Allele frequencies and evolutionary genetics of acaricide resistance-associated SNPs in *Rhipicephalus microplus* ticks from South Africa.

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I, **Samantha Reinecke** declare that the thesis/dissertation, which I hereby submit for the degree **MSc Biotechnology** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Summary

Rhipicephalus microplus constitutes a main vector for Asiatic babesiosis and anaplasmosis which are known to plague cattle. The lack of sufficient tick control strategies and management programs manifests into a severe economic burden threatening the sustainability of the livestock industry in South Africa, as well as globally. The principal method for tick control is the use of chemical acaricides. This form of tick control is, however, becoming less adequate due to an increase in the frequency of mutant alleles being selected in the population. This ultimately leads towards the development of resistance. In this study, a country-wide survey was conducted to determine the presence and frequency of mutant alleles of ticks resistant to the different chemical acaricide classes. Resistance to pyrethroids, formamidines and cyclodienes were investigated.

Results indicated that 58.8% of the tick population that was sampled was homozygous resistant to pyrethroids indicating that positive directional selection pressure is acting on the voltage-gated sodium channel. There was no significant presence of mutant alleles in the carboxylesterase gene conferring resistance to pyrethroids, or the GABA-gated chloride channel resulting in cyclodiene resistance. Results from this study represent the first report of two single nucleotide polymorphisms (SNPs) in the octopamine receptor in field populations of *R. microplus* ticks conferring resistance to amitraz, a formamidine. Gametic disequilibrium studies revealed a novel marker which was closely associated with these resistant alleles. A RFLP-based rapid diagnostic test was developed implementing this novel marker and shows great potential for future diagnosis. This is the first alternative suggestion for diagnosing amitraz resistance without the use of time-consuming and expensive bioassays.

This study provides the first comprehensive analysis of acaricide resistance in South Africa. Farmers will receive feedback regarding the resistance genotypes that were found on their farms. Along with advice from commercial acaricide suppliers, a tailored tick control program can be developed for each specific farm. This aims at minimizing the selection for resistance prolonging the use of current acaricides.

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A	Amitraz
ACh	Acetylcholine
AChE	Acetylcholinesterase
A	Adenine
AT	Adenine-thymine
AIT	Adult immersion test
bp	Base pair
BmAChE	*Boophilus microplus* acetylcholinesterase
BSA	Bovine serum albumin
C	Cytosine
Ca^{2+}	Calcium
°C	Degrees Celsius
CNS	Central nervous system
cAMP	Cyclic adenosine monophosphate
cyt	Cytochrome P450 resistance
DEM	Diethylmaleate
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
Est	Carboxylesterase resistance
F	Fipronil
FASTA	Fast-all
GABA	gamma-aminobutyric acid
GD	Gametic disequilibrium
Gbps	Giga base pairs
GPCR	G-protein coupled receptor
G	Guanine
GC	Guanine-cytosine

GSH	Glutathione-S-transferase
HWE	Hardy-Weinberg equilibrium
hrs	Hours
HCl	Hydrochloric acid
I_A	Index of association
IJ	Infective juvenile stage
ITS2	Internal transcribed spacer
IFMT	Isoleucine-phenylalanine-methionine-threonine
kb	Kilobases
LPT	Larval packet test
LTT	Larval tarsal test
ML	Macrocyclic lactone
mRNA	Messenger RNA
µM	Micromolar
µl	Microliter
mg	Milligram
ml	Milliliter
mM	Millimolar
min	Minutes
MAFFT	Multiple sequence alignment fast fourier transform
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NEB	New England biolabs
OP	Organophosphate
PHYLIP	Phylogeny inference package
PBO	Piperonyl butoxide
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
pmol	Picomole

Pty Ltd	Proprietary limited
RFLP	Restriction fragment length polymorphism
RNAse	Ribonuclease
RNA	Ribonucleic acid
sec	Seconds
SLIT	Shaw larval immersion test
SNP	Single nucleotide polymorphism
SDS	Sodium dodecyl sulfate
SNAP	Suit of nucleotide analysis programs
SP	Synthetic pyrethroid
Ti	Target site insensitive
T_a	Annealing temperature
Taq	*Thermus aquaticus*
TPP	Triphenylphosphate
TAE	Tris-acetate EDTA buffer
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl) aminomethane
T_m	Melting temperature
T	Thymine
U	Units

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Chapter 1: Acaricide resistance in *Rhipicephalus microplus* ticks.

1.1 The cattle tick, *Rhipicephalus microplus*

Rhipicephalus microplus is a hematophagous ectoparasite belonging to the Ixodidae family, with cattle being its primary host (Walker et al. 2003; Porto Neto *et al*. 2011). These blood-feeding arthropods are well known for the detrimental socio-economic impact they inflict on the cattle industry in terms of beef and dairy production (Jonsson, Davis, De Witt 2001; Jonsson 2006). Common names for *R. microplus* includes the cattle tick (CFSPH 2007), the southern cattle tick (CFSPH 2007) and the Asiatic blue tick (Horak 2009).

