essential part of evolutionary studies (ALBERT *et al.* 2010). Sampling can introduce bias and affect precision of results which can lead to unreliable conclusions about the evolutionary relationship and genetic diversity found within the target population (ALBERT *et al.* 2010).

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Ticks are distributed according to host abundance, specifically in relation to *R. microplus* and its occurrence on cattle farms. The local area movement of ticks is largely facilitated by host movement, such as cattle movement between communal dips, feeding lots and during trade. This generates a 'synthetic' distribution of tick populations since firstly, the ticks do not follow a natural distribution pattern as regulated by natural host movement and/or environmental factors and secondly, humans influence the distribution of ticks through the establishment of cattle farms and moving cattle between the different areas. Therefore, the sampling method for ticks cannot be assisted with distance software or sampling models, as suggested for many species that have a natural distribution according to their ecological niches (ALBERT *et al.* 2010; GUISAN *et al.* 2006; THOMAS *et al.* 2010; THUILLER *et al.* 2004).

Ticks are ectoparasites, dependent on a suitable host for nourishment. *Rhipicephalus microplus* is a one host tick which primarily parasitises cattle. Thus, its distribution will correlate with the host's distribution. Studies have shown that sampling ticks according to host density increases the probability of sampling the required tick species (BARANDIKA *et al.* 2006; DOBSON and RANDOLPH 2011; WILSON *et al.* 1985). Environmental sampling of ticks is recommended for research regarding tick abundance or distribution (CASTRO and CLOVER 2010; DOBSON *et al.* 2011; THUILLER *et al.* 2004), since ticks spend a large portion of their life cycle in vegetation during egg-laying or when searching for a host. Environmental sampling is however time consuming and labour intensive. Distribution or tick abundance was not the main focus of this research project and therefore host-based sampling was used as the primary method of tick sampling. Additional to the phylogenetic and phylogeographic study, an acaricide resistance analysis was conducted on the same sample pool, as required by funders Zoetis Pty. Ltd., but was not described in this proposal. Possible links between *R. microplus* population subdivision and acaricide resistance can be evaluated given the acaricide resistance results. Ticks were collected in provinces with high to medium cattle density as shown in Figure 16, but limited to the Republic of South Africa and Swaziland.

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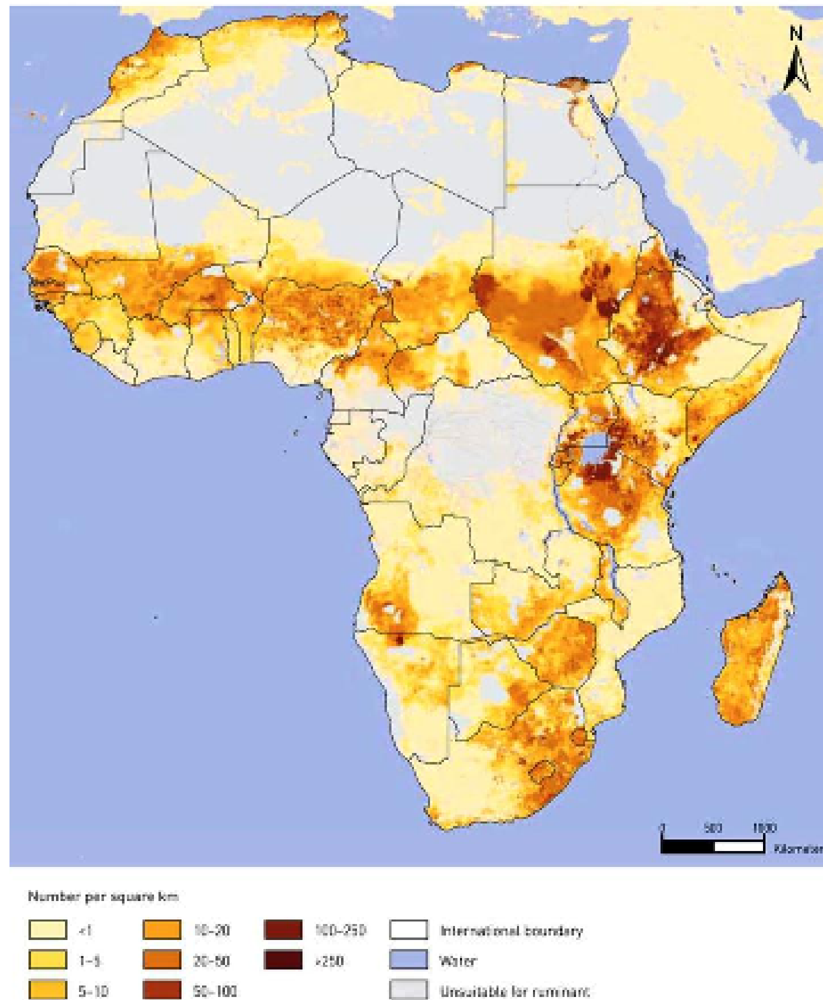

Figure 16: A map of observed cattle density in Africa, which includes numbers of cattle per square kilometre (WINT and ROBINSON 2007). This map was used to sample ticks in various provinces of South Africa with high-medium cattle density (high = 20-100 cattle/km^2, medium = 1-20 cattle/km^2).

The number of ticks required for this research project depended on the extent of the genetic variation within and between tick populations on a host, farm and provincial level. Koffi and colleagues (KOFFI *et al.* 2006a) have shown that there is no genetic differentiation in tick populations within a single cattle herd. This suggests that one cattle farm can be seen as one cattle herd. Therefore, we assumed that there would be limited genetic variation within cattle farms and increased genetic variation between cattle farms. Random sampling of cattle farms (one herd) within the provinces of South Africa would thus increase the representation of the genetic diversity (ALBERT *et al.* 2010). Additionally, cattle trade and movement is restricted by the political boundaries of the provinces of South Africa, which has an impact on the host availability for *R. microplus*. Consequently 'provinces' are used as a proxy for host availability, which is linked to the geographic location of the provinces.

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A 'sampling concept' was defined for the sampling method used to collect ticks. This assisted with downstream results and statistical analysis of the sampling method. Figure 17 represents the sampling concept for tick sample collection.

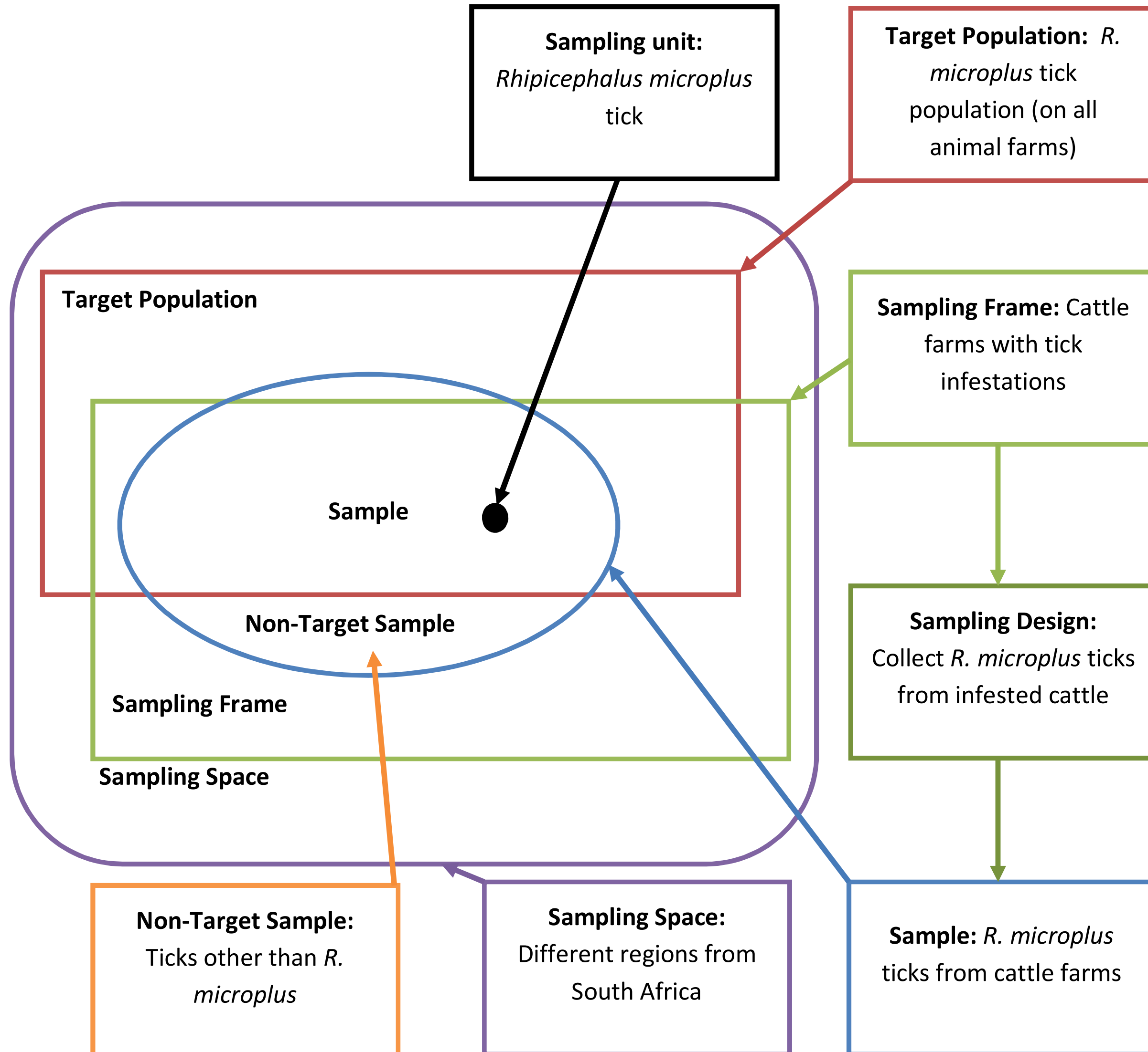

Figure 17: Sampling concepts for the sampling method applied to *R. microplus* ticks collected from different provinces of South Africa. Modified from Albert *et. al* (2010).

With reference to Figure 17, the sampling unit is described as the item to be measured in a biological study (ALBERT *et al.* 2010), such as an individual *R. microplus* tick. The sample unit is contained within a target population of ticks. These populations are found on a cattle farm, which represents the sampling frame. Random sampling according to host density is applied for tick sampling and is defined as the sampling design. The sample contains a representative population of *R. microplus* ticks collected from the cattle farms. The sampling space constitutes a geographical region (ALBERT *et al.* 2010), such as the different provinces of South Africa from which ticks were collected. Lastly, non-target samples were ticks collected which were not *R. microplus*, but was found within the same

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sampling frame. Both results and conclusions, with regards to tick sampling, can be clearly separated by defining each aspect of the sampling method.

4.7.3 Genomic DNA extraction

Genomic DNA extraction from mites and ticks are challenging, due to their small size (mites and immature of ticks), hard cuticles and their DNA being highly susceptible to degradation (HALOS *et al.* 2004; NAVAJAS and NAVIA 2010). Genomic DNA extraction from ticks relies largely upon extraction kits, which result in low quantities of DNA (CHAO *et al.* 2011; KOFFI *et al.* 2006a), or the use of hazardous chemicals during the extraction procedure (HALOS *et al.* 2004).

Aljanabi and colleagues (199) described an universal salt-based DNA extraction technique. It utilised a standard TEN (Tris-HCl, EDTA and NaCl) lysis buffer, with additional sodium chloride added during the extraction process (ALJANABI and MARTINEZ 1997). Similar genomic extraction techniques exist, which use a similar TEN lysis buffer, but the authors only use larvae for genomic DNA extraction and not adults (BARKER 1998; HILL and GUTIERREZ 2003).

The universal salt-based DNA extraction technique was optimised for genomic DNA extraction from *R. microplus* larvae, as well as male and female adults (OBERHOLSTER 2011). The genomic DNA recovered from male ticks was not degraded, yet female genomic DNA showed evidence of degradation (OBERHOLSTER 2011). Sodium lauryl sarcosinate was used to extract non-degraded DNA from ancient bones and museum samples (KRINGS *et al.* 1997; RICHARDS and SYKES 1995) and the addition of sarkosyl to the extraction protocol has yielded non-degraded DNA from several female adult *R. microplus* ticks (preliminary results, Tanzelle Oberholster 2013).

High concentrations of NaCl (0.1 - 5.0 M) added to a crude extract of genomic DNA and incubated on ice, caused proteins to aggregate and precipitate, in a process known as 'salting out' (EMILEY A. WILEY ; JOHN F. ROBYT and BERNARD J. WHITE). The proteins can then be removed from the crude extract by centrifugation at high speed (26 000 x g) and the supernatant was transferred to a new centrifugation tube (ROCHE DIAGNOSTICS GMBH 2007). The ice incubation and high speed centrifugation steps were added to the universal salt-based DNA extraction technique after RNA digestion. This prevented the retention of proteins in the purified DNA extract that may interfere with downstream reactions, such as PCR and sequencing. This allowed the extraction of high quality non-degraded genomic DNA from pooled larvae, individual male and female ticks for subsequent use in molecular procedures during this study.

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4.8 Final remarks

The evolution of *Rhipicephalus microplus* is a complex system of interplaying factors. Several biotic (eg. the specific life history traits and survival ability of the tick) and abiotic (eg. the climate, ecological and host-related environment where the ticks are present) are important. These factors contribute to the overall persistence and distribution of tick populations, the spread of resistance and disease and the ultimate impact of ticks on the South African economy. Figure 18 illustrates the factors involved in the hypothesised population structure and evolutionary dynamics of *R. microplus*.

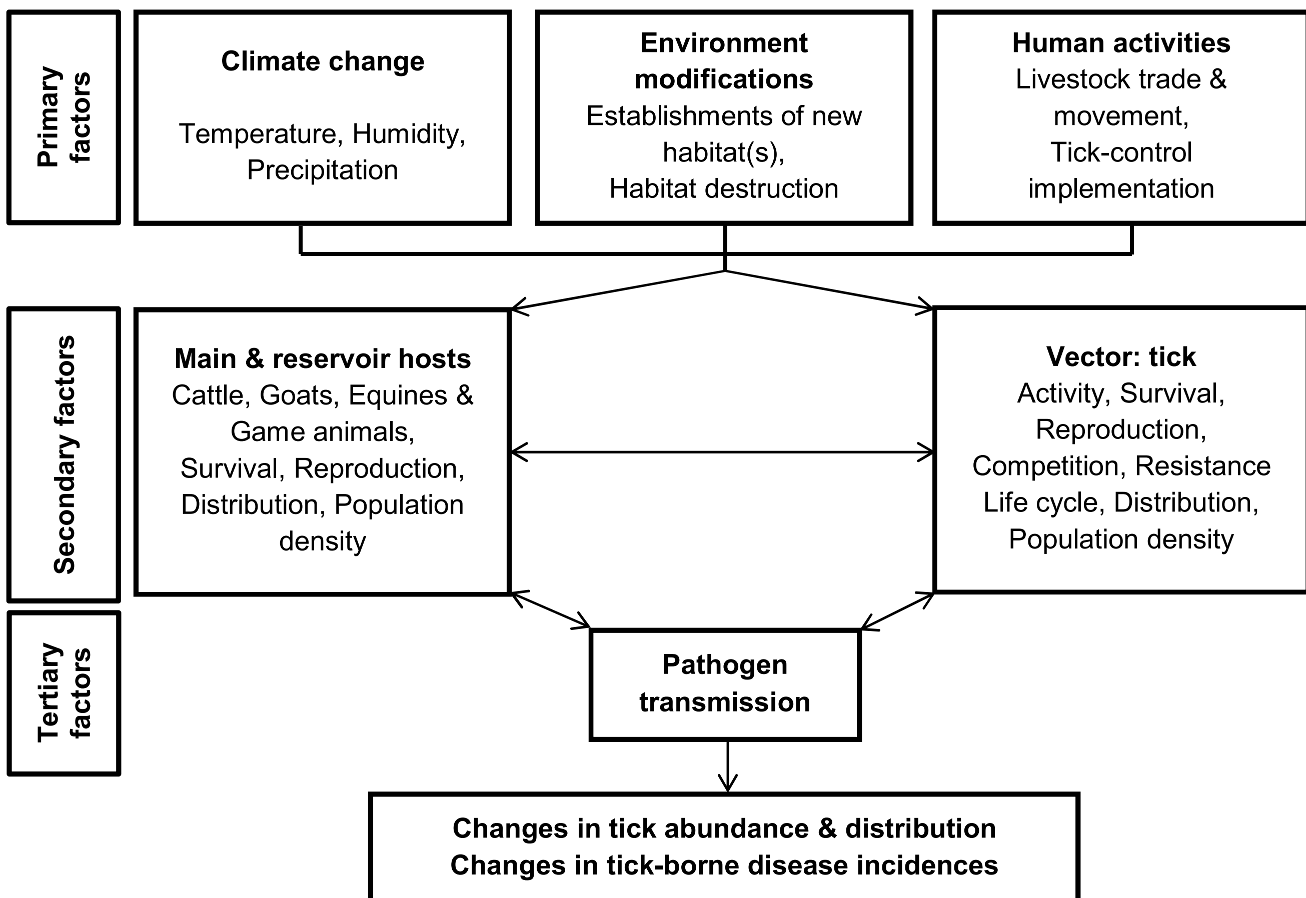

Figure 18: The complex interactive system of biotic and abiotic factors, both direct and indirect, that influence the population structure and evolutionary dynamics of *R. microplus* and its associated tick borne-diseases. The establishment of cattle farms or the burning of pastures to prevent larval survival was regarded as 'environment modifications'. Modified from Elsa Léger *et. al*. (2013); constructed with specific emphasis on *R. microplus*.

In consideration of all the previously discussed sections it is clear that *R. microplus* presents a threat to the farmer and consumer alike. Effective tick control for sustained periods without a fundamental understanding of the evolutionary behaviour of *R. microplus*, both at a genetic and phenotypic level, will have a low probability of success.

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4.9 Research question and aim

The main question addressed in the present study was: What are the evolutionary mechanisms governing the genetic diversity of the *R. microplus* population of South Africa?
This included investigating:

A) The level of genetic diversity (whether it is high or low),

B) The pattern of genetic diversity (whether the populations are structured or not) and,

C) The processes involved in shaping the genetic diversity (such as gene flow and subdivision).

4.10 Hypothesis

From the aims stated, the following hypotheses were developed for the *R. microplus* populations of South Africa.

A) *Rhipicephalus microplus* populations have a high level of genetic diversity.

B) *Rhipicephalus microplus* populations from South Africa have genetic structure. If so, it will be associated with either climate or geography, where 'province' are used as a proxy for arrangement of cattle (host) density. Furthermore, present cattle importation and movements influence the genetic diversity of *R. microplus* populations.

4.11 Objective

Determine the intraspecific genetic variation and population structure of *R. microplus* tick populations collected from the different provinces of South Africa and compare this to global tick strains through the use of nuclear (*ANT*, *D3*, *ITS2*) and mitochondrial (*COI*) sequences to determine the phylogeography of the tick populations of South Africa.

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Chapter 2: Genetic evaluation of *Rhipicephalus microplus* with nuclear and mitochondrial markers

T. Oberholster, P. Bloomer, C. Maritz-Olivier
Department of Genetics, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, 0002, South Africa.

2.1 Abstract

Background: There are a limited number of studies that have attempted to describe the within-species genetic variability of field-collected ticks on cattle farms by combining markers from both phylogenetic and phylogeographic disciplines. Currently, both acaricide resistance and varying tick vaccine efficacy levels are attributed to the high genetic diversity between and within tick populations, but without any supporting data. It has, therefore, become increasingly important to clarify the levels of genetic variation of these populations for the improved use and development of acaricide and tick vaccine control methods.

Purpose: The aim of this study is to determine the intraspecific variability of *Rhipicephalus microplus* samples from cattle farms in South Africa and Swaziland. Interspecific variability was also evaluated for *R. microplus* from different countries (West Africa, Brazil, America) and compared to other *Rhipicephalus* species (*R. annulatus*, *R. decoloratus, R. geigyi* and *R. australis*).

Methods: Adult female blood-fed ticks were collected from cattle farms across South Africa and Swaziland in collaboration with Zoetis Pty. Ltd. Several reference samples (larval, adult and NCBI GenBank sequences) were obtained for comparison. Ticks were identified with light microscopy and their identity confirmed using *ITS2*-RFLP analyses following genomic DNA extraction with a modified NaCl-based method. Phylogenetic analyses were performed on the *D3*, *ITS2* and *COI* sequences with character and distance methods in MEGA 5, and Bayesian inference in Mr. Bayes. Genealogical analyses were performed on the *ITS2* data in DNASP v5.

Results: The *D3* region provided no intra- or interspecific resolution, with a single polymorphic site among *R. microplus* populations and other *Rhipicephalus* species. The *ITS2* can be used as a species marker to distinguish between *R. microplus*, *R. decoloratus*, *R. annulatus* and *R. geigyi* at sequence level, but cannot distinguish between *R. microplus* and *R. australis*. The *COI* gene can be used as a species marker for the *Rhipicephalus* genus as it can distinguish between the cattle ticks as well as *R. microplus* and *R. australis*. The *COI* gene of *R. microplus* has 2 haplotypes. *R. microplus* populations from South Africa, West Africa and Brazil constitute haplotype 1, whereas *R. microplus* Deutsch strains (USA) constitute haplotype 2.

Conclusion: The history of geographical movement of *R. microplus* via cattle trade and importation is reflected in the population structure of *R. microplus* on a global scale. The movement and trade of cattle need to be monitored and restricted within countries and globally. This would prevent the

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introduction of new ticks species, resistant ticks and their associated tick-borne diseases into previously unoccupied areas. The consequences of continued inaction would be increased economic loss, which is already substantial, and the expanded occurrence of tick and tick-borne disease epidemics.

Keywords: *Rhipicephalus microplus*, *Rhipicephalus decoloratus*, *Rhipicephalus australis*, genetic diversity, phylogenetics, phylogeography, geographical distribution, genetic markers.

***Corresponding author:** Christine Maritz-Olivier, Tel: +27 (012) 420 3945. Fax: +27 (012) 362 5327. Postal address: Room 7-30, Agricultural Sciences Building, University of Pretoria, Hatfield, Pretoria, 0083, South Africa. Email: Christine.maritz@up.ac.za

2.2 Introduction

The *Rhipicephalus* genus contains a group of socio-economic important ticks, known as the cattle ticks, previously allocated to the *Boophilus* subgenus (HORAK 2009; MURRELL and BARKER 2003). The cattle ticks are a burden to cattle farmers, due to their direct influence on cattle condition and the spread of lethal tick-borne diseases (WALKER *et al.* 2003). In South Africa, the most prominent cattle ticks are *R. decoloratus*, a native and widely distributed species (NYANGIWE *et al.* 2011; TONETTI *et al.* 2009) and *R. microplus*, an invasive species imported during the late 1800 to early 1900 when the South African cattle industry suffered heavy mortalities due to the Rinderpest and East Coast Fever epidemics (HOWARD 1908; NYANGIWE *et al.* 2011).

Of the two *Rhipicephalus* (*Boophilus*) species in South Africa, *R. microplus* is of greater concern to the agricultural and veterinary sectors as it is the exclusive vector of *Babesia bovis*, the causative agent of lethal Asiatic babesiosis or redwater (POTGIETER and ELS 1977; TØNNENSEN 2002). Asiatic babesiosis is often misdiagnosed by cattle farmers as heartwater (*Cowdria ruminantium*) due to the neurological complications associated with the disease (OBEREM *et al.* 2006). *R. decoloratus* is a vector of African babesiosis (*B. bigemina*), which results is a milder disease in infected cattle (TERKAWI *et al.* 2011).

Rhipicephalus decoloratus maintains a large and continuous geographical distribution in South Africa (ESTRADA-PENA *et al.* 2006; OBEREM *et al.* 2006), but is more often associated with the arid regions of South Africa. By contrast, *R. microplus* has a discontinuous distribution along the coast line and the more temperate regions of South Africa (ESTRADA-PENA *et al.* 2006; NYANGIWE *et al.* 2011; OBEREM *et al.* 2006). Recently, *R. microplus* has increased its geographical range into previously unsuitable habitats (NYANGIWE *et al.* 2011; TONETTI *et al.* 2009) and has been recorded on reservoir hosts, such as goats (DE MATOS *et al.* 2009; NYANGIWE and HORAK 2007) and gemsbok (TONETTI *et al.* 2009). This has allowed *R. microplus* to spread into areas previously unoccupied and has resulted in the displacement of *R. decoloratus* from several areas, such as the Free State (TONETTI *et al.* 2009),

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Limpopo (TØNNENSEN *et al.* 2004) and the Eastern Cape Provinces (NYANGIWE *et al.* 2011; NYANGIWE *et al.* 2013a). Given that *R. microplus* is the vector of Asiatic babesiosis, for which South African cattle are immunologically naïve, it presents an increased economic threat to cattle farming in South Africa.

Acaricides are proving less effective as a tick control strategy, due to its overuse and misuse, which results in the accumulation of acaricide resistance in tick populations (BUDELI *et al.* 2009; LI *et al.* 2007). Acaricide resistance is also introduced to new areas due to the movement of cattle during trade and the importation of cattle from abroad. Vaccines provide a viable alternative to acaricides, but have been observed to have variable efficacy and reports of possible vaccine resistance have also increased (GARCIA-GARCIA *et al.* 2000; PARIZI *et al.* 2009). The rapid accumulation of resistance and the adaptation of *R. microplus* to unsuitable habitats may be attributed to the high genetic diversity and pangamy mating structure of tick populations (CHEVILLON *et al.* 2007a; CHEVILLON *et al.* 2007b; CUTULLÉ *et al.* 2010).

Although well documented for *R. australis* from New Caledonia (CHEVILLON *et al.* 2013), which may serve as a comparative tick population, genetic diversity studies of *R. microplus* is lacking. The aim of this study was to determine the intraspecific variability of *Rhipicephalus microplus* samples from cattle farms in South Africa and Swaziland. The interspecific variability was also evaluated for *R. microplus* from different countries (West Africa, Brazil, America) and compared to other *Rhipicephalus* species (*R. annulatus*, *R. decoloratus, R. geigyi* and *R. australis*). Several markers were selected for the present study: the *D3* region within the *28S rRNA* gene has proven to be phylogenetically useful in the study of oribatid mites (MARK MARAUN *et al.* 2004), several species of *Ixodes* and *Dermacentor* ticks as well as *R. sanguineus* (ANSTEAD *et al.* 2011). The *ITS2* sequence, also within the *28S rRNA* gene, has been shown to be highly variable for several tick species, including rhipicephaline ticks (CHAO *et al.* 2011; HLINKA *et al.* 2002). The *ITS2* sequence has been characterised for *R. australis (MURRELL et al. 2001a)* and *R. microplus* (LEMPEREUR *et al.* 2010). The *COI* gene has been used to resolve relationships within *Rhipicephalus* (MURRELL *et al.* 2001b), its variable number of haplotypes in *R. appendiculatus* (YSSOUF *et al.* 2011), makes it an excellent candidate for intraspecific studies of *R. microplus*. *Rhipicephalus australis* from New Caledonia represents the only cattle tick for which extensive microsatellite markers have been developed (KOFFI *et al.* 2006b). These microsatellites can be evaluated as cross-species markers for *R. microplus*.

The determination of the genetic variability and structure *R. microplus* populations will assist with the identification of areas with high genetic diversity in South Africa. This variability can be correlated, in the future, with acaricide resistance (such as with *R. australis* (CUTULLÉ *et al.* 2009)) and may assist with the use and development of improved acaricide control strategies. High resolution information on genetic diversity should significantly improve tick vaccine design and implementation to target specific *R. microplus* populations based on their genetic diversity (POLAND *et al.* 2007).

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In this study we report on the intra – and interspecific genetic variability of *R. microplus* populations from South Africa and its comparison to *R. microplus* from other countries (West Africa, Brazil and USA) as well as other *Rhipicephalus* species (*R. decoloratus*, *R. annulatus*, *R geigyi* and *R. australis*). We present molecular evidence for in support of the reinstatement of *R. australis* as a separate species. We also report on the current geographical distribution of *R. microplus* and *R. decoloratus* in South Africa.

2.3 Materials & Methods

2.3.1 Tick collection and inventory

Rhipicephalus microplus and *R. decoloratus* ticks were collected from 112 farms, which were randomly distributed in high and medium cattle density areas (WINT and ROBINSON 2007) (Figure 16 and 19). Each farmer was also required to complete a questionnaire to provide supporting information for the tick sampling. Ticks were collected in collaboration with Zoetis Pty. Ltd. and their representatives, who visited the farms, explained the project, the process for collecting ticks and obtained consent.

Ticks were collected from the Gauteng, KwaZulu-Natal, Mpumalanga, Free State, North West and Eastern Cape Provinces of South Africa, as well as Swaziland. On each farm approximately 50 ticks were randomly collected (mostly blood-fed non-engorged adult *R. microplus* and *R. decoloratus* females) with specific attention to tick attachment sites on the cattle dewlap, shoulders, flanks, belly and the tip of the tail. The farmers were instructed to record the origin of the ticks by farm, by GPS co-ordinates and the date obtained. They were further provided with the necessary equipment and compounds to store tick samples under 70% ethanol. Ticks were identified, catalogued and returned to storage under 70% ethanol until further use.

Additional samples from ClinVet International (Bloemfontein, Free State) and the Mnisi Research Area (provided by Dr Ivan G. Horak and Dr Hein Stoltz, Department of Veterinary Tropical Diseases, University of Pretoria, South Africa) were included in the study as outgroups, hence there are no questionnaire data available for these and they are not indicated in the sampling collections. The ClinVet samples represented a *R. microplus* colony strain and constitute the main strain used for vaccine development in our group (Ticks and Tick-borne Disease Program, University of Pretoria). Additional genomic DNA samples were provided for *R. australis* (Prof Ala E. Lew-Tabor, Center for Comparative Genomics, Murdoch University, Australia); *R. microplus* (Deutsch strain) from USA Texas-Mexican border (Dr Felix Guerrero, USDA-ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, USA); *R. microplus*, *R. decoloratus*, *R. annulatus* and *R. geigyi* from Benin, West Africa

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(Prof M. Madder, Department of Veterinary Tropical Diseases, University of Pretoria, South Africa); and *R. microplus* from Brazil (Prof Isabel K. Ferreira de Miranda Santos, Department of Biochemistry and Immunology, University of São Paulo, Brazil).

The tick questionnaire was used to gather relevant information about the types of hosts present on each farm, parasite load, environmental conditions of the cattle farm and the movement of cattle (for trade or to communal dip tanks). A copy of the questionnaire is provided in Appendix B.

The questionnaire information was data captured and processed to provide insight into the geographical and climate distribution patterns of *R. microplus* and *R. decoloratus* ticks, background information on cattle breeds present on the farms, comparative information on additional non-cattle hosts, comparative parasite loads and prevalence of different acaricides in use.

2.3.2 Map construction

The GPS co-ordinates of 103 farms were entered into the ArcGIS Online mapping software available at www.arcgis.com. The exact GPS co-ordinates of farms 37-39, 53-55, 62, 65 and 70 are currently being verified. The absence of exact GPS data meant that these farms could not be added to the map. The GPS omitted farms are could be classified by province and as such the data for these farms are included in the results for provincial distribution.

2.3.3 Ratio comparison between *R. microplus* and *R. decoloratus* ticks

The prevalence of each of the species in each of the provinces was compared by calculating the relative occurrence of each of the species per province from the sampling data. The formulas used per province were:
Number of *R. microplus*: observations/Σ (*R. microplus*, *R. decoloratus*) expressed as a percentage.
Number of *R. decoloratus*: observations/Σ (*R. microplus*, *R. decoloratus*) expressed as a percentage.

2.3.4 Tick identification

Ticks collected were identified using the descriptions and example pictures provided by the tick identification guides; Tick Photodatabase (MADDER and HORAK 2010), Diseases and Parasites of Cattle, Sheep and Goats in South Africa (OBEREM *et al.* 2006) and Ticks of Domestic Animals in Africa: a Guide to Identification of Species (WALKER *et al.* 2003). The ticks were identified visually into genera and thereafter were identified to species level under a light microscope. Species identification was confirmed by the use of species-specific restriction fragment sizes generated by PCR-RFLPs of the *ITS2* sequence of the *Rhipicephalus* ticks (LEMPEREUR *et al.* 2010).

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2.3.5 Genomic DNA extraction

Adult tick genomic DNA was isolated with a modified NaCl-based genomic DNA extraction method. Briefly, a whole adult tick was homogenised in lysis buffer (final concentration of 0.5 M EDTA and 0.5% sodium lauryl sarcosinate) with a pestle in a stainless steel mortar. The homogenate was transferred to a microcentrifuge-tube and 400 µl of sterile NaCl DNA extraction solution (final concentration of 0.4 M NaCl, 60 mM Tris-HCl, 12 mM EDTA, 0.01% SDS, pH 7.0) was added. Proteinase K was added to the solution at a final concentration of 0.06 mg/µl and the solution vortexed for 30 seconds. Following overnight incubation at 55°C, the Proteinase K was inactivated at 65°C for 20 minutes. RNase A was added at a final concentration of 0.01 mg/ul and incubated at 37°C for 15 minutes, before 360 µl of 5 M NaCl was added, vortexed thoroughly and incubated on ice for 5 minutes. Thereafter samples were centrifuged for 20 min at 25 500 x g (15-25°C). The supernatant was transferred to fresh tubes and equal volumes of isopropanol were added to each sample, mixed and incubated at -20°C for 1 hour. After centrifugation (20 min, room temperature at 10 000 x g), the pellet was washed with 70% EtOH, dried and finally re-suspended in 50 µl 1x TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Genomic DNA was analysed on a 1% agarose gel. DNA concentrations were determined by gel analysis using the ImageLab® software supplied by BioRad® GelDoc system.

All tick identification was confirmed using the *ITS2*-RFLP after genomic DNA extraction (LEMPEREUR *et al.* 2010). After PCR amplification and gel analyses, the remainder of the *ITS2* product (23 µl, approximately 1000 ng) was digested with *MspI* enzyme (New England Biolabs) at a final concentration of 0.12 U/µl with 1x NEB Buffer (New England Biolabs) and a final concentration of 0.1 mg/ml BSA (Promega). The reactions were made up to a final volume of 50 µl and incubated for 2 hours at 37°C. Banding patterns were analysed on a 3% agarose gel and the ImageLab® software supplied by BioRad® GelDoc system.

2.3.6 Nuclear and Mitochondrial sequences

Several tick samples were analysed for the nuclear *D3* (ANSTEAD *et al.* 2011), ITS2 (LEMPEREUR *et al.* 2010) and mitochondrial *COI* sequences (APANASKEVICH *et al.* 2011; FOLMER *et al.* 1994) with the EconoTaq PCR Master Mix (Lucigen™). Initially, a designed set of *COI* primers (RmCOIF and RmCOIR) from contigs provided by Paula Moolhuijzen (Center for Comparative Genomics, Murdoch University, Australia) and Dr Felix Guerrero (USDA-ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, USA) was used for sequencing. Due to problematic amplification of certain samples with the designed set, the *COI* primers described by Folmer (1994) were alternatively used. Table 13 supplies the PCR primers used for the nuclear and mitochondrial sequences. Specific primers

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for ticks have been designed for the *ANT* gene (CAREW *et al.* 2009) using the sequence of *R. microplus* retrieved from the VectorBase website (https://www.vectorbase.org/) as an additional genetic marker. *COI* and *ANT* primers were designed using the Oligo Primer Analysis Software v 7.0.

The nuclear and mitochondrial amplifications were optimised according to the Taguchi method as described by Cobb and Clarkson (COBB and CLARKSON 1994). PCR products were analysed with 2% agarose gels. PCR product was purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and concentrations were determined by gel analysis using the ImageLab® software supplied by BioRad® GelDoc system. Sequencing of *D3* and *ITS2* samples were done by Macrogen Europe and *COI* samples were sequenced at the DNA Sequencing Faculty of the University of Pretoria using standard dye termination sequencing (APPLIED-BIOSYSTEMS 2002).

Pseudogene analyses of the *COI* gene was evaluated using a BLAST-X search on the *COI* dataset. The sequences were translated (frame +1) using the Invertebrate Mitochondrial Codon Table and the EMBOSS Transeq online software (http://www.ebi.ac.uk/Tools/st/emboss_transeq/).

2.3.7 Phylogenetic analysis

Sequence data of nuclear and mitochondrial genes were analysed using BioEdit Sequence Alignment Editor (HALL 1999). Reference sequences for each were obtained from the NCBI GenBank database and included in the analyses. Sequence alignments were performed using the online MAFFT 7.0 multiple alignment program for amino acid or nucleotide sequences (http://mafft.cbrc.jp/alignment/server/index.html). Initial phylogenetic analysis was conducted in MEGA 5 (TAMURA *et al.* 2011) with distance and character methods such as Neighbour Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP). Additional refined analyses were conducted using maximum likelihood in PHYML 3.0 (GUINDON *et al.* 2010) and Bayesian analyses in Mr. Bayes (RONQUIST *et al.* 2012), with models estimated from JModelTest (POSADA 2008). Only the Maximum Likelihood (MEGA 5) and Bayesian analysis results are shown (all other phylogenetic trees provided in Appendix B section 2). The node support was determined using 1000 bootstrap replicates. Branch support above 75 for NJ, ML and MP was not rejected. Posterior probability support equal to and above 0.95 was not rejected.

Allele networks were constructed for the *R. microplus* populations of South Africa with TCS 2.21 (CLEMENT *et al.* 2000) and Network 4.6.1.1 (Fluxus Technology) based on statistical parsimony and a Median Joining algorithm, respectively. Allele networks were based on geographical sampling and climate classifications according to the Köppen-Geiger climate classification system of South Africa (CONRADIE 2012).

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2.3.8 Population genetic analysis

Haplotype determination, polymorphism and neutrality tests were conducted on the *ITS2* dataset of *R. microplus* sequences from South Africa. Haplotype scoring and tests were conducted using the DNA Sequence Polymorphism (DNASP) v5.10.01 software package with the Generate: Haplotype Data File, Analysis: Polymorphic Sites and Overview: Polymorphic Data functions (LIBRADO and ROZAS 2009).

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2.4 Results

2.4.1 Tick collection and farmer's questionnaire database

Data collected and provided by the farmer's questionnaire was captured in the Tick Questionnaire database (Electronic Excel File on Appendix CD).

Cross-breeds represented the most popular cattle breed on farms (37.5%), followed by Bonsmara (26.8%) and Holstein-Friesland (14.3%) cattle. The cross-breeds and Bonsmara cattle have *Bos indicus* genetic influence and are more resistant to ticks and tick-borne diseases than Holstein-Frieslands (*Bos taurus*), which constitute superior milk producing breeds in this survey. Cattle farmers either dip their cattle once a year, twice a year or more than five times a year and no consensus existed for the frequency of dipping. The pour on application method for acaricides was the most widely used (48%). It represents the least expensive, least time-consuming and most convenient (no specialist equipment required) method to cattle farmers. The majority of the farms from which ticks were collected were located in Highveld regions (44%), a large biome type of South Africa. Sheep (39%), goats (12%), game animals (13%) and horses (8%) represent additional hosts on farms (other than cattle) and are likely to host and serve as reservoirs for *R. microplus* ticks. Ticks (24%) represented nearly a quarter of the parasites on cattle farms, followed by flies (23%), worms (20%), mites (13%) and other (20%). Other (20%) represented largely unspecified parasites and may include lice, fleas and haematophagous midges.

The total number of ticks collected from 112 cattle farms was 3182. Figure 19 shows the geographical distributions of *R. microplus* and *R. decoloratus* on cattle farms in South Africa (larger maps are provided in Appendix B). The target ticks represented 66.15% of the total ticks collected, where 665 were *R. microplus* and 1440 were *R. decoloratus*. The remaining 1077 ticks belonged to a different genus; such as *Amblyomma* and *Hyalomma*, or represented non-target *Rhipicephalus* ticks; such as *R. evertsi evertsi* or *R. appendiculatus*, or were damaged (heads or hypostomes missing). Table 14 represents the number of *R. microplus* and *R. decoloratus* ticks per province and the comparisons of *R. microplus* and *R. decoloratus* ratios (Section 2.3.3).

The distribution of *R. microplus* is discontinuous and is mostly in the temperate parts of South Africa, From Table 14, it is evident that *R. microplus* is highly prevalent in Swaziland (77.5% and 3.44), whereas *R. decoloratus* is highly prevalent in the North West Province (97.44% and 38.13). The abundance of *R. microplus* in Swaziland can be partly attributed to its warm temperate climate and suggests that the trade or movement of cattle between South Africa and Swaziland needs to be monitored for repeated *R. microplus* introductions into South Africa. The North West Province is an arid steppe, for which it is assumed that *R. microplus* has not completely adapted to yet and therefore *R.*

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decoloratus maintains a dominant presence in the North West. *Rhipicephalus microplus* has been collected from the arid region of Mnisi, and provides further evidence that *R. microplus* may be adapting to arid environments. This trend needs to be monitored given the propensity of *R. microplus* to build on small foothold invasions. The Western Cape, Gauteng, KwaZulu-Natal, Eastern Cape and Mpumalanga Provinces have similar ratios for *R. microplus* and *R. decoloratus.* These provinces have approximately 60% *R. decoloratus* presence (approximately 80% for Mpumalanga Province). Farms in these areas are within the warm temperate regions of South Africa and the abundance result is consistent with the expectation of dual infestation in cattle herds with both *R. microplus* and *R. decoloratus*. The movement of cattle on the farms surveyed during this study was limited, restricted to cattle movements in the last two years, and mainly occurred between dip tanks and other farms within close proximity (approximately 50 km). This type of distribution may lead to a stepping stone model for the population structure of *R. microplus*, and also for *R. decoloratus,* in South Africa.

2.4.2 Genomic DNA extraction

Genomic DNA was extracted from adult female blood-fed ticks from the field. Genomic DNA was usually degraded. Figure 20 represents genomic DNA isolated from farms 41 (sample 41:1 – 41:5 in lanes 2 - 6) and 42 (sample 42:1 - 42:5 in lanes 7 - 11). The negative control constituted extraction without tick tissue to ensure that solutions were not contaminated. The degradation of genomic DNA could be attributed to tick biology, where tick organs are degraded prior to ovipositioning, or to handling of the samples before they arrive at the laboratory. Individual adult identification was confirmed with *ITS2*-RFLP (Figure 21).

2.4.3 Optimisations

All amplification reactions were optimised with the Taguchi PCR method. Table 15 illustrates the use of nine reactions with different PCR reagent concentrations to facilitate the isolation and optimisation of gene sequence with the Taguchi method. Further optimisations were facilitated by varying cycles and temperature for the nuclear and mitochondrial sequences. Table 16 and Figure 22 provide the final optimised reaction conditions for the genetic markers.

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2.4.4 *D3* sequence

A total of 25 *D3* sequences were obtained: 21 *R. microplus* from South Africa; 2 *R. microplus* from West Africa and 1 *R. annulatus* from West Africa. An additional sequence was downloaded from the NCBI GenBank database, *R. sanguineus* (AF062986) as a reference from the original study (same primer sequences). Farms 7, 8 and 9 are from the Eastern Cape, 17 from Swaziland, 18 and 42 from Western Cape, 26 and 46 from Mpumalanga and 41 from KwaZulu-Natal. The *D3* dataset includes the ClinVet strain as well.

The *D3* sequence had only one variable site with either a T or a C at position 97 in the sequence. Some sequences are heterozygous for this position (Y). This variable site is also present in *R. sanguineus*, which indicates that the *D3* region provides no phylogenetic resolution for the *Rhipicephalus* genus. Figure 23 illustrates the *D3* alignment with the single variable site at position 97. Therefore, the *D3* region is highly conserved and is not suitable as a genetic marker for intra- or interspecific diversity of the *Rhipicephalus* genus.

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2.4.5 *ITS2* sequence

A total of 86 *ITS2* sequences were obtained: 82 *R. microplus* (77 South Africa, 2 West Africa, 1 Australia, 1 USA and 1 NCBI sequence from Brazil); a single *R. annulatus* (1 West Africa); outgroups *R. decoloratus* (1 South Africa, 1 West Africa) and *R. geigyi* (1 West Africa). The *ITS2* sequence of *R. microplus* was highly variable for samples from different continents, but similar for *R. microplus* sequences from South Africa. The *ITS2* sequence of *R. geigyi* contained 3 base pair insertions, whereas *R. decoloratus* contained 2 insertions in the same region, when compared to *R. microplus*. Additionally *R. geigyi* had a 63 base pair deletion when compared to *R. microplus* and *R. decoloratus*. *R. microplus* contains 3 insertions at the end of the sequence, not present in the other species. *R. microplus* from the USA contained a single base pair insertion (G) not found in any other *R. microplus*, *R. decoloratus* or *R. geigyi* sequences (position 124 in Table 17, highlighted in grey). These indels will form the main basis of sequence variation between the species for haplotype allocations and phylogenetic inference (Appendix B 2.2).

The *ITS2* sequence dataset had 11 haplotypes. South Africa contained 8 haplotypes, where haplotypes 1-8 were shared between South African ticks. Haplotype 1 contained most of the sequences (47 of 72, 65%) and Haplotypes 9-11 are unique. Table 17 indicated the haplotypes and their differences for *R. microplus* and *R. annulatus*.

Allele networks were constructed for the *R. microplus* dataset (including *R. annulatus*), excluding *R. decoloratus* and *R. geigyi* as they do not link within the network with reference to Table 17. Figures 24 and 25 indicated the allele networks per location (origin of tick collected) and based on climate (Köppen-Geiger climate classification system of South Africa).

The haplotype networks for the *ITS2* sequence were reticulated with all linkages equally likely due to multiple mutations at a single site (position 92). Non-reticulated networks as per location are provided (Appendix B 2.2). Both Figures 24 and 25 showed that the *R. microplus* tick populations of South Africa had no structure, neither according to geography (location/province) or climate. The sequence of *R. australis* represented haplotype 1 and groups within the South African network. The haplotype networks indicate that Brazil and USA (Deutsch) isolates are genetically distinct from South Africa and West Africa. The *ITS2* sequence of *R. annulatus* links within the haplotype network, whereas *R. decoloratus* and *R. geigyi* do not as they are too divergent (Figure E, Appendix B 2.2). This suggests that the *ITS2* sequence of *R. australis* and *R. annulatus* may evolve similarly to that of *R. microplus* or have a shared ancestral polymorphism. Additional markers are needed to distinguish between the three species.

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The haplotypes with single character or mutational changes (Hap 2-6 and 8) were removed from the phylogenetic analyses to improve the resolution of the trees after additional outgroups failed to improve resolution. Haplotypes 1, 7, 9-11 had the highest pairwise distances within the *ITS2* dataset (Appendix B 2.2). The phylogenetic analyses conducted on Haplotypes 1, 7, 9-11 indicated that *R. microplus* and *R. annulatus* are each other's closest relatives, followed by *R. decoloratus* and *R. geigyi*. The USA (Deutsch) strain groups outside the monophyletic clade of *R. microplus* sequences from South Africa, West Africa and Brazil. This trend is also observed for the ITS2 phylograms, which have identical branching patterns (Appendix B 2.2).

The populations of the ITS2 dataset were derived from their geographical location, such as country and province; South Africa (Mpumalanga, Eastern Cape, KwaZulu-Natal, Gauteng, Swaziland, Western Cape and Mnisi), West Africa, and Non-African (USA, Brazil and AUS) (Appendix B 2.2). Outgroups were designated as *R. decoloratus* and *R. geigyi*. Additional analyses of *ITS2* sequence polymorphism with neutrality statistics confirmed low variability of between the *R. microplus* sequences from South Africa, where invariable sites constituted 372 of the total 376 sites and only 4 sites were polymorphic (single variants and parsimony informative sites) (Appendix B 2.2).

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2.4.6 *COI* sequence

A total of 25 sequences were used for evaluating the *COI* gene: five *R. microplus* (2 South Africa, 2 USA and 1 Brazil); two *R. australis* (1 sequenced and 1 NCBI GenBank sequence, AF132827); two *R. annulatus* (1 sequenced and 1 NCBI GenBank sequence, AF132825); two *R. decoloratus* from West Africa (1 sequenced and 1 NCBI GenBank sequence, AF132826); two *R. geigyi* (1 sequenced and 1 NCBI GenBank sequence, AY008680); three *Ixodes* spp. (*I. rubicundus*, GU437875; *I. pilosus*, GU437874 and *I. granulatus*, AB231673) and nine *Hyalomma* spp. (2 *H. aegyptium*, JX394190 and JX394193; 2 *H. detritum*, EU827696 and EU827695; 2 *H. dromedarii*, AJ437065 and AJ437072; 2 H. *lusitanicum*, EU827735 and EU827715; and 1 *H. truncatum*, AF132824). The *Ixodes* and *Hyalomma* spp. represented outgroups for the *COI* dataset.

Rhipicephalus microplus from South Afirca, West Africa and Brazil are identical in the *COI* alignment (Appendix B 2.3). *Rhipicephalus microplus* from USA (Deutsch strain) contained a G at position 282 in the alignment, whereas other *R. microplus* sequences contained an A. The mutation was synonymous and did not increase the genetic distance between the two groups (0% pair-wise distance, Appendix B 2.3). The *COI* sequence clearly differentiates *R. microplus* from *R. australis* (previously, *R. microplus* from Australia, also represented by AF132827) with 26 base pair substitutions and a pairwise distance of 12% (Appendix B 2.3). This supports *R. australis* as a separate species from *R. microplus*, but additional markers and further morphological studies are required to confirm this. The *R. annulatus* sample sequenced during the study had a different haplotype to the exiting sequence on the NCBI GenBank database (AF132825). The sequenced *R. annulatus* has 5 base pair substitutions; a T at position 66, A at position 168, A at position 291, G at position 378 and an A at position 396 in the alignment. The sequence, AF132825, has a C, G, G, A, C at the respective positions in the alignment. This would suggest that the *R. annulatus* from West African may have multiple *COI* haplotypes. The *COI* gene is also different for each tick species present in the alignment and is therefore a good species marker for ticks. Figure 26 represents the Maximum Likelihood phylogram of the *COI* gene with Bayesian posterior probability superimposed on the phylogram. Additional phylograms were constructed (NJ, MP, ML and Bayesian inference) with identical branching patterns. The models and phylogenetic tree for each method (NJ, MP, ML and Bayesian inference) are provided (Appendix B 2.3).

Rhipicephalus microplus from South Africa, West Africa and Brazil represented haplotype 1, whereas *R. microplus* from the USA represented haplotype 2. *R. microplus*, *R. autralis* and *R. annulatus* represented a monophyletic clade, Clade I, whereas *R. decoloratus* and *R. geigyi* represented another monophyletic clade, Clade II. *R. microplus* and *R. australis* had a pairwise distance of 12% at gene level, but were identical at amino acid level (Appendix B 2.3).

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Pseudogene (numts) evaluation of the *COI* gene was facilitated by the use of two primers sets. The primer sets are located in and amplified different regions of the *COI* gene (Appendix B 2.3). The sequences amplified from the two primer sets were identical for 32 *R. microplus COI* sequences. This generated a large overlap region of 711 bp and allowed several long consensus sequences to be constructed from the two primer sets, namely sequences 26-1, 26-4 and 41-2. No co-amplified sequences were observed in the *COI* chromatograms and no "*COI*-like" BLAST-X hits on the NCBI GenBank database were found for the *COI* dataset (Appendix B 2.3). Protein BLAST hits were identical to the translated *COI* dataset with no stop codons or indels present in the amino acid sequence (Appendix B 2.3). Therefore, it can be concluded that the *COI* dataset used during this study does not contain pseudogene representatives.

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2.4.7 ANT sequence

Primers were designed for the *ANT* sequence by using the *R. microplus* homologue TC20518 retrieved from the VectorBase website (Appendix B 2.4). Amplification with the *ANT* primers yielded a product approximately 1200 bp, larger than the predicted 739 bp. This may be due to intron(s) within the amplified sequence. The *ANT* sequence proved difficult to amplify, likely due to its low copy number in the nuclear genome.

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2.5 Discussion

Although *R. microplus* and *R. decoloratus* ticks were collected throughout South Africa, the exact geographical distribution of both ticks cannot be determined as the collections were not extensive enough and excluded environmental sampling. As distribution or tick abundance was not the main focus of this study, host-based sampling was used as the primary method of tick sampling. This study illustrated that *R. microplus* has maintained its distribution and has become established after its introduction into new areas, such as in Swaziland and the Eastern Cape Province. It also provided further evidence for the spread of *R. microplus* into unsuitable habitats, such as the arid regions of the Mnisi Research Area ((ROBBERTSE 2014) In Press) and Namibia (NYANGIWE *et al.* 2013b). These trends would indicate that *R. microplus* will likely become more prevalent in arid regions in the future, such as the North West and Northern Cape provinces, especially with the aid of spread via cattle movement between communal dip tanks and during trade.

The historical importation and movement of cattle is reflected in both the global and national population structure of *R. microplus*. The south-eastern Cape was the initial introduction point of *R. microplus* into South Africa (HOWARD 1908; NYANGIWE *et al.* 2011). The Eastern Cape populations displayed the greatest number of unique *ITS2* haplotypes, suggesting an older population, which may coincide with *R. microplus* historical introduction. *The ITS2* haplotype network indicates that cattle movement plays a large role in homogenising the *R. microplus* populations of South Africa, Brazil and West Africa, which is also evident for the *COI* gene.

The shared *COI* haplotype between South Africa, Brazil and West Africa suggests that cattle trade between these countries may homogenise *R. microplus* populations and facilitate gene flow. Gene flow from Brazil to West Africa is facilitated by the introduction of *R. microplus* into West Africa (Benin) from Brazil on Girolando cattle, where the first records of *R. microplus* in Benin were made in 2007 (MADDER and HORAK 2010; MADDER *et al.* 2007). Subsequent trade of cattle between South Africa, Brazil and West Africa would result in a genetically similar population when cattle ticks mix and breed. These suggestions are made based on the current evidence and knowledge of *R. microplus* and as such no evidence to the contrary exists. The *COI* gene represents a species marker for the *Rhipicephalus* genus and can genetically distinguish between all the cattle ticks, including *R. microplus* (Canestrini) and *R. australis* (Fuller). With the reinstatement of *R. australis*, previously published articles of *R. microplus* from Australia and New Caledonia need to be regarded as *R. australis*.

The *COI* gene also displayed little genetic variance given that *R. microplus* populations are large on cattle farms and *R. australis* (comparative species) has a high genetic diversity (CHEVILLON *et al.* 2007b; CUTULLÉ *et al.* 2010; KOFFI *et al.* 2006a). The *COI* gene did however indicate genetic distinctiveness for *R. microplus* samples from the different continents evaluated (Clade I vs. Clade II),

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suggesting resolution at a higher 'continental' or 'global' intraspecific scale than a 'local' intraspecific scale. The mitochondrial genome is assumed to evolve according to the neutral theory of molecular evolution, where it is free of recombination and adaptive evolution is rare (GALTIER *et al.* 2009). This allows population genetic diversity to increase with population size (MEIKLEJOHN *et al.* 2007). The lack of *COI* resolution in *R. microplus* across South Africa may be due to several factors that violate the assumptions of the evolutionary mechanisms that govern the mitochondrial genome.

NUMTs (nuclear copies of mitochondrial DNA) also known as pseudogenes are the result of illegitimate recombination events that insert mitochondrial copies of genes into the nuclear genome (LEITE 2012). Traditionally these copies lose their original coding function and become non-functional upon insertion into the nuclear genome allowing the copy to accumulate mutations and evolve independently from its mitochondrial counterpart (LEITE 2012). It may result in population homogenisation when mitochondrial copies are inserted into a region of the nuclear genome that is under positive selection. The nuclear copy may become linked to the mitochondrial copy and drive its fixation whilst retaining its original function. The validity of whether pseudogenes may drive mitochondrial genes to fixation in large populations, as seen with the two *COI* haplotypes of *R. microplus*, requires investigation. It was determined that our dataset did not contain COI haplotypes since no co-amplified sequences were observed in the *COI* chromatograms, no "*COI*-like" BLAST-X hits on the NCBI GenBank database were found for the *COI* dataset, the protein BLAST hits were identical to the translated *COI* dataset with no stop codons and no indels present in the amino acid sequence (Appendix B 2.3).

It has been shown that the *COX* genes, specifically *COI*, has a lower divergent rate that cytochrome *b* and may suggest the influence of positive selection on the function of the *COX* genes (MEIKLEJOHN *et al.* 2007). Adaptive evolution (such as through direct selection for increased or more flexible respiratory function) (BAZIN *et al.* 2006; GALTIER *et al.* 2009) and adaptive introgression (due to environmental pressures) (GALTIER *et al.* 2009) may drive the fixation of certain allele variants in large populations.

Bazin and colleagues (2006) suggested that decreased genetic diversity can also be a result of purifying selection against deleterious mutations (known as background selection) that occurs at linked loci and through hitch-hiking events that fix allele variants (BAZIN *et al.* 2006). Yet, the generation of an independent relationship between genetic diversity and population size was reasoned improbable by the same authors (BAZIN *et al.* 2006).

Population bottlenecks (the introduction of a small founding population and subsequent expansion) (BAZIN *et al.* 2006) or selective sweeps (removal of genetic variation around a positively selected site and subsequent fixation) (MEIKLEJOHN *et al.* 2007) may also generate low genetic diversity in large populations. Maternally transmitted endosymbionts are common in invertebrates and mimic the effects of population bottlenecks through selective sweeps, such as with the *Wolbachia* system (BAZIN *et al.*

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2006; GALTIER *et al.* 2009; WILLIAM *et al.* 1995). The lack of evidence for such a tick endosymbiont does not suggest that it may not be an unknown factor driving allele fixation of the mitochondrial genome.

Another model has been suggested for decreased genetic diversity in large invertebrate populations that has a similar effect to population bottlenecks and carries a signature of positive selection (MEIKLEJOHN *et al.* 2007) in the absence of recombination (BAZIN *et al.* 2006; GILLESPIE 2000). The concept of linked selection and genetic hitch-hiking was first introduced by Gillespie (2000) as the "pseudohitchhiking model", later referred to as "genetic draft" (BAZIN *et al.* 2006; GILLESPIE 2000; MEIKLEJOHN *et al.* 2007) and separately investigated by Baton (2000) as genetic hitchhiking (BARTON 2000). A closely linked neutral locus experiences stochastic dynamics that resemble genetic drift as induced by another locus under selection (GILLESPIE 2000). Only a fraction of these replacement substitutions are selected for and the rest hitch-hike to fixation (MEIKLEJOHN *et al.* 2007). This can be seen as "random drift in infinite populations" (GILLESPIE 2000) and results in a level or negative relationship between population size and genetic diversity (BAZIN *et al.* 2006). Genetic draft is also maximised in the mitochondrial genome due to its gene-dense and non-recombining nature in animals (BAZIN *et al.* 2006). Flaxman and colleagues (2013) provide a method with which to test the effects of selection, genetic hitchhiking and gene flow on natural populations (FLAXMAN *et al.* 2013) and provides a valuable resource to investigate genetic hitchhiking in *R. microplus* during future studies.

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2.6 Conclusion

We have illustrated the use of several markers to investigate the intraspecific and interspecific genetic diversity of *R. microplus*. The *D3* region, although used successfully as a species marker for *Ixodes* and *Dermacentor* species (ANSTEAD *et al.* 2011), does not resolve the intraspecific phylogeny of *R. microplus.* Therefore, the *D3* region is not a species marker for the *Rhipicephalus* genus as the sequences for *R. microplus* and *R. sanguineus* are identical. The *ITS2* showed multiple 'similar' haplotypes for South African *R. microplus* and the lack of variation was confirmed using neutrality tests. It is observed that *R. annulatus* and Brazilian *R. microplus* group within the South African haplotype network and warrants further investigation as soon as additional samples of *R. annulatus* from West Africa and *R. microplus* from Brazil become available. Thus, the *ITS2* is a suitable candidate for interspecific phylogenetic and phylogeographic studies of cattle ticks. The *COI* indicated that *R. microplus* is genetically similar in Africa and Brazil. Also, *R. annulatus* from West Africa may have multiple *COI* haplotypes and is likely due to the importation of cattle from Brazil, as with *R. microplus*, but requires further investigation with samples from both regions.

We have shown that *R. microplus* populations from South Africa have low genetic diversity (*ITS2* and *COI* genes), which has resulted in a population with no genetic structure (neither according to geographic or climate). This may suggest that the markers used were unsuitable for the analyses or that *R. microplus* populations had not experienced enough time to become genetically distinct, since its introduction in 1908 (HOWARD 1908; NYANGIWE *et al.* 2011). The shared *COI* haplotype between South Africa, Brazil and West Africa suggests that on-going cattle trade has an impact on genetic diversity at regional and global scales, where the Deutsch strain from the USA has an unique haplotype due to its isolation from other strains. Additional studies with microsatellite markers, might indicate population structure according to acaricide resistance or vectoral competence (with *Babesia bovis*) as for *R. australis* (CHEVILLON *et al.* 2013) and *R. appendiculatus* (KANDUMA *et al.* 2012) tick populations, respectively. We have also illustrated the importance of cattle trade monitoring and restrictions to prevent the introduction of new tick species, resistant ticks and their associated tick-borne diseases into unoccupied areas, which may result in epidemic outbreaks. We have further established that the process, trade and cattle movement, whereby *R. microplus* achieves a foothold in a population where it was not observed previously, must be monitored. Preventing the establishment of foothold *R. microplus* populations may assist greatly in limiting its spread to previously unoccupied areas.

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2.7 Figures and Tables

Table 13: PCR primers to amplify selected nuclear and mitochondrial genes in *R. microplus* (ANSTEAD *et al.* 2011; APANASKEVICH *et al.* 2011; FOLMER *et al.* 1994; LEMPEREUR *et al.* 2010).

Locus	Forward Primer 5' → 3'	Reverse Primer 5 → 3'
ITS2[*]	GCCGTCGACTCGTTTTGA	TCCGAACAGTTGCGTGATAAA
D3[†]	GCGGCGAGTAGGTCGGTAACC	ACGTCAGAATCGCTTCGGA
COI[‡]	GGTCAACAAATCATAAAGATATTGG	TAAACTTCAGGGTGACCAAAAAATCA
COI[a]	CCTAAACTTCAGCCATTTTACCG	TGTCTTGTCAATAGGAGCAGTAT
ANT[b]	ACTGCTTTGTCCGAATCCCC	GGTCCCTAGAATATGAACGCCTTG

[*] *ITS2* as described by Lempereur and colleagues (LEMPEREUR *et al.* 2010).
[†] *D3* as described by Anstead and colleagues (ANSTEAD *et al.* 2011).
[‡] *COI* as described by Beati and Keiranst (APANASKEVICH *et al.* 2011; FOLMER *et al.* 1994)
[a & b] Primers designed for the study (RmCOIF and RmCOIR)

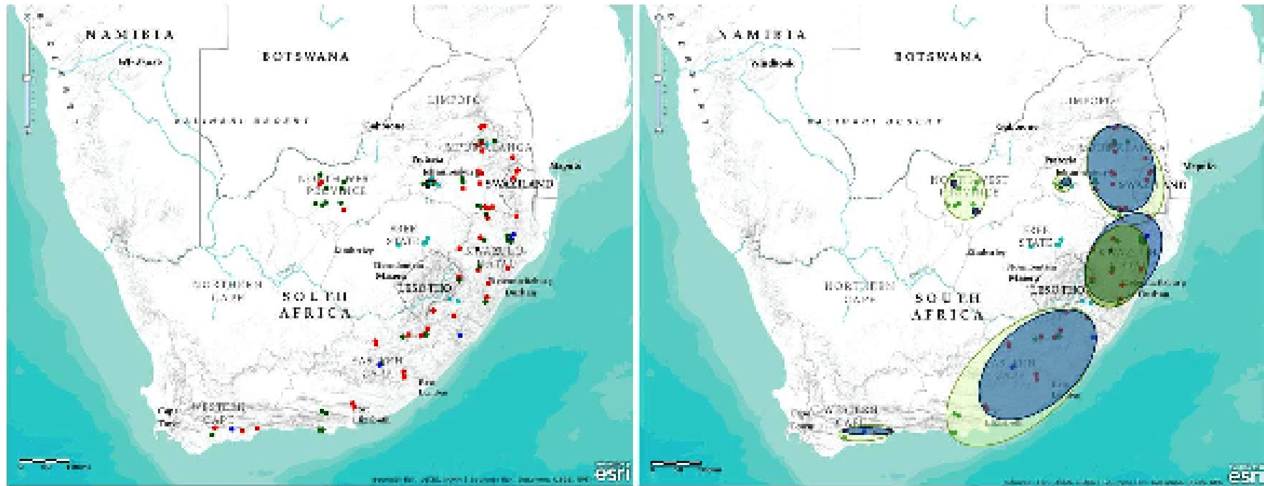

Figure 19: ArcGIS™ Maps (www.arcgis.com) indicating the sampling points of cattle farms throughout South Africa in high and medium cattle density areas. Legend: Farms with only *R. microplus* are indicated in blue (Rm), farms with only *R. decoloratus* are indicated in green (Rd), farms with both *R. microplus* and *R. decoloratus* are indicated in red (Both), farms with no *R. microplus* or *R. decoloratus* ticks are indicated in black (None) and farms for which ticks have not been identified are indicated in turquoise (NA). Left: Sampling point map indicating exact GPS locations of cattle farms and the tick species collected on these. Right: Distribution map indicating the relative distributions of *R. microplus* (blue) and *R. decoloratus* (green) throughout the sampling points.

Table 14: The prevalence of *R. microplus* and *R. decoloratus* on collected cattle farms in each province, see Sheet 1: Database for calculations.

Province	Percentage *R. microplus* (*Rm*)	Percentage *R. decoloratus* (*Rd*)	Ratio *Rm*:*Rd*	Ratio *Rd*:*Rm*
Mpumalanga	17.52%	82.48%	0.21	4.71
Eastern Cape	34.64%	65.36%	0.53	1.89
KwaZulu-Natal	41.61%	58.39%	0.71	1.40
Gauteng	33.33%	66.67%	0.50	2.00
Swaziland[a]	77.50%	22.50%	3.44	0.29
Western Cape	36.05%	63.95%	0.56	1.77
North West	2.56%	97.44%	0.03	38.13

[a] Not a province of South Africa but a region chosen for this study and treated similar to a province. Data based on limited samples and results may be biased.

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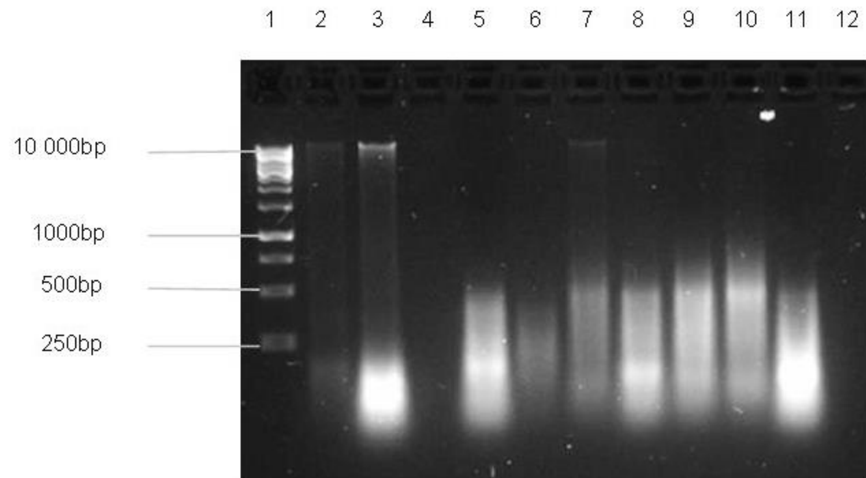

Figure 20: Genomic DNA isolations from adult female blood-fed ticks, *R. microplus*. GeneRuler 1kbp DNA Ladder (lane1); samples with some non-degraded DNA (lanes 2, 3 and 7); samples with degraded DNA (lanes 4 - 6, 8 - 11) and the negative control (lane 12).

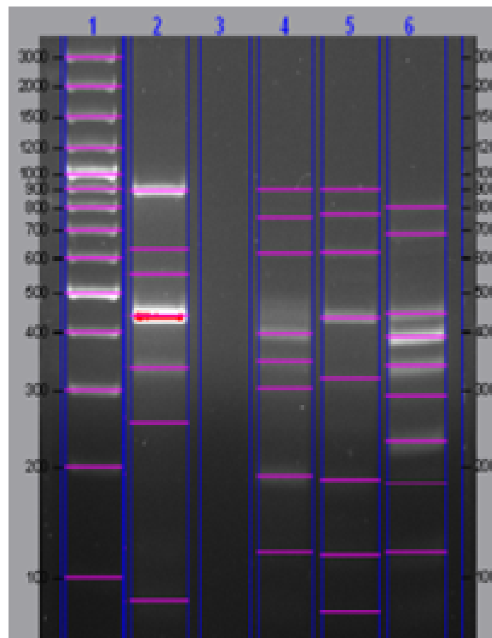

Figure 21: Specific banding pattern of the *ITS2*-RFLP which allowed identification of *Rhipicephalus* species. GeneRuler 100bp DNA ladder (lane 1), *R. microplus* banding pattern (lane 2), No sample (lane 3), *R. decoloratus* banding pattern (lane 4), *R. annulatus* banding pattern (lane 5) and *R. geigyi* banding pattern (lane 6). Image produced with BioRad® ImageLab® software.

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Table 15: The Taguchi PCR reagent concentrations and grid reaction set-up. Adapted from the original Taguchi grid (COBB and CLARKSON 1994).

Template	Amount	Unit	Primer	Amount	Unit	$MgCl_2$	Amount	Unit
A	50	ng	**A**	2.5	pmol	**A**	1.5	mM
B	75	ng	**B**	5	pmol	**B**	2	mM
C	100	ng	**C**	10	pmol	**C**	2.5	mM
Reaction		**T**	**P**	**M**		**T**	**P**	**M**
	1	A	A	A	**AAA**	50	2.5	1.5
	2	A	B	B	**ABB**	50	5	2
	3	A	C	C	**ACC**	50	10	2.5
	4	B	A	B	**BAB**	75	2.5	2
	5	B	B	C	**BBC**	75	5	2.5
	6	B	C	A	**BCA**	75	10	1.5
	7	C	A	C	**CAC**	100	2.5	2.5
	8	C	B	A	**CBA**	100	5	1.5
	9	C	C	B	**CCB**	100	10	2

T: template, P: primer, M: magnesium chloride

Table 16: PCR reagents and conditions used to amplify selected nuclear and mitochondrial genes in *Rhipicephalus microplus*.

Sequence	Size of product (bp)	Template concentration (ng)	Primer concentration (pmol)	Annealing Temperature (T^a, °C)	Cycling conditions (of T_a)	$MgCl_2$ (mM)
D3	300	300	10	48	30sec, 30 cycles	1.5
ITS2	700	300	10	58	45sec, 40 cycles	1.5
***COI* (Designed)**	1200	300	15	56	25sec, 30 cycles	1.5
***COI* (Folmer)**	600	300	10	50	30sec, 35 cycles	1.5
ANT	1200	300	15	56	25sec, 35 cycles	1.5

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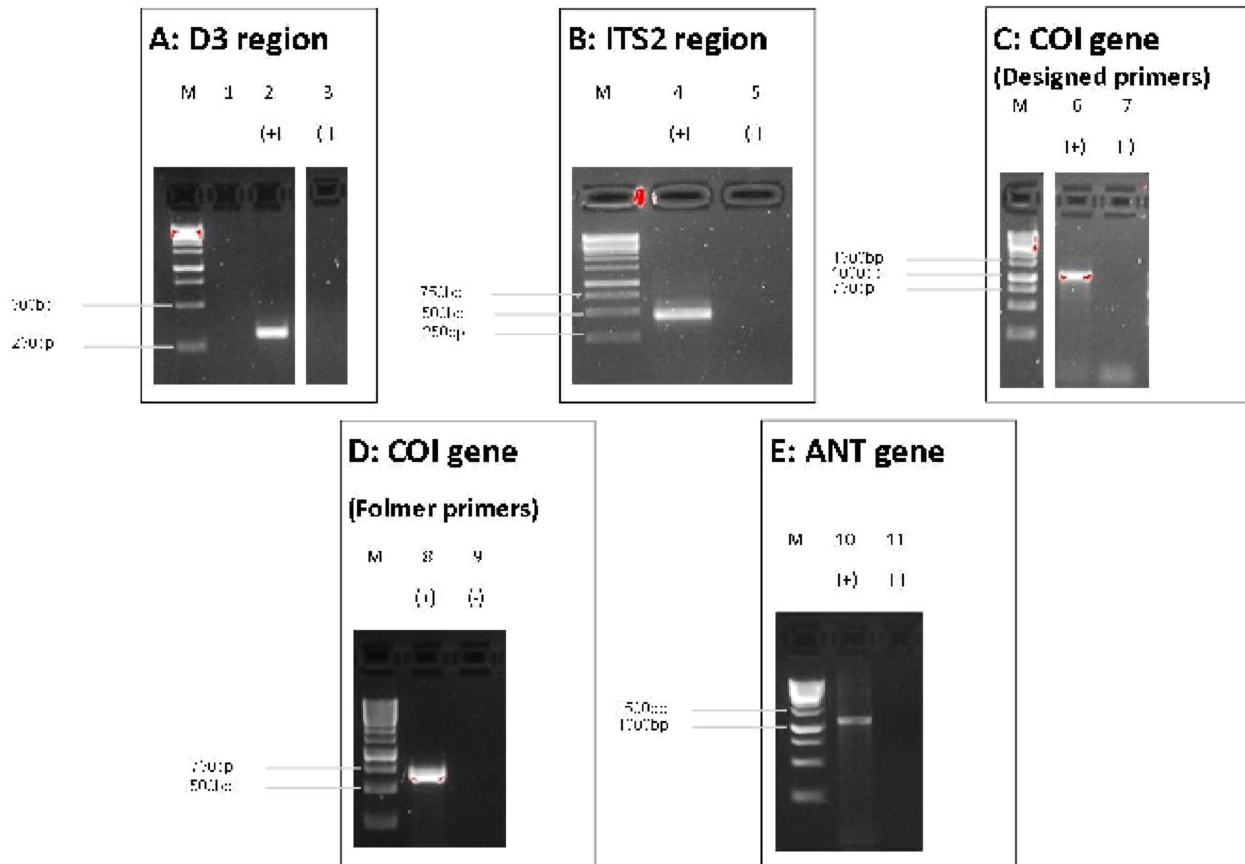

Figure 22: Optimised amplification for nuclear and mitochondrial genes. 22A: Molecular weight markers (M), no sample (lane 1), optimised *D3* (lane 2), negative control for *D3* (lane 3). **22B:** optimised *ITS2* (lane 4), negative control for *ITS2* (lane 5). **22C:** optimised *COI* with designed primers (lane 6), negative control for *COI* (lane 7). **22D:** optimised *COI* with Folmer primers (lane 8), negative control for *COI* (lane 9). **22E:** optimised *ANT* (lane 10) and negative control for *ANT* (lane 11). All markers were amplified with the ClinVet strain and is designated as the positive control for the remained of PCR reactions.

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                        10         20         30         40         50         60
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
7-1             ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~ATGTGC GCGAGTCAAT GGGTCTCCCG   26
7-2             ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~C TAAC...... .......... ..........   31
7-3             ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~.... .......... ..........   24
8-1             ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~C...... .......... ..........   27
8-2             ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~...... .......... ..........   26
8-3             ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~TC TAAC...... .......... ..........   32
9-2             ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~.... .......... ..........   24
9-3             ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~..... .......... ..........   25
17-1            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~..... .......... ..........   25
17-3            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~..... .......... ..........   25
18-1            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~GTC TAAC...... .......... ..........   33
18-2            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~TC TAAC...... .......... ..........   32
26-1            ~~~~~~~~~~ ~~~~~~CGGA CCAAGGAGTC TAAC...... .......... ..........   44
26-2            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~TC TAAC...... .......... ..........   32
41-2            ~~~~~~~~~~ ~~~~~~CGGa CCAAGGAGTC TAAC...... .......... ..........   44
42-1            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ TAAC...... .......... ..........   30
42-2            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~..... .......... ..........   25
42-3            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~C TAAC...... .......... ..........   31
46-3            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~.... .......... ..........   24
46-5            ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~...... .......... ..........   26
ClinVet         ~~~TCCACCC CTGAACCGGA CCAAGGAAGT CTAC...... .......... ..........   57
G70_RM (WA)     ---------- ---------- --------~~ -------... .......... ..........   23
R_mic_WA        ~~CCCCAGGT CTGAAACGGA CCAAGGAGTC TAAC...... .......... ..........   58
R_ann_WA        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~.... .......... ..........   24
R_san_AF062986  GACCCGTCTT GAAACACGGA CCAAGGAGTC TAAC...... .......... ..........   60

                        70         80         90        100        110        120
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
7-1             AAACCCAATG GCGCAATGAA ACGTGAAGGC CCCTAGCGGG CTGCGTTGCG ATCCCGGACC   86
7-2             .......... .......... .......... .......... .......... ..........   91
7-3             .......... .......... .......... .......... .......... ..........   84
8-1             .......... .......... .......... ......T... .......... ..........   87
8-2             .......... .......... .......... .......... .......... ..........   86
8-3             .......... .......... .......... ......y... .......... ..........   92
9-2             .......... .......... .......... ......y... .......... ..........   84
9-3             .......... .......... .......... .......... .......... ..........   85
17-1            .......... .......... .......... .......... .......... ..........   85
17-3            .......... .......... .......... .......... .......... ..........   85
18-1            .......... .......... .......... ......y... .......... ..........   93
18-2            .......... .......... .......... ......y... .......... ..........   92
26-1            .......... .......... .......... .......... .......... ..........  104
26-2            .......... .......... .......... ......T... .......... ..........   92
41-2            .......... .......... .......... ......T... .......... ..........  104
42-1            .......... .......... .......... .......... .......... ..........   90
42-2            .......... .......... .......... .......... .......... ..........   85
42-3            .......... .......... .......... .......... .......... ..........   91
46-3            .......... .......... .......... .......... .......... ..........   84
46-5            .......... .......... .......... .......... .......... ..........   86
ClinVet         .......... .......... .......... .......... .......... ..........  117
G70_RM (WA)     .......... .......... .......... ......y... .......... ..........   83
R_mic_WA        .......... .......... .......... .......... .......... ..........  118
R_ann_WA        .......... .......... .......... .......... .......... ..........   84
R_san_AF062986  .......... .......... .......... ......T... .......... ..........  120

                       130        140        150        160        170        180
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
7-1             GCACAGGGGT CCGATAAAGG GCGCAGCAAC GGCCCGTCCC AGGCGCTCAC ACGTCGCCGG  146
7-2             .......... .......... .......... .......... .......... ..........  151
7-3             .......... .......... .......... .......... .......... ..........  144
8-1             .......... .......... .......... .......... .......... ..........  147
8-2             .......... .......... .......... .......... .......... ..........  146
8-3             .......... .......... .......... .......... .......... ..........  152
9-2             .......... .......... .......... .......... .......... ..........  144
9-3             .......... .......... .......... .......... .......... ..........  145
17-1            .......... .......... .......... .......... .......... ..........  145
17-3            .......... .......... .......... .......... .......... ..........  145
18-1            .......... .......... .......... .......... .......... ..........  153
18-2            .......... .......... .......... .......... .......... ..........  152
26-1            .......... .......... .......... .......... .......... ..........  164
26-2            .......... .......... .......... .......... .......... ..........  152
41-2            .......... .......... .......... .......... .......... ..........  164
42-1            .......... .......... .......... .......... .......... ..........  150
42-2            .......... .......... .......... .......... .......... ..........  145
42-3            .......... .......... .......... .......... .......... ..........  151
46-3            .......... .......... .......... .......... .......... ..........  144
46-5            .......... .......... .......... .......... .......... ..........  146
ClinVet         .......... .......... .......... .......... .......... ..........  177
G70_RM (WA)     .......... .......... .......... .......... .......... ..........  143
R_mic_WA        .......... .......... .......... .......... .......... ..........  178
R_ann_WA        .......... .......... .......... .......... .......... ..........  144
R_san_AF062986  .Y........ .......... .......... .......... .......... ..........  180
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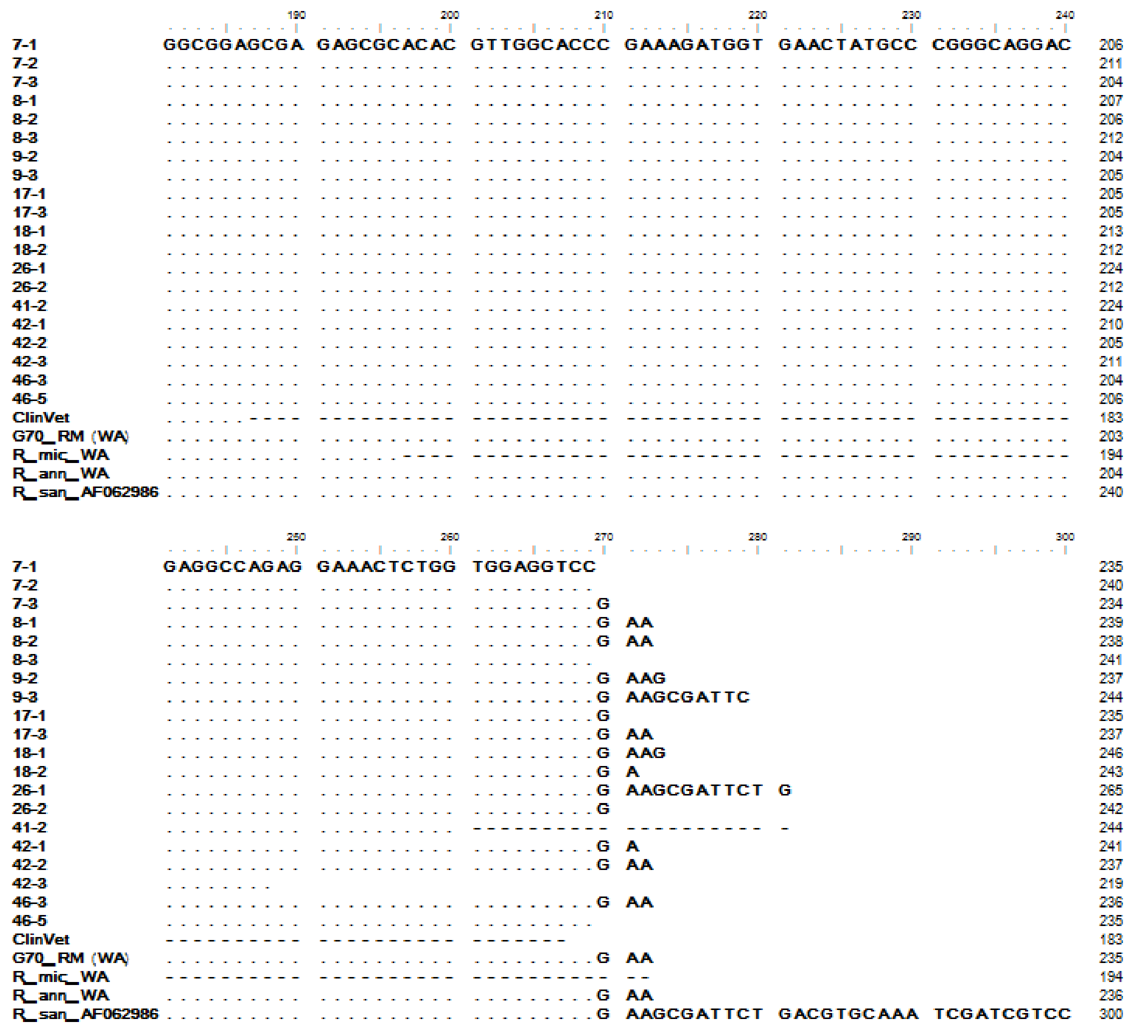

Figure 23: The *D3* alignment for sequences from South Africa and West Africa. Numbers indicate: Farm reference (7) and tick sample (1) from South Africa. G70_RM (WA) and R_mic_WA are *R. microplus* sequences from Benin, West Africa. R_ann_WA is a *R. annulatus* sequence from Benin, West Africa. West African tick samples were provided by Prof M. Madder (Department of Veterinary Tropical Diseases, University of Pretoria, South Africa) and sequenced during the study at the University of Pretoria. *R. sanguineus* (AF062986) represents a GenBank sequence from Anstead *et al.* (2011). The first 30 base pairs are highly variable due to incomplete primer extension of some sequences. The (.) represent identical base pairs to the top row and (-) indicates gaps in the alignment.

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Table 17: The 11 haplotypes of the *ITS2* sequence and the positions of the base pair changes. (·) indicates base pairs that are the same as the top row and (-) indicates a deletion. Grey indicates G insertion in the *R. microplus* Deutsch strain not present in the other haplotypes.

Haplotype/Position	25	34	71	92	124	134	152	175	214	223	235	261	284	285	323	338	357
Hap 1 (SA & WA)*	G	G	A	C	-	C	C	A	G	C	G	G	G	G	A	C	G
Hap 2 (SA)	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
Hap 3 (SA)	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	A
Hap 4 (SA)	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 5 (SA)	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 6 (SA)	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 7 (SA & WA)	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
Hap 8 (SA)	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 9 (USA)	.	A	.	.	G	.	.	.	.	T	.	.	.	.	T	T	.
Hap 10 (Brazil U97714)	.	.	.	.	.	.	.	T	C	.	C	C	C	C	.	.	.
Hap 11 (WA)+	.	.	G	.	.	A	A	.	.	.	.	.	.	.	.	.	.

* Haplotype 1 includes the ClinVet strain and *R. australis.*
\+ Haplotype 11 represents the *R. annulatus* sequence

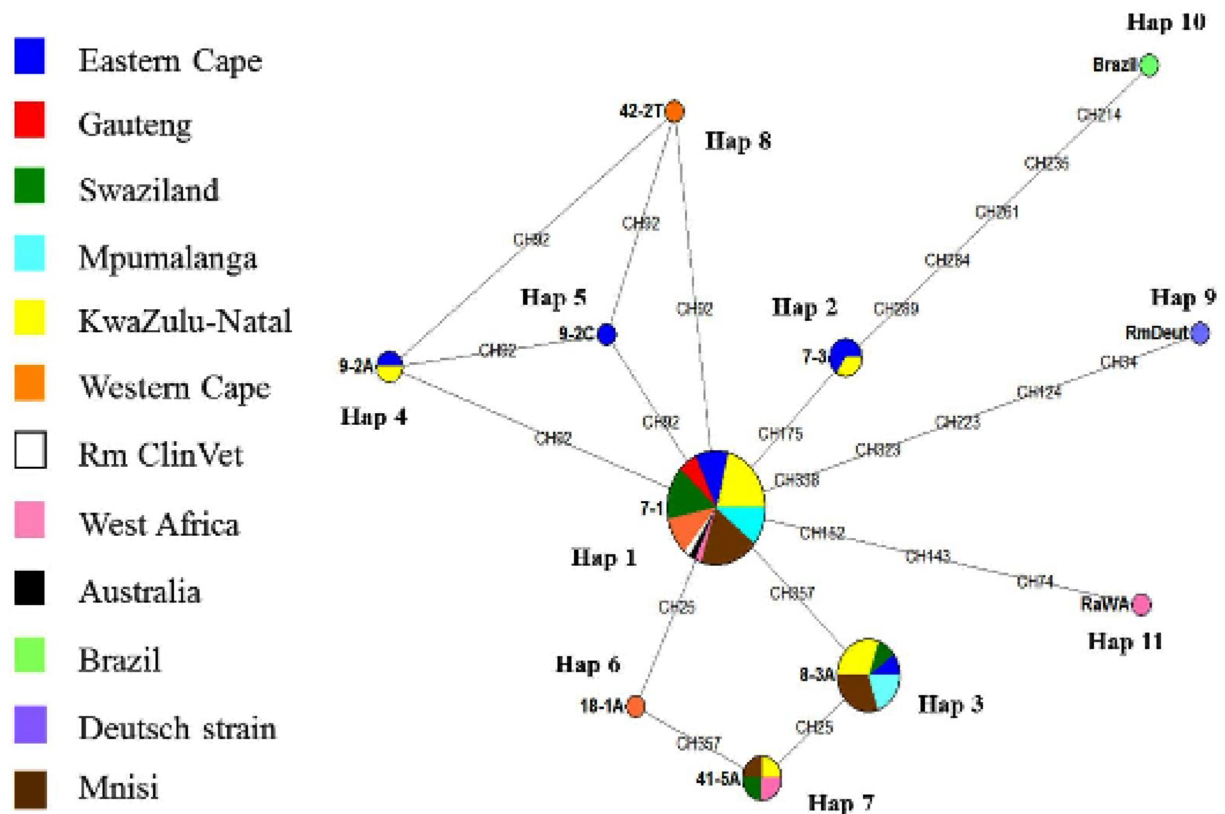

Figure 24: The Median Joining haplotype network for the *ITS2* sequences from South Africa based on location (province or country) where ticks were collected. Network constructed with 95% confidence and character mutations are indicated. *R. annulatus* (Hap 1 and 11) link within allele network, whereas *R. decoloratus* and *R. geigyi* do not. Numbers indicate farm reference and tick sample (7-1) and sequences with one heterozygous base pair are indicated (e.g. 18-1A and 18-1G, with an A or G at position 22).

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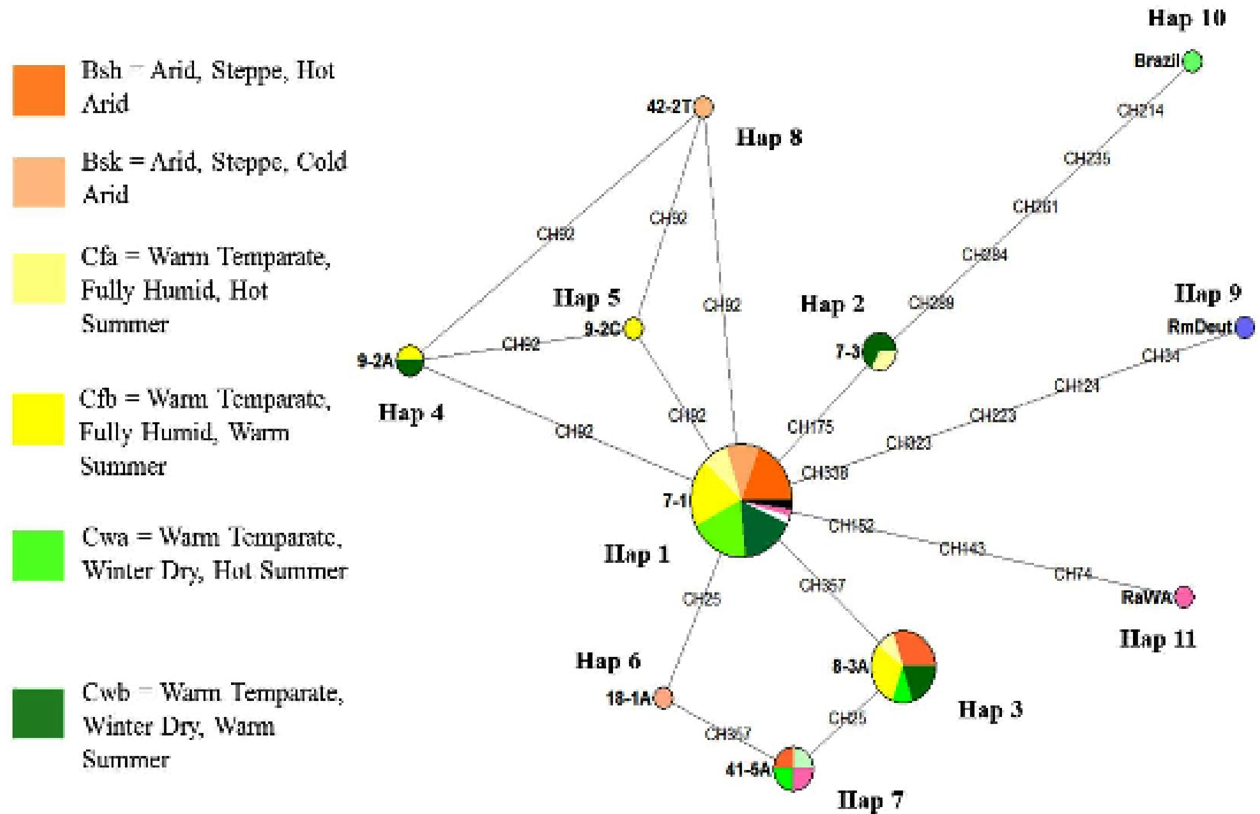

Figure 25: The Median Joining haplotype network for the *ITS2* sequences from South Africa based as per ecology (Köppen-Geiger climate classification system of South Africa). Network constructed with 95% confidence and character mutations are indicated. *R. annulatus* (Hap 1 and 11) link within allele network, whereas *R. decoloratus* and *R. geigyi* do not. Numbers indicate farm reference and tick sample (7-1) and sequences with one heterozygous base pair are indicated (e. g. 18-1A and 18-1G, with an A or G at position 22).

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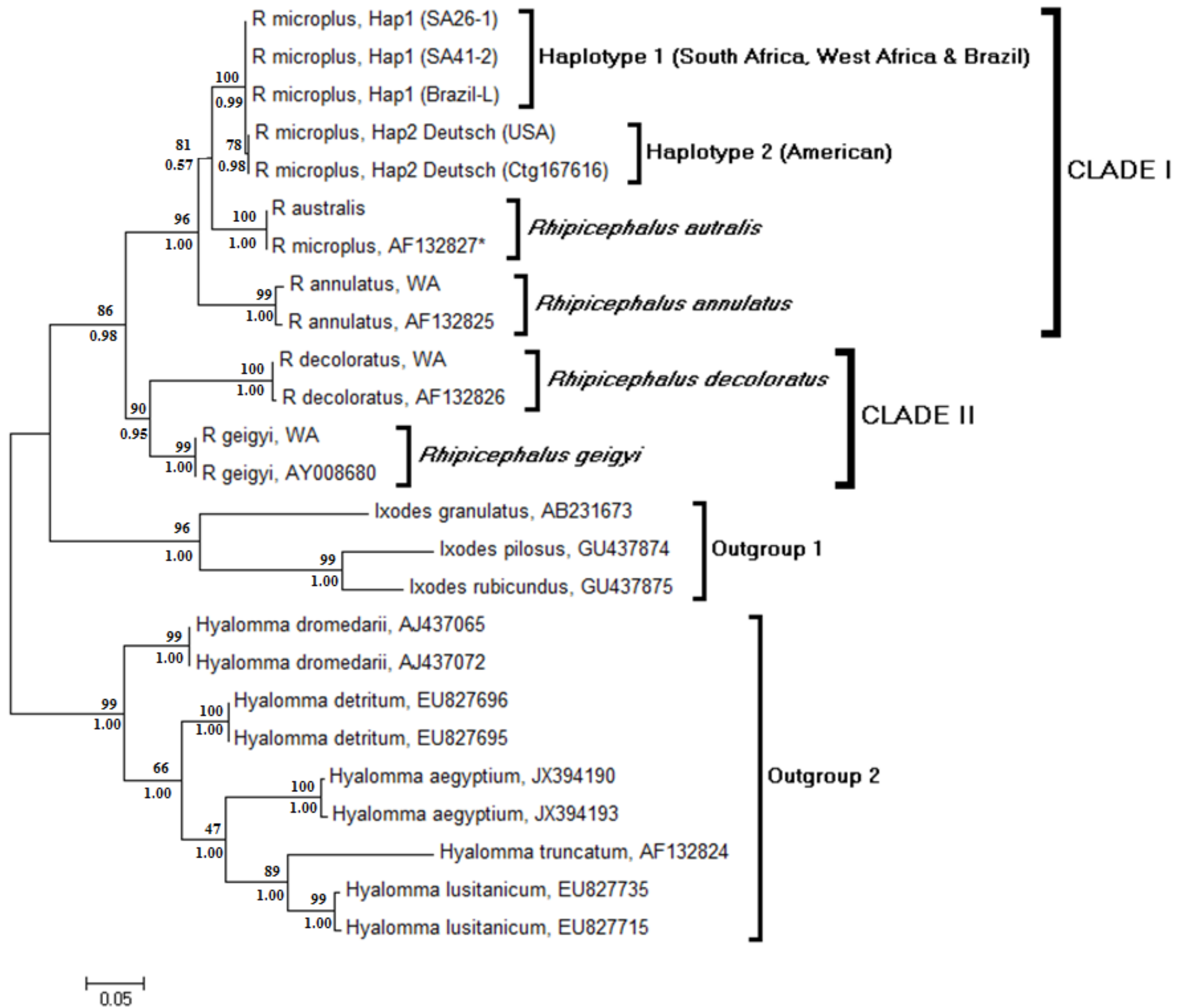

Figure 26: Maximum Likelihood phylogram of the *COI* gene sequences. The phylogenetic tree was constructed with the General Time Reversible model (GTR with a 2.0 gamma parameter), nearest-neighbour-interchange (NNI) and 1000 bootstraps replicates. Maximum Likelihood bootstrap support shown above branch nodes and Bayesian posterior probability shown below branch nodes. (*) indicates sequence AF132827 as *R. microplus* collected from Australia, and it therefore likely *R. ausralis* given its grouping in the phylogram.

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Chapter 3: Concluding Discussion

The agricultural industry is witnessing a global human trade-assisted expansion of the Acari class (NAVAJAS and OCHOA 2013), of which *R. microplus* and *R. australis* have been the most successful tick species (CHEVILLON *et al.* 2013; MADDER *et al.* 2012; MADDER *et al.* 2011). The unique ability of these two species to adapt and acquire acaricide resistance is at the foundation of this expansion (CHEVILLON *et al.* 2013; RODRIGUEZ-VIVAS *et al.* 2011). *R. microplus* in particular has been significantly assisted in this growth through cattle trade. The invasive capacity of *R. microplus* poses a current threat to cattle farming globally and urgent steps are required to stem its geographical and acaricide resistance spread into new areas. Genetic and ecological studies on the invasion capacity of *R. microplus* will assist with the management and prevention of tick and acaricide resistance spread. This study reveals some of the mechanisms involved in shaping the genetic population structure of *R. microplus* and concludes that cattle trade plays a pivotal role. This is not to be confused with local factors that contribute to genetic variation at farm level, such as humidity and temperature. The adaptation to new habitats will largely be facilitated and exaggerated by cattle trade or movements at a national level.

Rhipicephalus microplus populations experience gene flow that allows populations to become genetically similar. Gene flow is more apparent at a global scale (Brazilian, West African and South African representatives vs. the Deutsch strain) than at a national scale where no defined population structure exists. Global homogenisation is facilitated by the on-going and extensive global cattle trade. The Deutsch strain has been maintained at the USDA-ARS Cattle Fever Tick Research Laboratory in Mission (Texas) as a colony strain (HILL *et al.* 2009) since its collection during the cattle fever outbreaks of the 1890s in South Texas (LOHMEYER *et al.* 2011; POUND *et al.* 2010). This may have resulted in the isolation of this colony from the other natural field *R. microplus* strains and has allowed it to diverge over time, due to its inbreeding and the lack of environmental constraints that influence selection pressure on the population. The Deutsch strain in relation to *R. microplus* from Brazil, West Africa and South Africa can be seen as a subdivided population at a global scale. Therefore, when considering the global *R. microplus* population, gene flow via cattle trade and the isolation of certain strains can result in an ‘Island model’ for the global gene flow (with reference to Section 2.2). In contrast, on a national level in South Africa, cattle trade is limited to farms within approximately 50 km from one another and can result in a ‘stepping-stone model’ for national/regional gene flow (with reference to Section 2.2). These models allow for some definition of *R. microplus* populations, but they remain largely unstructured. This can be due to either the low variability of the markers analysed or that *R. microplus* population have not experienced sufficient time to diverge.

Lack of population structure is evident for many tick species. *Ixodes holocyclus* (SONGA et al. 2011), *Ixodes cornuatus* (SONGA *et al.* 2011), *Rhipicephalus appendiculatus* (YSSOUF *et al.* 2011) and *Amblyomma hebraeum* (CANGI *et al.* 2013) do not readily structure with traditional ecological

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parameters. *Hyalomma rufipes* (CANGI *et al.* 2013) and the *R. sanguineus* species-group (DANTAS-TORRES *et al.* 2013) have been suggested to be divided into northern and southern lineages according to geographical locations and eco-regions respectively. The northern and southern lineages remain unstructured within their allocated groups. *Hyalomma rufipes* also displayed heterozygosity for the *ITS2* region. This complicates population structure and suggests that the *ITS2* region may not be a suitable genetic marker for intraspecific studies of ticks and may suggest that tick populations generally structure according to different parameters than geography or climate.

Microsatellite loci (with reference to Section 4.6) may present alternative markers to investigate the population structure of *R. microplus*. Studies of *R. australis* populations from New Caledonia (DE MEEÛS *et al.* 2010; KOFFI *et al.* 2006b) has suggested that ticks may structure according to acaricide resistance status (resistant vs. susceptible). In contrast, *R. australis* from Australia does not have any population structure (including acaricide resistance status), but a study has revealed that population variation occurs at farm level (CUTULLÉ *et al.* 2009). It is expected that microsatellites have the potential to elucidate the population structure of *R. microplus*, especially when combined with Next Generation Sequencing (NGS) technologies.

To expand upon the conclusions in this dissertation and to develop further insight, NGS technologies can be used. NGS are cost effective and time efficient (DAVEY *et al.* 2011; ELSHIRE *et al.* 2011; WIT *et al.* 2012). They allow pooling of many samples through multiplexing, the use of adaptors on individual sequences and the generation of large number of sequencing results (DAVEY *et al.* 2011; ELSHIRE *et al.* 2011; WIT *et al.* 2012). Genotyping-by-sequencing (GBS) (ELSHIRE *et al.* 2011), Restriction site-associated DNA tags (RAD-tags) (EMERSON *et al.* 2010; HOHENLOHE *et al.* 2010) and Complexity Reduction of Polymorphic Sequences (CRoPS) (ORSOUW *et al.* 2007) allow rapid and accurate studies of genetic variation of non-model organisms (DAVEY *et al.* 2011; EMERSON *et al.* 2010). These include organisms with large genomes that have little or no prior genome knowledge (DAVEY *et al.* 2011; EMERSON *et al.* 2010). These NGS techniques are based on the same principle. Firstly, the genome complexity is reduced by restriction enzyme digestion (DAVEY *et al.* 2011). Secondly, multiplex identifier sequences (MIDs) or short barcode identifier sequences are ligated to the digested sequences (DAVEY *et al.* 2011). Thirdly, samples are pooled and fragments of a certain size are selected (RAD-tags and CRoPS) (DAVEY *et al.* 2011). Lastly, PCR yields sequence information of multiple individuals identified by their adaptor sequences (MIDs) (DAVEY *et al.* 2011). Draw backs of RAD-tags, GBS and CRoPS is that the manual gel size-selection steps limit reproducibility and throughput, sequence coverage may be difficult to estimate since these are non-target techniques while null alleles may hinder data analyses and skewed diversity estimates (MCCORMACK *et al.* 2013). Adaptors (MIDs) also require exact design in order to assign unique barcodes to each pooled individual (DAVEY *et al.* 2011) and the appropriate RE needs to be chosen (does not cut frequently and produce 2 to 3 bp overhangs) to digest the genome and ensure efficient adaptor ligation (ELSHIRE *et al.* 2011). Table 18 provides a

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summary of the NGS technologies that can be combined with microsatellite and SNP genome-wide analyses.

Table 18: Summary of the advantages and disadvantages of Next Generation Sequencing technologies; RAD-tags, CRoPS and GBS (DAVEY *et al.* 2011; ELSHIRE *et al.* 2011; EMERSON *et al.* 2010; HOHENLOHE *et al.* 2010; MCCORMACK *et al.* 2013; ORSOUW *et al.* 2007).

Technology	Application	Reads	Coverage	Advantages	Considerations
RAD-Tag	Adapted from microarrays for NGS. SNP mapping	Short reads (50/150 bp around RE site)*	High sequencing coverage	Population or individual sample studies. Pooled samples decrease associated costs.	Doesn't provide gametic phase or haplotype sequence information. 300ng of DNA required per sample.
CRoPS	Adapted from AFLPs. SNP mapping	Long reads	High sequencing coverage	Population or individual sample study. MID for tagging.	Doesn't provide gametic phase or haplotype sequence information. 300ng of DNA required per sample.
GBS	Recombination studies QTLs if parents known. SNP mapping	Short reads*	Low-coverage per individual sample	Simpler protocol than RAD-tag or CRoPS. Lower cost per sample. Feasible for high-diversity, large genome species.	Doesn't provide gametic phase or haplotype sequence information. Results seen as dominant markers. Libraries without adaptors can be kept for DNA sequencing. More complex analyses. 100ng of DNA per sample.

* Increased with Ion-torrent technology (up to 500bp in total) (MCCORMACK *et al.* 2013).

NGS technology is highly suited to phylogenetic and population genetic studies of agriculturally important species, which often have a low number of individuals in the representative population (MCCORMACK *et al.* 2013). Non-genic regions (repeat regions and SNP variants) are mainly studied with NGS (WIT *et al.* 2012), but can be modified to include genic regions (such as the *COI* gene) (EMERSON *et al.* 2010). NGS also allows intraspecific investigation of populations with recent divergent time scales (MCCORMACK *et al.* 2013) and permits the use of microsatellites markers in combination with the technology.

Additionally, hybrid studies may be conducted with the combinational use of microsatellites and NGS technology (given that the parents are known). This may allow studies of *R. microplus* hybrids in South Africa and to determine the level and direction of hybridisation (personal communication Prof. Ivan Horak and Dr. Maxime Madder). Knowledge of these hybrid dynamics may assist with clarifying hybrid diversity, adaptation, acaricide resistance and the impact of hybrids on tick control strategies with tick vaccines.

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The expected results of this study was that the *R. microplus* populations of South Africa would have a high genetic diversity based on what was observed for *R. australis* (CUTULLÉ *et al.* 2009; KOFFI *et al.* 2006b). It was expected that the populations will group with geography or climate. Furthermore we wanted to determine whether cattle trade or movement would influence the structure or genetic diversity of *R. microplus* populations.

It is clear from the observations made within this study that historical cattle trade has been a large factor in determining the current genetic diversity of *R. microplus* in South Africa. It would seem that the initial and continuous importations of *R. microplus* cattle from abroad, specifically Brazil, has resulted in a population with mixed haplotypes (*ITS2* region) and no apparent spatial structure (*ITS2* region and *COI* gene). Free movement of cattle geographically inside South Africa facilitates the homogenisation of *R. microplus* populations. The *COI* gene is regarded as the gold standard for species identification (HAJIBABAEI *et al.* 2007; WAUGH 2007) and it has been applied successfully in the intraspecific diversity studies of *Ixodes* spp. (SONGA *et al.* 2011) and *R. appendiculatus* (YSSOUF *et al.* 2011), yielding multiple haplotypes. This allowed the identification of the import origin of the ticks (YSSOUF *et al.* 2011), but the populations had no apparent structure (SONGA *et al.* 2011; YSSOUF *et al.* 2011). However, for *R. microplus*, the *COI* indicated that the region in which the *COI* gene resides potentially evolves under genetic draft and/or genetic hitch-hiking and resulted in two haplotypes only. The one haplotype is from the Deutsch strain of America and the other for South African, West African and Brazilian specimens. The *COI* gene is suitable as a species marker where there is clear distinction amongst the cattle ticks but not for the intraspecific studies of *R. microplus*. The existence of different *COI* haplotypes for the *R. annulatus* samples analysed might merit its use as an intraspecific marker for the other cattle ticks.

Other than the difference of evolutionary mechanisms that differ in *R. microplus* from other tick species that display greater variation, several factors may contribute to the bias and erroneous classification of genetic diversity. One example will be the poor resolution of phylogenetic analyses or the use of single method phylogenetic analyses that is prominent in several genetic studies of ticks, such as (DANTAS-TORRES *et al.* 2013) and (BURLINI *et al.* 2010). Another example would be the incorrect identification of the species use in the genetic study and may result in a larger apparent genetic diversity, such as the sequences deposited on the NCBI GenBank database of *R. microplus* sequences from China.

R. microplus has not been found in China, but unpublished reports from China suggests that *R. microplus* may be present (JF758630 and JF758636 in the NCBI GenBank database). Unpublished *COI* gene sequences of *R. microplus* from China were submitted during 2011 to the NCBI GenBank database. These sequences (JF758630.1 and JF758636.1) were added to the preliminary *COI* gene tree of this study, but were removed from the analyses after they grouped with *R. annulatus* sequence (AF132825) (Appendix B 2.3). *R. annulatus* is not distributed in China, only in northern Africa and South America (ESTRADA-PENA *et al.* 2006; LOHMEYER *et al.* 2011; WALKER *et al.* 2003). This might

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suggest that *R. annulatus* was introduced to China through cattle trade with northern Africa and/or South America. Additional sample collections and a thorough investigation with morphological characteristics and molecular markers are required to confirm this occurrence.

Recently *R. microplus* was collected from cattle on four cattle farms in Namibia during March to April of 2013 (NYANGIWE *et al.* 2013b). It is speculated that the infested cattle were imported from South Africa before the trade-ban of cloven-hoofed animals was applied in November 2010 (due to foot-and-mouth disease outbreaks in South Africa) (NYANGIWE *et al.* 2013b). It is also possible that these cattle might have been illegally imported after November 2010. It raises the question of whether *R. microplus* was collected from infested cattle recently imported from South Africa or whether *R. microplus* has been able to reproduce and sustain viable populations in arid Namibia. Additional investigations are required to confirm the presence and maintenance of *R. microplus* populations in Namibia. These investigations will have to establish the introduction date of the cattle to Namibia from which samples are collected. It would be another example of the subtropical *R. microplus* adapting to hot arid regions if *R. microplus* is able to sustain a foothold in Namibia and spread throughout the country. This re-enforces the concept that strict trade and quarantine regulations of cattle are required and should be enforced to prevent the introduction and spread of *R. microplus* to new unoccupied areas. Quarantine regulations, of course, would recognise the threat from an unchecked spread of *R. microplus* and the diseases it vectors.

The interaction between South Africa and Namibia illustrates the importance of identifying the establishment of foothold populations at an early stage. This study has demonstrated that *R. microplus* gains a foothold in new areas mostly through trade. Foothold populations then expand through the unique adaptations characterising this species to not only establish viable populations but also would displace *R. decoloratus* as a dominant species. The effect is that cattle resistance to native ticks (R. *decoloratus*) and tick-borne diseases (*B. bigemina*) are exchanged for a tick species and its associated diseases for which they are immunologically naïve (*R. microplus* and *B. bovis*). It is logical to identify and eradicate foothold populations rather than to control established *R. microplus* populations. The quarantine measures proposed in this dissertation has the objective to prevent the establishment of *R. microplus* foothold populations, which generally occurs through trade, and the Texas-Mexican border experience (LOHMEYER *et al.* 2011; POUND *et al.* 2010) has shown that this can be successfully applied.

The linked distribution of *R. microplus* to the cattle trade and the complex mechanisms governing the evolution of this tick species has made the study of its genetic diversity challenging. This emphasises the need for reliable genetic markers and the management of cattle movement in order to prevent further impact of this tick to the societies and the economies of the world.

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Reference List

2009 Classification and Identification of Cattle Breeds, pp. in *Mt MorrisBeef Cattle Seminar*. UW-Extension.

ABERCROMBIE, M., C. J. HICKMAN and M. L. JOHNSON, 2004 The Penguin Dictionary of Biology, pp. 753 in *The Penguin Dictionary of Biology*, edited by M. THAIN and M. HICKMAN. Penguin Books, London.

ALBERT, C. C. H., N. G. YOCCOZ, T. C. EDWARDS JR, C. H. GRAHAM, N. E. ZIMMERMANN *et al.*, 2010 Sampling in ecology and evolution - bridging the gap between theory and practice. Ecography **33:** 1028-1037.

ALJANABI, S. M., and I. MARTINEZ, 1997 Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res **25:** 4692-4693.

ANIMAL GENETICS TRAINING RESOURCE (AGTR), Cattle Breeds, pp. ILRI-SLU.

ANSTEAD, C. A., C. N. KRAKOWETZ, A. S. MANN, K. A. SIM and N. B. CHILTON, 2011 An assessment of genetic differences among ixodid ticks in a locus within the nuclear large subunit ribosomal RNA gene. Mol Cell Probe **25:** 243-248.

APANASKEVICH, D. A., I. G. HORAK, C. A. MATTHEE and S. MATTHEE, 2011 A New Species Of Ixodes (Acari: Ixodidae) From South African Mammals. J Parasitol **97:** 389-398.

APPLIED-BIOSYSTEMS, 2002 BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol
pp. 1-72. Appliad Biosystems, USA.

AU-IBAR, 2011 The Eradication of Rinderpest from Africa: A Great Milestone, pp. 8, Nairobi, Kenya.

AVISE, J. C., 1994 *Molecular Markers, Natural History and Evolution*. Chapman & Hall, London.

AVISE, J. C., 2000 *Phylogeography: The History and Formation of Species*. Harvard University Press, London.

AVISE, J. C., 2009 Phylogeography: retrospect and prospect. J Biogeogr **36:** 3-15.

BAFFI, M. A., G. R. L. DE SOUZA and C. U. VIEIRA, 2007 Identification of point mutations in putative carboxylesterase and their association with acaricide resistance in *Rhipicephalus (Boophilus) microplus*. Vet Parasitol **148:** 301-309.

BARANDIKA, J. F., E. BERRIATUA, M. BARRAL, R. A. JUSTE and P. ANDA, 2006 Risk factors associated with ixodid tick species distributions in the Basque region in Spain. Med Vet Entomol **20:** 177-188.

BARKER, G. C., 2002 Microsatellite DNA: A tool for population genetic analysis. T Roy Soc Trop Med H **96:** S1/21-S21/24.

BARKER, S. C., 1998 DISTINGUISHING SPECIES AND POPULATIONS OF RHIPICEPHALINE TICKS WITH ITS 2 RIBOSOMAL RNA. J Parasitol **84:** 887-892.

BARKER, S. C., and A. MURRELL, 2002 Phylogeny, evolution and historical zoogeography of ticks: a review of recent progress. Exp Appl Acarol **28:** 55-68.

BARKER, S. C., and A. MURRELL, 2004 Systematics and evolution of ticks with a list of valid genus and species names. Parasitology **129:** S15-S36.

BARRETT, L. G., P. H. THRALL, J. J. BURDON and C. C. LINDE, 2008 Life history determines genetic structure and evolutionary potential of host–parasite interactions. Trends Ecol Evol **23:** 678-685.

BARTON, N. H., 2000 Genetic hitchhiking. Phil Trans R Soc B **355:** 1553-1562.

BAZIN, E., S. GLÉMIN and N. GALTIER, 2006 Population Size Does Not Influence Mitochondrial Genetic Diversity in Animals. Science **312:** 570-572.

BEATI, L., and J. E. KEIRANST, 2001 Analysis of the systematic relationships among ticks of the genera *Rhipicepalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial 12S Ribosomal DNA gene sequences and morphological characters. J Parasitol **87:** 32-78.

BELLGARD, M. I., P. M. MOOLHUIJZEN, F. D. GUERRERO, D. SCHIBECI and M. RODRIGUEZ-VALLE, 2012 CattleTickBase: An integrated Internet-based bioinformatics resource for *Rhipicephalus (Boophilus) microplus*. Int J Parasitol **42:** 161–169.

BHARGAVA, A., and F. F. FUENTES, 2010 Mutational Dynamics of Microsatellites. Mol Biotechnol **44:** 250-266.

BIGALKE, R. D., 1980 The Control of ticks and tick-borne diseases in South Africa., pp. 20-21 in *Zimbabwe Veterinary Journal*.

BLACK, W. C., and J. PIESMAN, 1994 Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc Natl Acad Sci USA **91:** 10034-10038.

BROUQUI, P., 2011 Arthropod-Borne Diseases Associated with Political and Social Disorder. Annu Rev Entomol **56:** 357-374.

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BRYANT, D., A. ROYCHOUDHURY, R. BOUCKAERT, J. FELSENSTEIN and N. ROSENBERG, 2011 Exact coalescent likelihoods for unlinked markers in finite-sites mutation models. arXiv:1109.3525v1[q-bio.PE]**:** 1-17.

BUDELI, M. A., K. A. NEPHAWE, D. NORRIS, N. W. SELAPA, L. BERGH *et al.*, 2009 Genetic parameter estimates for tick resistance in Bonsmara cattle. S Afr J Anim Sci **39:** 321-327.

BURLINI, L., K. T. R. S. TEIXEIRA, M. P. J. SZABO' and K. T. M. FAMADAS, 2010 Molecular dissimilarities of *Rhipicephalus sanguineus* (Acari: Ixodidae) in Brazil and its relation with samples throughout the world: is there a geographical pattern? Exp Appl Acarol **50:** 361–374.

BUSHIAZZO, E., and N. J. GEMMELL, 2006 The rise, fall and renaissance of microsatellites in eukaryotic genomes. BioEssays **28:** 1040-1050.

CAMPBELL, N. J. H., and S. C. BARKER, 1999 The Novel Mitochondrial Gene Arrangement of the Cattle Tick, *Boophilus microplus*: Fivefold Tandem Repetition of a Coding Region. Mol Biol Evol **16:** 732-740.

CANGI, N., I. G. HORAK, D. A. APANASKEVICH, S. MATTHEE, L. C. B. G. D. NEVES *et al.*, 2013 The Influence of Interspecific Competition and Host Preference on the Phylogeography of Two African Ixodid Tick Species. Plos One **8:** e76930.

CAREW, M., M. SCHIFFER, P. UMINA, A. WEEKS and A. HOFFMANN, 2009 Molecular markers indicate that the wheat curl mite, *Aceria tosichella* Keifer, may represent a species complex in Australia. B Entomol Res **99:** 479–486.

CASTILLO, A., H. BUDAK, R. K. VARSHNEY, G. DORADO, A. GRANER *et al.*, 2008 Transferability and polymorphism of barley EST-SSR markers used for phylogenetic analysis in Hordeum chilense. BMC Plant Biol **8:** 1-9.

CASTRO, M. B., and J. R. CLOVER, 2010 A comparison of visual and flagging methods for estimating adult *Ixodes pacificus* (Acari: Ixodidae) tick abundance. J Vector Ecol **35:** 435-438.

CENTERS FOR DISEASE CONTROL & PREVENTION, pp. Index: (http://www.cdc.gov/).

CFSPH, 2007 *Rhipicephalus (Boophilus) microplus*, pp. 1-3. The Centre for Food Security & Public Health, Iowa.

CHAO, L.-L., W.-J. WU and C.-M. SHIH, 2011 Species identification of Ixodes granulatus (Acari: Ixodidae) based on internal transcribed spacer 2 (ITS2) sequences. Exp Appl Acarol **54:** 51-63.

CHAPUIS, M.-P., and A. ESTOUP, 2007 Microsatellite Null Alleles and Estimation of Population Differentiation. Mol Biol Evol **24:** 621-631.

CHAUVIN, A., E. MOREAU, S. BONNET, O. PLANTARD and L. MALANDRIN, 2009 *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. Vet Res **40:** doi: 10.1051/vetres/2009020.

CHEN, A. C., H. HE and R. B. DAVEY, 2007 Mutations in a putative octopamine receptor gene in amitraz-resistant cattle ticks. Vet Parasitol **148:** 379-383.

CHEVILLON, C., S. DUCORNEZ, T. DE MEEUˆS, B. B. KOFFI, H. GAI¨ *et al.*, 2007a Accumulation of acaricide resistance mechanisms in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) populations from New Caledonia Island. Vet Parasitol **147:** 276–288.

CHEVILLON, C., M. D. GARINE-WICHATITSKY, N. BARRE, S. DUCORNEZ and T. D. MEEÛS, 2013 Understanding the genetic, demographical and/or ecological processes at play in invasions: lessons from the southern cattle tick *Rhipicephalus microplus* (Acari: Ixodidae). Exp Appl Acarol.

CHEVILLON, C., B. B. KOFFI, N. BARRE´, P. DURAND, C. L. ARNATHAU *et al.*, 2007b Direct and indirect inferences on parasite mating and gene transmission patterns Pangamy in the cattle tick *Rhipicephalus (Boophilus) microplus*. Infect Genet Evol **7:** 298-304.

CHIGAGURE, N. N., G. D. BAXTER and S. C. BARKER, 2000 Microsatellite loci of the cattle tick *Boophilus microplus* (Acari: Ixodidae). Exp Appl Acarol **24:** 951-956.

CHITIMIA, L., R. Q. LIN, I. COSOROABA, P. BRAILA, H. Q. SONG *et al.*, 2009 Molecular characterization of hard and soft ticks from Romania by sequences of the internal transcribed spacers of ribosomal DNA. Parasitol Res **105:** 907–911.

CLEMENT, M., D. POSADA and K. CRANDALL, 2000 TCS: a computer program to estimate gene genealogies. Mol Ecol **9:** 1657-1660.

COBB, B. D., and J. M. CLARKSON, 1994 A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. Nucleic Acids Res **22:** 3801-3805.

CONRADIE, D. C. U., 2012 South Africa's Climatic Zones: Today, Tomorrow, pp. 1-9 in *International Green Building Conference and Exhibition*. CSIR, Pretoria, South Africa, Sandton, South Africa.

CRANDALL, K. A., and A. R. TEMPLETON, 1993 Empirical Tests of Some Predictions From Coalescent Theory With Applications to Intraspecific Phylogeny Reconstruction. Genetics **134:** 959-969.

© University of Pretoria

CRESPI, B., and P. NOSIL, 2013 Conflictual speciation: species formation via genomic conflict. Trends Ecol Evol **28:** 48-57.

CRISCIONE, C. D., R. POULIN and M. S. BLOUIN, 2005 Molecular ecology of parasites: elucidating ecological and microevolutionary processes. Mol Ecol **14:** 2247-2257.

CRUICKSHANK, R. H., 2002 Molecular markers for the phylogenetics of mites and ticks. Syst Appl Acar **7:** 3-14.

CUMMING, G. S., 1999 Host distributions do not limit the species ranges of most African ticks (Acari: Ixodida). B Entomol Res **89:** 303-327.

CUTULLÉ, C., N. N. JONSSON and J. SEDDON, 2009 Population structure of Australian isolates of the cattle tick *Rhipicephalus (Boophilus) microplus*. Vet. Parasitol. **161:** 283-291.

CUTULLÉ, C., N. N. JONSSON and J. M. SEDDON, 2010 Multiple paternity in *Rhipicephalus (Boophilus) microplus* confirmed by microsatellite analysis. Exp Appl Acarol **50:** 51-58.

DABERT, M., W. WITALINSKI, A. KAZMIERSKI, Z. OLSZANOWSKI and J. DABERT, 2010 Molecular phylogeny of acariform mites (Acari, Arachnida): Strong conflict between phylogenetic signal and long-branch attraction artifacts. Mol Phylogenet Evol **56:** 222-241.

DAFF, 2010 A PROFILE OF THE SOUTH AFRICAN BEEF MARKET VALUE CHAIN, pp. 1-60. Department of Agriculture, Forestry & Fisheries, South Africa.

DAFF, 2011a A PROFILE OF THE SOUTH AFRICAN BEEF MARKET VALUE CHAIN, pp. 1-57. Department of Agriculture, Forestry and Fisheries, South Africa.

DAFF, 2011b A PROFILE OF THE SOUTH AFRICAN DAIRY MARKET VALUE CHAIN, pp. 1-109. Department of Agriculture, Forestry & Fisheries, South Africa.

DAFF, 2011/12 Economic Review of the South African Agriculture, pp. 1-10. Department of Agriculture, Forestry & Fisheries, South Africa.

DAFF, 2011/2012 Annual Report, pp. Department Agriculture, Forestry and Fisheries, South Africa.

DAFF, 2012 Trends in the agricultural sector pp. 1-67. Department Agriculture, Forestry and Fisheries, South Africa.

DANTAS-TORRES, F., M. S. LATROFA, G. ANNOSCIA, A. GIANNELLI, A. PARISI *et al.*, 2013 Morphological and genetic diversity of *Rhipicephalus sanguineus* sensu lato from the New and Old Worlds. Parasites & Vectors **6:** 213. doi:210.1186/1756-3305-1186-1213.

DAVEY, J. W., P. A. HOHENLOHE, P. D. ETTER, J. Q. BOONE, J. M. CATCHEN *et al.*, 2011 Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nat Rev Genet **12:** 499-510.

DAVEY, R. B., J. M. POUND and L. M. COOKSEY, 1994 Comparative reproduction and nonparasitic development of *Boophilus microplus* and hybridized *Boophilus* ticks (Acari: Ixodidae) under natural field conditions in subtropical South Texas. Exp Appl Acarol **18:** 185-200.

DE CLERCQ, E. M., S. O. VANWAMBEKE, M. SUNGIRAI, S. ADEHAN, R. LOKOSSOU *et al.*, 2012 Geographic distribution of the invasive cattle tick *Rhipicephalus microplus*, a country-wide survey in Benin. Exp Appl Acarol.

DE LA FUENTE, J., 2003 The fossil record and the origin of ticks (Acari:Parasitiformes: Ixodida). Exp Appl Acarol **29:** 331-344.

DE LA FUENTE, J., C. ALMAZAN, M. CANALES, J. MANUEL PE´REZ DE LA LASTRA, K. M. KOCAN *et al.*, 2007 A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Anim Health Res Rev **8:** 23-28.

DE LA FUENTE, J., J. C. GARCIA-GARCIA, D. M. GONZÁLEZ, G. IZQUIERDO and M. E. OCHAGAVIA, 2000 Molecular analysis of *Boophilus* spp. (Acari: Ixodidae) tick strains. Vet Parasitol **92:** 209-222.

DE MATOS, C., C. SITOE, L. NEVES, J. O. NOTHLING and I. G. HORAK, 2009 The comparative prevalence of five ixodid tick species infesting cattle and goats in Maputo Province, Mozambique. Onderstepoort J Vet Res **76:** 201-208.

DE MEEÛS, T., B. B. KOFFI, N. BARRE´, M. DE GARINE-WICHATITSKY and C. CHEVILLON, 2010 Swift sympatric adaptation of a species of cattle tick to a new deer host in New Caledonia. Infect Genet Evol **10:** 976–983.

DEPARTMENT OF ANIMAL SCIENCE, Breeds of Livestock: Cattle (*Bos*), pp. in *http://www.ansi.okstate.edu/breeds/cattle/*. Oklahoma State University.

DOBSON, A. D. M., and S. E. RANDOLPH, 2011 Modelling the effects of recent changes in climate, host density and acaricide treatments on population dynamics of Ixodes ricinus in the UK. J Appl Ecol **48:** 1029-1037.

DOBSON, A. D. M., J. R. F. THOMAS and S. E. RANDOLPH, 2011 A modified matrix model to describe the seasonal population ecology of the European tick *Ixodes ricinus*. J Appl Ecol **48:** 1017-1028.

© University of Pretoria

DUNLOP, J. A., 2010 Geological history and phylogeny of Chelicerata. Arthropod Struct & Dev **39:** 124-142.

DUNLOP, J. A., 2011 THE FATE AND STATUS OF THE SUPPOSED FOSSIL TICK IXODES TERTIARIUS SCUDDER, 1885. Acarologia **51:** 399-404.

ELLEGREN, H., 2004 Microsatellites: Simple sequences with complex evolution. Nat Rev Genet **5:** 435-444.

ELSA LÉGER, G. L. VOURC'H, L. VIAL, C. CHEVILLON and K. D. MCCOY, 2013 Changing distributions of ticks: causes and consequences. Exp Appl Acarol **59:** 219-244.

ELSHIRE, R. J., J. C. GLAUBITZ, Q. SUN, J. A. POLAND, K. KAWAMOTO *et al.*, 2011 A Robust, Simple Genotyping-by-Sequencing (GBS) Approach for High Diversity Species. Plos One **6:** e19379. doi:19310.11371/journal.pone.0019379.

EMERSON, K. J., C. R. MERZ, J. M. CATCHEN, P. A. HOHENLOHE, W. A. CRESKO *et al.*, 2010 Resolving postglacial phylogeography using high-throughput sequencing. Proc Natl Acad Sci USA **107:** 16196–16200.

EMILEY A. WILEY, *Current Protocols: Essential Laboratory Techniques*. John Wiley & Sons Inc., Canada.

ESTRADA-PENA, A., A. BOUATTOUR, J. L. CAMICAS, A. GUGIELMONE, I. G. HORAK *et al.*, 2006 The known distribution and ecological preferences of the tick subgenus *Boophilus* (Acari: Ixodidae) in Africa and Latin America. Exp Appl Acarol **38:** 219-235.

ESTRADA-PEÑA, A., V. NARANJO, K. ACEVEDO-WHITEHOUSE, A. J. MANGOLD, K. M. KOCAN *et al.*, 2009 Phylogeographic analysis reveals association of tick-borne pathogen, *Anaplasma marginale*, MSP1a sequences with ecological traits affecting tick vector performance. BMC Biol **7:** 57.

ESTRADA-PEÑA, A., C. SÁNCHEZ ACEDO, J. QUÍLEZ and E. DEL CACHO, 2005 A retrospective study of climatic suitability for the tick *Rhipicephalus (Boophilus) microplus* in the Americas. Global Ecol Biogeogr **14:** 565-573.

ESTRADA-PEÑA, A., J. M. VENZAL, S. NAVA, A. MANGOLD, A. A. GUGLIELMONE *et al.*, 2012 Reinstatement of *Rhipicephalus* (*Boophilus*) *australis* (Acari: Ixodidae) with Redescription of the Adult and Larval Stages. J Med Entomol **49:** 794-802.

FLAXMAN, S. M., J. L. FEDER and P. NOSIL, 2013 GENETIC HITCHHIKING AND THE DYNAMIC BUILDUP OF GENOMIC DIVERGENCE DURING SPECIATION WITH GENE FLOW. Evolution **67:** 2577–2591.

FOLMER, O., M. BLACK, W. HOEH, R. LUTZ and R. VRIJENHOEK, 1994 DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotech **3:** 294-299.

GADALETA, A., A. GIANCASPRO, S. ZACHEO, D. NIGRO, S. L. GIOVE *et al.*, 2011 Comparison of genomic and EST-derived SSR markers in phylogenetic analysis of wheat. Plant Genet Resources **9:** 243-246.

GALTIER, N., B. NABHOLZ, S. GLÉMIN and G. D. D. HURST, 2009 Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Mol Ecol **18:** 4541–4550.

GARCIA-GARCIA, J. C., C. MONTERO, M. REDONDO, M. VARGAS and M. CANALES, 2000 Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, Boophilus microplus. Vaccine **18:** 2275-2287.

GASPARIN, G., M. MIYATA, L. L. COUTINHO, M. L. MARTINEZ, R. L. TEODORO *et al.*, 2007 Mapping of quantitative trait loci controlling tick [*Riphicephalus (Boophilus) microplus*] resistance on bovine chromosomes 5, 7 and 14. Anim Genet **38:** 453-459.

GILLESPIE, J. H., 2000 Genetic Drift in an Infinite Population: The Pseudohitchhiking Model. Genetics **155:** 909-919.

GOLDSTEIN, D. B., and D. D. POLLOCK, 1997 Launching Microsatellites: A Review of Mutation Processes and Methods of Phylogenetic Inference. J Hered **88:** 335-342.

GRADY, J. M., and J. M. QUATTRO, 2001 Using Character Concordance to Define Taxonomic and Conservation Units. Conserv Biol **13**.

GRIFFITHS, A. J. F., S. R. WESSLER, S. B. CARROLL and J. DOEBLEY, 2010 *Introduction to Genetic Analysis*. W. H. Freeman and Company, New York.

GUERRERO, F. D., K. G. BENDELE, A. C. CHEN, A. Y. LI, R. J. MILLER *et al.*, 2007 Serial analysis of gene expression in the southern cattle tick following acaricide treatment of larvae from organophosphate resistant and susceptible strains. Insect Mol Biol **16:** 49-60.

GUERRERO, F. D., P. MOOLHUIJZEN, D. G. PETERSON, S. BIDWELL and E. CALER, 2010 Reassociation kinetics-based approach for partial genome sequencing of the cattle tick, *Rhipicephalus (Boophilus) microplus*. BMC Genomics **11**.

GUINDON, S., J.-F. DUFAYARD, V. LEFORT, M. ANISIMOVA, W. HORDJIK *et al.*, 2010 New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Syst Biol **59:** 307-321.

© University of Pretoria

GUISAN, A., O. BROENNIMANN, R. ENGLER, M. VUST and N. G. YOCCOZ, 2006 Using Niche-Based Models to Improve the Sampling of Rare Species. Conserv Biol **20:** 501-511.

GUZINSKI, J., K. M. SAINT, M. G. GARDNER, S. C. DONNELLAN and C. M. BULL, 2008 Development of microsatellite markers and analysis of their inheritance in the Australian reptile tick, *Bothriocroton hydrosauri*. Mol Ecol Resources **8:** 443-445.

HAJIBABAEI, M., G. A. C. SINGER, P. D. N. HEBERT and D. A. HICKEY, 2007 DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet **23:** 167-172.

HALL, T. A., 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser **41:** 95-98.

HALLIBURTON, R., 2004 *Introduction to population genetics*. Pearson/Prentice Hall.

HALOS, L., T. JAMAL, L. VIAL, R. MAILLARD, A. SUAU *et al.*, 2004 Determination of an efficient and reliable method for DNA extraction from ticks. Vet Res **35:** 709-713.

HANSKI, I., 1991 Metapopulation dynamics: brief history and conceptual domain. Biol J Linn Soc **42:** 3-16.

HENRIOUD, A. N., 2011 Towards sustainable parasite control practices in livestock production with emphasis in Latin America. Vet Parasitol **180:** 2-11.

HERNANDEZ, R., F. D. GUERRERO, J. E. GEORGE and G. G. WAGNER, 2002 Allele frequency and gene expression of a putative carboxylesterase-encoding gene in a pyrethroid resistant strain of the tick *Boophilus microplus*. Insect Biochem Molec **32:** 1009-1016.

HERNANDEZ, R., H. HE, A. C. CHEN, S. D. WAGHELA, G. W. IVIE *et al.*, 2000 Identification of a point mutation in an esterase gene in different populations of the southern cattle tick, *Boophilus microplus*. Insect Biochem Molec **30:** 969-977.

HEY, J., and C. A. MACHADO, 2003 THE STUDY OF STRUCTURED POPULATIONS — NEW HOPE FOR A DIFFICULT AND DIVIDED SCIENCE. Nat Rev Genet **4:** 535-543.

HILL, C. A., F. D. GUERRERO, J. P. V. ZEE, N. S. GERACI, J. G. WALLING *et al.*, 2009 The position of repetitive DNA sequence in the southern cattle tick genome permits chromosome identification. Chromosome Res **17:** 77-89.

HILL, C. A., and J. A. GUTIERREZ, 2003 A method for extraction and analysis of high quality genomic DNA from ixodid ticks. Med Vet Entomol **17:** 224-227.

HLINKA, O., A. MURRELL and S. C. BARKER, 2002 Evolution of the secondary structure of the rRNA internal transcribed spacer 2 (ITS2) in hard ticks (Ixodidae, Arthropoda). Heredity **88:** 275–279.

HOHENLOHE, P. A., S. BASSHAM, P. D. ETTER, N. STIFFLER, E. A. JOHNSON *et al.*, 2010 Population Genomics of Parallel Adaptation in Threespine Stickleback using Sequenced RAD Tags. Plos Genet **6:** e1000862. doi:1000810.1001371/journal.pgen.1000862.

HOOGSTAAL, H., 1985 Argasid and nuttalliellid ticks as parasites and vectors. Adv Parasitol **24:** 135-238.

HOPE, M., M. MENZIES and D. KEMP, 2010 Identification of a Dieldrin Resistance-Associated Mutation in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). J Econ Entomol **103:** 1355-1359.

HORAK, I. G., 2009 A century of tick taxonomy in South Africa. Onderstepoort J Vet Res **76:** 69-74.

HORAK, I. G., L. J. FOURIE and L. E. O. BRAACK, 2005 Small mammals as hosts of immature ixodid ticks. Onderstepoort J Vet Res **72:** 255–261.

HORAK, I. G., N. NYANGIWE, C. DE MATOS and L. NEVES, 2009 Species composition and geographic distribution of ticks infesting cattle, goats and dogs in a temperate and in a subtropical region of south-east Africa. Onderstepoort J Vet Res **76:** 263-276.

HOWARD, C. W., 1908 *A list of the ticks of South Africa, with descriptions and keys to all the forms known*. Annals of the Transvaal Museum

HOWELL, C. J., J. B. WALKER and E. M. NEVILL, 1978 *Ticks, mites and insects infesting domestic animals in South Africa*. Science Bulletin, no. 393, Department of Agricultural Technical Services, South Africa, Pretoria.

HUANG, W., A. NADEEM, B. ZHANG, M. BABAR and M. SOLLER, 2012 Characterization and Comparison of the Leukocyte Transcriptomes of Three Cattle Breeds. Plos One **7:** e30244.

HUSON, D. H., and D. BRYANT, 2006 Application of Phylogenetic Networks in Evolutionary Studies. Mol Biol Evol **23:** 254–267.

HUSON, D. H., and C. SCORNAVACCA, 2011 A Survey of Combinatorial Methods for Phylogenetic Networks. Genome Biol Evol **3:** 23-35.

INDIGENOUSBREEDS.CO.ZA, South African Indigenous Breeds: Indigenous & Locally Deveped Cattle Breeds, pp.

© University of Pretoria

JOHN F. ROBYT, and BERNARD J. WHITE, *Biochecmical Techniques: Theory and Practise*. Brooks/Cole Publishing Company, Monterey, California.

JOHNSON, N. A., and J. LACHANCE, 2012 The genetics of sex chromosomes: evolution and implications for hybrid incompatibility. Ann N Y Acad Sci **1256:** E1-E22.

JONSSON, N. N., C. CUTULLÉ, S. W. CORLEY and J. M. SEDDON, 2010 Identification of a mutation in the para-sodium channel gene of the cattle tick *Rhipicephalus microplus* associated with resistance to flumethrin but not to cypermethrin Int J Parasitol **40:** 1659-1664.

KAIN, D. E., F. A. H. SPERLING, H. V. DALY and R. S. LANE, 1999 Mitochondrial DNA sequence variation in *Ixodes pacificus* (Acari: Ixodidae). Heredity **83:** 378-386.

KANDUMA, E. G., J. M. MWACHARO, J. D. SUNTER, I. NZUKI, S. MWAURA *et al.*, 2012 Micro- and minisatellite-expressed sequence tag (EST) markers discriminate between populations of *Rhipicephalus appendiculatus*. Tick and Tick-borne Diseases **3:** 128–136.

KAWAZOE, K., A. KAWAKITA, Y. KAMEDA and M. KATO, 2008 Redundant species, cryptic host-associated divergence, and secondary shift in Sennertia mites (Acari: Chaetodactylidae) associated with four large carpenter bees (Hymenoptera: Apidae: Xylocopa) in the Japanese island arc. Mol Phylogenet Evol **49**.

KING, R. C., and W. D. STANSFIELD, 2002 A Dictionary of Genetics, pp. 1-530 in *A Dictionary of Genetics*. Oxford University Press, New York.

KLOMPEN, J. S. H., W. C. B. IV, J. E. KEIRANS and D. E. NORRIS, 2000 Systematics and Biogeography of Hard Ticks,a Total Evidence Approach. Cladistics **16:** 79-102.

KOFFI, B. B., T. DE MEEÛS, N. BARRE, P. DURAND and C. ARNATHAU, 2006a Founder effects, inbreeding and effective sizes in the Southern cattle tick: the effect of transmission dynamics and implications for pest management. Mol Ecol **15:** 4603-4611.

KOFFI, B. B., A. M. RISTERRUCCI, D. JOULIA, P. DURAND and N. BARRE, 2006b Characterization of polymorphic microsatellite loci within a young Boophilus microplus metapopulation. Mol Ecol Notes **6:** 502-504.

KREBS, C. J., 1994 Ecology: The Experimental Analysis of Distribution and Abundance., pp. Harper Collins, New York.

KREITMAN, M., and J. M. COMERON, 1999 Coding sequence evolution. Curr Opin Genet Dev **9:** 637–641.

KRINGS, M., A. STONE, R. W. SCHMITZ, H. KRAINITZKI and M. STONEKING, 1997 Neandertal DNA Sequences and the Origin of Modern Humans. Cell **90:** 19-30.

LABRUNA, M. B., V. NARANJO, A. J. MANGOLD, C. THOMPSON, A. ESTRADA-PEÑA *et al.*, 2009 Allopatric speciation in ticks: genetic and reproductive divergence between geographic strains of *Rhipicephalus (Boophilus) microplus*. BMC Evol. Biol. **9:** 46.

LATIF, A. A., J. F. PUTTERILL, D. G. D. KLERK, R. PIENAAR and B. J. MANS, 2012 *Nuttalliella namaqua* (Ixodoidea: Nuttalliellidae): First Description of the Male, Immature Stages and Re-Description of the Female. Plos One **7:** e41651.

LEITE, L. A. R., 2012 Mitochondrial pseudogenes in insect DNA barcoding: differing points of view on the same issue. Biota Neotrop **12:** 301-308.

LEMEY, P., M. SALEMI and A.-M. VANDAMME (Editors), 2009 *The Phylogenetic Handbook*. Cambrige University Press, United Kingdom.

LEMPEREUR, L., D. GEYSEN and M. MADDER, 2010 Development and validation of a PCR–RFLP test to identify African *Rhipicephalus (Boophilus)* ticks. Acta Trop **114:** 55-58.

LI, A. Y., A. C. CHEN, R. J. MILLER, R. B. DAVEY and J. E. GEORGE, 2007 Acaricide resistance and synergism between permethrin and amitraz against susceptible and resistant strains of *Boophilus microplus* (Acari: Ixodidae). Pest Manag Sci **63:** 882-889.

LIBRADO, P., and J. ROZAS, 2009 DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics **25:** 1451-1452.

LITTLE, D. A., 1963 THE EFFECT OF CATTLE TICK INFESTATION ON THE GROWTH RATE OF CATTLE. Aust J Parasitol **39:** 6-10.

LOHMEYER, K. H., J. M. POUND, M. A. MAY, D. M. KAMMLAH and R. B. DAVEY, 2011 Distribution of *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus (Boophilus) annulatus* (Acari: Ixodidae) Infestations Detected in the United States Along the Texas/Mexico Border. J Med Entomol **48:** 770-774.

© University of Pretoria

LYNEN, G., P. ZEMAN, C. BAKUNAME, G. D. GIULIO, P. MTUI *et al.*, 2008 Shifts in the distributional ranges of *Boophilus* ticks in Tanzania: evidence that a parapatric boundary between *Boophilus microplus* and *B. decoloratus* follows climate gradients. Exp Appl Acarol **44:** 147-164.

MADDER, M., S. ADEHAN, R. DE DEKEN, R. ADEHAN and R. LOKOSSOU, 2012 New foci of *Rhipicephalus microplus* in West Africa. Exp Appl Acarol**:** 1-6.

MADDER, M., and I. G. HORAK, 2010 Tick Photodatabase, pp. in *African Tick of Veterinary Importance*. Institute of Tropical Medicine Antwerpen-Belgium and the University of Pretoria (http://www.itg.be/photodatabase/African_ticks_files/index.html), Belgium and Pretoria.

MADDER, M., E. THYS, L. ACHI, A. TOURE´ and R. DE DEKEN, 2011 Rhipicephalus (Boophilus) microplus: a most successful invasive tick species in West-Africa. Exp Appl Acarol **53:** 139-145.

MADDER, M., E. THYS, D. GEYSEN, C. BAUDOUX and I. HORAK, 2007 *Boophilus microplus* ticks found in West Africa. Exp Appl Acarol **43:** 233-234.

MALLET, J., 1995 A species definition for the modern synthesis. Trends Ecol Evol **10:** 294-299.

MANAMELA, D. M. S., 2001 THE HISTORY OF TICKS AND TICK-BORNE DISEASES IN CATTLE IN NATAL AND ZULULAND (KWAZULU-NATAL) FROM 1896 TO THE PRESENT., pp. 87 in *Environment and Development*. University of Natal, Pietermaritzburg.

MANGOLD, A. J., M. D. BARGUES and S. MAS-COMA, 1998 18S rRNA gene sequences and phylogenetic relationships of European hard-tick species (Acari: Ixodidae). Parasitol Res **84:** 31-37.

MARK MARAUN, MICHEAL HEETHOFF, KATJA SCHNEIDER, STEFAN SCHEU, GERD WEIGMANN *et al.*, 2004 Molecular phylogeny of oribatid mites (Oribatida, Acari): evidence for multiple radiations of parthenogenetic lineages. Exp Appl Acarol **33:** 183-201.

MCCAULEY, L. A. R., D. L. ERDNER, S. NAGAI, M. L. RICHLEN and D. M. ANDERSON, 2009 BIOGEOGRAPHIC ANALYSIS OF THE GLOBALLY DISTRIBUTED HARMFUL ALGAL BLOOM SPECIES *ALEXANDRIUM MINUTUM* (DINOPHYCEAE) BASED ON rRNA GENE SEQUENCES AND MICROSATELLITE MARKERS. J Phycol **45:** 454-463.

MCCORMACK, J. E., S. M. HIRD, A. J. ZELLMER, B. C. CARSTENS and R. T. BRUMFIELD, 2013 Applications of next-generation sequencing to phylogeography and phylogenetics. Mol Phylogenet Evol **66:** 526–538.

MCCOY, K. D., 2008 THE POPULATION GENETIC STRUCTURE OF VECTORS AND OUR UNDERSTANDING OF DISEASE EPIDEMIOLOGY. Parasite **15:** 444-448.

MCLAIN, D. K., 2001 Evolution of transcript structure and base composition of rDNA expansion segment D3 in ticks. Heredity **87:** 544- 557.

MCLAIN, D. K., J. LI and J. H. OLIVER, 2001 Interspecific and geographical variation in the sequence of rDNA expansion segment D3 of Ixodes ticks (Acari: Ixodidae). Heredity **86:** 234-242.

MEIKLEJOHN, C. D., K. L. MONTOOTH and D. M. RAND, 2007 Positive and negative selection on the mitochondrial genome. Trends Genet **23:** 259-263.

MENDESA, M. C., C. K. P. LIMAA, A. H. C. NOGUEIRAA, E. YOSHIHARAB, D. P. CHIEBAOC *et al.*, 2011 Resistance to cypermethrin, deltamethrin and chlorpyriphos in populations of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from small farms of the State of São Paulo, Brazil. Vet Parasitol **178:** 383–388.

MOOLHUIJZEN, P. M., A. E. LEW-TABOR, J. A. T. MORGAN, M. R. VALLE and D. G. PETERSON, 2011 The complexity of *Rhipicephalus (Boophilus) microplus* genome characterised through detailed analysis of two BAC clones. BMC Research Notes **4:** 1-16.

MORGAN, J. A. T., S. W. CORLEY, L. A. JACKSON, A. E. LEW-TABOR, P. M. MOOLHUIJZEN *et al.*, 2009 Identification of a mutation in the para-sodium channel gene of the cattle tick *Rhipicephalus (Boophilus) microplus* associated with resistance to synthetic pyrethroid acaricides Int J Parasitol **39:** 775-779.

MURRELL, A., and S. C. BARKER, 2003 Synonymy of Boophilus Curtice, 1891 with Rhipicephalus Koch, 1844 (Acari: Ixodidae). Syst Parasitol **56:** 169-172.

MURRELL, A., N. J. H. CAMPBELL and S. C. BARKER, 2000 Phylogenetic Analyses of the Rhipicephaline Ticks Indicate That the Genus *Rhipicephalus* Is Paraphyletic. Mol Phylogenet Evol **16:** 1-7.

MURRELL, A., N. J. H. CAMPBELL and S. C. BARKER, 2001a Recurrent gains and losses of large (84–109 bp) repeats in the rDNA internal transcribed spacer 2 (ITS2) of rhipicephaline ticks. Insect Mol Biol **10:** 587–596.

MURRELL, A., N. J. H. CAMPBELL and S. C. BARKER, 2001b A Total-Evidence Phylogeny of Ticks Provides Insights into the Evolution of Life Cycles and Biogeography. Mol Phylogenet Evol **21:** 244-258.

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NAVAJAS, M., and B. FENTON, 2000 The application of molecular markers in the study of diversity in acarology: a review. Exp Appl Acarol **24:** 751-774.

NAVAJAS, M., and D. NAVIA, 2010 DNA-based methods for eriophyoid mite studies: review, critical aspects, prospects and challenges. Exp Appl Acarol **51:** 257–271.

NAVAJAS, M., and R. OCHOA, 2013 Integrating ecology and genetics to address Acari invasions. Exp Appl Acarol **59:** 1-10.

NORRIS, D. E., J. S. H. KLOPMEN and W. C. BLACK, 1999 Comparison of the Mitochondrial 12S and 16S Ribosomal DNA Genes in Resolving Phylogenetic Relationships Among Hard Ticks (Acari: Ixodidae). Ann Entomol Soc Am **92:** 117-129.

NORVAL, R. A. I., and N. J. SHORT, 1984 Acarology VI, pp. 1242-1246, edited by D. A. GRIFLITHS and C. E. BOWMAN. Ellis Horwood, Chichester.

NORVAL, R. A. I., and R. W. SUTHERST, 1986 ASSORTATIVE MATING BETWEEN *BOOPHILUS DECOLORATUS* AND *BOOPHILUS MICROPLUS* (ACARI: IXODIDAE). J Med Entomol **23:** 459-460.

NOUREDDINE, R., A. CHAUVIN and O. PLANTARD, 2011 Lack of genetic structure among Eurasian populations of the tick *Ixodes ricinus* contrasts with marked divergence from north-African populations. Int J Parasitol **41:** 183–192.

NYANGIWE, N., S. GONI, L. P. HERVÉ-CLAUDE, I. RUDDAT and I. G. HORAK, 2011 Ticks on pastures and on two breeds of cattle in the Eastern Cape province, South Africa. Onderstepoort J Vet Res **78:** 1-9.

NYANGIWE, N., A. HARRISON and I. G. HORAK, 2013a Displacement of *Rhipicephalus decoloratus* by *Rhipicephalus microplus* (Acari: Ixodidae) in the Eastern Cape Province, South Africa. Exp Appl Acarol**:** 10.1007/s10493-10013-19705-10497.

NYANGIWE, N., and I. G. HORAK, 2007 Goats as alternative hosts of cattle ticks. Onderstepoort J Vet Res **74:** 1-7.

NYANGIWE, N., C. MATTHEE, I. HORAK and S. MATTHEE, 2013b First record of the pantropical blue tick *Rhipicephalus microplus* in Namibia. Exp Appl Acarol **DOI 10.1007/s10493-013-9717-3**.

OBEREM, P., D. ODENDAAL, P. T. OBEREM, M. G. S. SNYMAN, L. LUDWIG *et al.*, 2006 *Diseases and Parasites of Cattle, Sheep and Goats in South Africa*. Afrivet Bussiness Management, Pretoria.

OBERHOLSTER, T., 2011 The preliminary study of the Genetic Diversity of the southern cattle tick *Rhipicephalus microplus* from South Africa, pp. 44 in *Genetics*. University of Pretoria, Pretoria.

OLIVER, J. H., 1989 BIOLOGY AND SYSTEMATICS OF TICKS (ACARI:IXODIDA). Annu Rev Ecol Syst **20:** 397-430.

ORSOUW, N. J. V., R. C. J. HOGERS, A. JANSSEN, F. YALCIN, S. SNOEIJERS *et al.*, 2007 Complexity Reduction of Polymorphic Sequences (CRoPS): A Novel Approach for Large-Scale Polymorphism Discovery in Complex Genomes. Plos One **2:** e1172. doi:1110.1371/journal.pone.0001172.

PARIZI, L. F., P. C. POHL, A. MASUDA and I. DA SILVA VAZ JUNIOR, 2009 New approaches toward anti-*Rhipicephalus (Boophilus) microplus* tick vaccine. Rev Bras Parasitol Vet **18:** 1-7.

PASCOAL, J. D. O., M. D. P. AMORIM, M. M. MARTINS, C. MELO, E. L. D. S. JÚNIOR *et al.*, 2013 Ticks on birds in a savanna (Cerrado) reserve on the outskirts of Uberlândia, Minas Gerais, Brazil. Rev Bras Parasitol Vet **8:** 1-7.

PESTICIDE RESISTANCE FACILITY, 2010 Acaricide Resistance Report, pp. University of the Free State, South Africa.

PHALATSI, M. S., L. J. FOURIE and I. G. HORAK, 2004 Larval biology of *Rhipicephalus (Boophilus) decoloratus* (Acarina: Ixodidae) in Free State Province, South Africa. Onderstepoort J Vet Res **71:** 327–331.

PIPER, E. K., N. N. JONSSON, C. GONDRO, A. E. LEW-TABOR, P. MOOLHUIJZEN *et al.*, 2009 Immunological Profiles of *Bos taurus* and *Bos indicus* Cattle Infested with the Cattle Tick, *Rhipicephalus (Boophilus) microplus*. Clin & Vac Immunol **16:** 1074–1086.

POLAND, G. A., I. G. OVSYANNIKOVA, R. M. JACOBSON and D. I. SMITH, 2007 Heterogeneity in Vaccine Immune Response: The Role of Immunogenetics and the Emerging Field of Vaccinomics. Clin Pharmacol Ther **82:** 653-664.

PORTO NETOA, L. R., N. N. JONSSON, M. J. D'OCCHIO and W. BARENDSE, 2011 Molecular genetic approaches for identifying the basis of variation in resistance to tick infestation in cattle. Vet Parasitol **180:** 165-172.

POSADA, D., 2008 jModelTest: Phylogenetic Model Averaging. Mol Biol Evol **25:** 1253–1256.

POTGIETER, F. T., and H. J. ELS, 1977 Light and electron microscopic observations on the development of Babesia bigemina in larvae, nymphae and non-replete females of *Boophilus decoloratus*. Onderstepoort J Vet Res **44:** 213-232.

POUND, J. M., J. E. GEORGE, D. M. KAMMLAH, K. H. LOHMEYER and R. B. DAVEY, 2010 Evidence for Role of White-Tailed Deer (Artiodactyla: Cervidae) in Epizootiology of Cattle Ticks and Southern Cattle Ticks (Acari: Ixodidae)

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in Reinfestations Along the Texas/Mexico Border in South Texas: A Review and Update. J Econ Entomol **103:** 211-218.

QUELOZ, V., A. DUO, T. N. SIEBER and C. R. GRUNIG, 2010 Microsatellite size homoplasies and null alleles do not affect species diagnosis and population genetic analysis in a fungal species complex. Mol Ecol Resources **10:** 348-367.

RAHBARI, S., and S. NABIAN, 2007 The First Report of *Rhipicephalus* (*Boophilus*) *kohlsi* (Hoogstraal and Kaiser 1960) from Wild Goats (*Capra hircus aegagrus*) in Iran Iranian J Parasitol **2:** 53-56.

RICHARDS, M. B., and B. C. SYKES, 1995 Authenticating DNA Extracted From Ancient Skeletal Remains. J Archaeol Sci **22:** 291-299.

ROBBERTSE, L., 2014 Acaricide resistance and genetic diversity of Rhipicephalus microplus in the Mnisi area. Tick and Tick-borne Diseases.

ROCHE DIAGNOSTICS GMBH, 2007 *Nucleic Acid Isolation and Purification*.

RODRIGUEZ-VIVAS, R. I., A. J. TREES, J. A. ROSADO-AGUILAR, S. L. VILLEGAS-PEREZ and J. E. HODGKINSON, 2011 Evolution of acaricide resistance: Phenotypic and genotypic changes in field populations of *Rhipicephalus (Boophilus) microplus* in response to pyrethroid selection pressure. Int J Parasitol **41:** 895–903.

RONQUIST, F., M. TESLENKO, P. V. D. MARK, D. L. AYRES, A. DARLING *et al.*, 2012 MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice across a Large Model Space. Syst Biol **61:** 1-4.

ROY, L., A. P. G. DOWLING , C. M. CHAUVE and T. BURONFOSSE, 2009 Delimiting species boundaries within Dermanyssus Dugès, 1834 (Acari:Dermanyssidae) using a total evidence approach. Mol Phylogenet Evol **50:** 446-470.

SCHÄFFER, S., T. PFINGSTL, S. KOBLMÜLLER, K. A. WINKLER and C. STURMBAUER, 2010 Phylogenetic analysis of European Scutovertex mites (Acari, Oribatida, Scutoverticidae) reveals paraphyly and cryptic diversity: A molecular genetic and morphological approach. Mol Phylogenet Evol **55**.

SCHILTHUIZEN, M., M. C. W. G. GIESBERS and L. W. BEUKEBOOM, 2011 Haldane's rule in the 21st century. Heredity **107:** 95-102.

SCHÖNHOFER, A. L., and J. MARTENS, 2010 Hidden Mediterranean diversity: Assessing species taxa by molecular phylogeny within the opilionid family Trogulidae (Arachnida, Opiliones). Mol Phylogenet Evol **54:** 59-75.

SCHUMAKER, T. T. S., and D. M. BARROS, 1994 Notes on the biology of *Amblyomma dissimile* Kock, 1844 (Acari: Ixodida) on *Bufo marinus* (Linnaeus, 1758) from Brazil. Mem Inst Oswaldo Cruz **89:** 29-31.

SHAO, R., S. C. BARKER, H. MITANI, Y. AOKI and M. FUKUNAGA, 2005 Evolution of Duplicate Control Regions in the Mitochondrial Genomes of Metazoa: A Case Study with Australasian Ixodes Ticks. Mol Biol Evol **22:** 620–629.

SHORT, N. J., R. B. FLOYD, R. A. I. NORVAL and R. W. SUTHERST, 1989 Development Rates, Fecundity and Survival of Developmental Stages of the Ticks *Rhipicephalus appendiculatus*, *Boophilus decoloratus* and *B. microplus* under Field Conditions in Zimbabwe. Exp Appl Acarol **6:** 123-141.

SIMUUNZA, M., H. BILGIC, T. KARAGENC, M. SYAKALIMA, B. SHIELS *et al.*, 2011 Population genetic analysis and sub-structuring in *Babesia bovis*. Mol Biochem Parasitol**:** doi:10.1016/j.molbiopara.2011.1002.1002.

SONENSHINE, D. E., 1991 *Biology of Ticks*. Oxford University Press, New York.

SONGA, S., R. SHAOB, R. ATWELLA, S. BARKERB and D. VANKAN, 2011 Phylogenetic and phylogeographic relationships in *Ixodes holocyclus* and *Ixodes cornuatus* (Acari: Ixodidae) inferred from COX1 and ITS2 sequences. Int J Parasitol.

SPICKETT, A. M., and J. R. MALAN, 1978 Genetic incompatibility between *Boophilus decoloratus* (Koch, 1844) and *Boophilus microplus* (Canestrini, 1888) and hybrid sterility of Australian and South African *Boophilus microplus* (Acarina: Ixodidae). Onderstepoort J Vet Res **45:** 149-153.

TAKAHASI, K. R., 2009 Coalescent under the evolution of coadaptation. Mol Ecol **18:** 5018–5029.

TAKEZAKI, N., and M. NEI, 2008 Empirical Tests of the Reliability of Phylogenetic Trees Constructed With Microsatellite DNA. Genetics **178:** 385-392.

TAMURA, K., D. PETERSON, N. PETERSON, G. STECHER, M. NEI *et al.*, 2011 MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Bio Evol**:** 1-18.

TEMPLETON, A. R., 2006 *Population Genetics and Microevolutionary Theory*. John Wiley & Sons, Inc, New Jersey.

TERKAWI, M. A., O. M. M. THEKISOE, C. KATSANDE, A. A. LATIF, B. J. MANS *et al.*, 2011 Serological survey of *Babesia bovis* and *Babesia bigemina* in cattle in South Africa. Vet Parasitol **182:** 337-342.

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THEILER, G., 1962 The Ixodoidea parasites of vertebrates in Africa south of the Sahara., pp. 154-159. Division of Veterinary Services, Onderstepoort (mimeographed).

THOMAS, L., S. T. BUCKLAND, E. A. REXSTAD, J. L. LAAKE and S. STRINDBERG, 2010 Distance software: design and analysis of distance sampling surveys for estimating population size. J Appl Ecol **47:** 5-14.

THOMPSON, G. D., R. L. OSBURN, R. B. DAVEY, R. O. DRUMMOND and M. A. PRICE, 1981 Hybrid sterility in cattle ticks (Acari: Ixodidae) Experientia **37:** 127-128.

THUILLER, W., L. BROTONS, M. B. ARAUJO and S. LAVOREL, 2004 Effects of restricting environmental range of data to project current and future species distributions. Ecography **27:** 165-172.

TONETTI, N., M. BERGGOETZ, C. RU¨ HLE, A. M. PRETORIUS and L. GERN, 2009 Ticks and tick-borne pathogens from wildlife in the Free State Province, South Africa. J Wildlife Dis **45:** 437-446.

TØNNENSEN, M. H., 2002 DISTRIBUTION OF *BOOPHILUS MICROPLUS* AND *BOOPHILUS DECOLORATUS* AND ASSOCIATED OCCURRENCE OF *BABESIA* IN CATTLE IN THE SOUTPANSBERG REGION, NORTHERN PROVINCE, SOUTH AFRICA, pp. in *Department of Veterinary Tropical Diseases*. University of Pretoria, Pretoria.

TØNNENSEN, M. H., B. L. PENZHORN, N. R. BRYSON, W. H. STOLTSZ and T. MASIBIGIRI, 2004 Displacement of *Boophilus decoloratus* by *Boophilus microplus* in the Soutpansberg region, Limpopo Province, South Africa. Exp Appl Acarol **32:** 199-208.

TSAGKARAKOU, A., T. VAN LEEUWEN, J. KHAJEHALI, A. ILIAS, M. GRISPOU *et al.*, 2009 Identification of pyrethroid resistance associated mutations in the para sodium channel of the two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae). Insect Mol Biol **18:** 583-593.

ULLMANN, A. J., C. M. R. LIMA, F. D. GUERRERO, J. PIESMAN and W. C. BLACK, 2005 Genome size and organization in the blacklegged tick, *Ixodes scapularis* and the Southern cattle tick, *Boophilus microplus*. Insect Mol Biol **14:** 217–222.

VAN DJIK, C. J., 2008 CURRENT PRACTICES FOR THE SALE AND DISTRIBUTION OF VETERINARY DRUGS IN AFRICA TO FARMERS, pp. 1-10 in *OIE Conference on veterinary medicinal products in Africa*, Dakar.

VAN NIEKERK, D. J., L. J. FOURIE and I. G. HORAK, 2006 Birds as hosts of immature ixodid ticks in Free State Province, South Africa. Onderstepoort J Vet Res **73:** 123-130.

VATSYA, S., and C. L. YADAV, 2011 EVALUATION OF ACARICIDE RESISTANCE MECHANISMS IN FIELD POPULATIONS OF *RHIPICEPHALUS (BOOPHILUS) MICROPLUS* COLLECTED FROM INDIA. Int J Acarol **37:** 405–410.

VUURE, C. (Editor), 2005 *Retracing the Aurochs: History, Morphology and Ecology of an Extinct Wild Ox*. Pensoft Publishers, Sofia-Moscow.

WALKER, A. R., 2011 Eradication and control of livestock ticks: biological, economic and social perspectives. Parasitology **138:** 945–959.

WALKER, A. R., A. BOUATTOUR, J. L. CAMICAS, A. ESTRADA-PEÑA, I. G. HORAK *et al.*, 2003 *Ticks of Domestic Animals in Africa: a Guide to Identification of Species*. Bioscience Reports, Edinburgh.

WAPLES, R. S., and O. GAGGIOTII, 2006 What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. Mol Ecol **15:** 1419-1439.

WAUGH, J., 2007 DNA barcoding in animal species: progress, potential and pitfalls. Bioessays **29:** 188–197.

WEDDERBURN, P. A., T. D. JAGGER, B. MCCARTAN and A. G. HUNTER, 1991 DISTRIBUTION OF BOOPHILUS SPECIES TICKS IN SWAZILAND. Trop Anim Health Prod **23:** 167-171.

WILLIAM, J., O. BALLARD and M. KREITMAN, 1995 Is mitochondrial DNA a strictly neutral marker? TREE **10:** 485-488.

WILSON, M. L., G. H. ADLER and A. SPIELMAN, 1985 Correlation Between Abundance of Deer and That of the Deer Tick, *Ixodes dammini* (Acari: Ixodidae). Ann Entomol Soc Am **78:** 172-176.

WINT, W. G. R., and T. P. ROBINSON, 2007 Gridded livestock of the world, pp. 17. FOA, Rome.

WIT, P. D., M. H. PESPENI, J. T. LADNER, D. J. BARSHIS, F. SENECA *et al.*, 2012 The simple fool's guide to population genomics via RNA-Seq: an introduction to high-throughput sequencing data analysis. Mol Ecol Resources **12:** 1058–1067.

YSSOUF, A., E. LAGADEC, A. BAKARI, C. FORAY, F. STACHURSKI *et al.*, 2011 Colonization of Grande Comore Island by a lineage of *Rhipicephalus appendiculatus* ticks. Parasites & Vectors **4:** 1-8.

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