Controversial name allocations to tick species resulted in the re-evaluation of tick systematics based on both molecular and morphological data, rather than phenotypic assessment alone. One such hesitant yet eminent change was that of *Boophilus microplus* and *Boophilus decoloratus* to *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus (Boophilus) decoloratus*, respectively (Horak, Camicas, Keirans 2002). The re-classification of the genus *Boophilus* as a sub-generic appellation of *Rhipicephalus* was concluded after considerable molecular and morphological evidence indicated the paraphyletic relationship between *Rhipicephalus* and its sub-genus *Rhipicephalus* (*Boophilus*) (Barker, Murrell 2004). Barker and Murrell (2004) proposed a hypothetical phylogenetic tree (Figure 1.1) based on nucleotide sequences (12S rRNA, 16S rRNA and cytochrome C oxidase I,) and phenotypes acquired from several references. It is worthy to note that some genes and proteins from *R. microplus* have not been re-named and are still referred to as *Boophilus* in this study to maintain uniformity with that which is published.

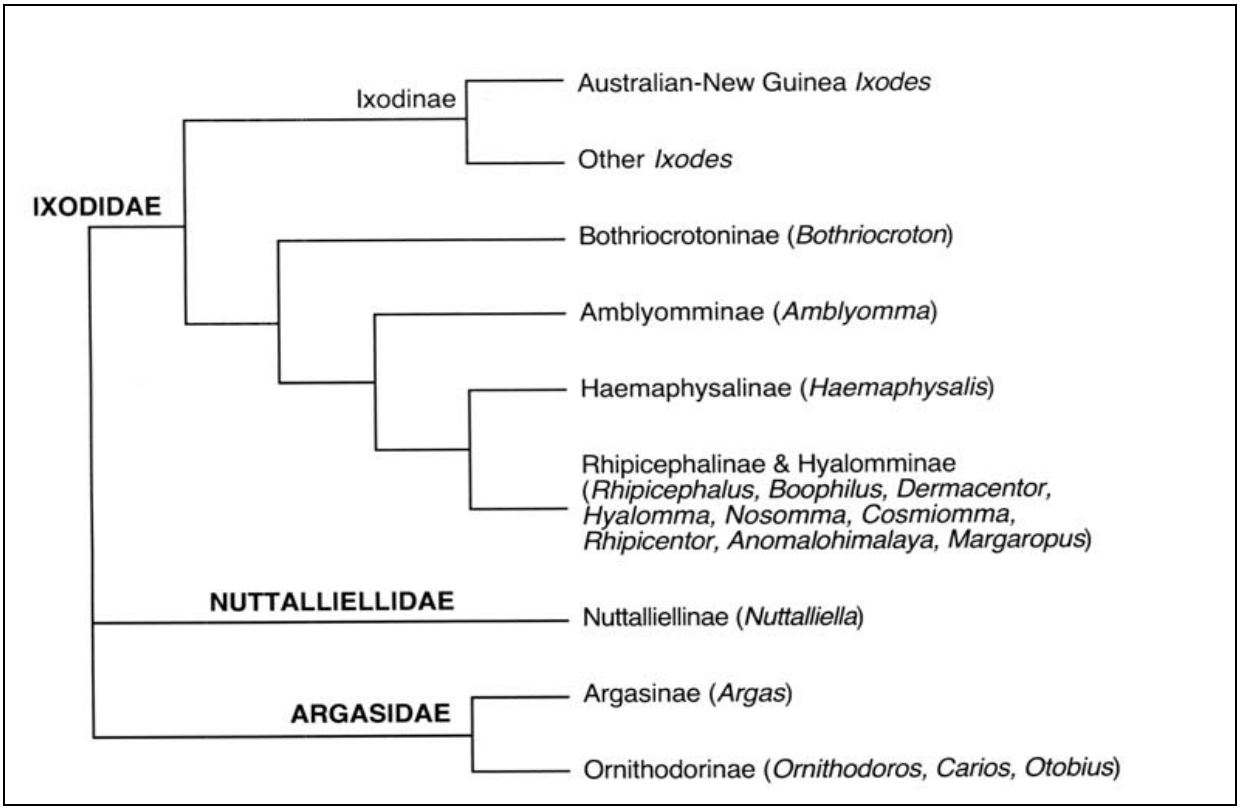


Figure 1:1 Phylogenetic tree constituting subfamilies of ticks. *Boophilus* is now a sub-genus of the genus *Rhipicephalus.* Taken from (Barker, Murrell 2004).

The family Argasidae lacks the scutum (females) or conscutum (males) from their dorsal surfaces giving them the characteristic description of 'soft ticks'. In contrast, the family Ixodidae embodies a distinctive 'hard plate' on its dorsal surface earning them the colloquial description as 'hard ticks'. The family, Nuttalliellidae, remained vague over the years due to the lack of sample collection and only a single valid species name, *Nuttalliella namaqua*, is known (Walker *et al.* 2003; Barker, Murrell 2004). Sequencing of the *N. namaqua* mitochondrial genome confirmed the phylogenetic relationship displayed between families iterating that *N. namaqua* represented the closest relative to the ancestral tick lineage. Blood-feeding of the ancestral tick lineage was also shown to encompass the unique characteristics displayed by both hard and soft tick families (Mans *et al.* 2011; Mans *et al.* 2012).

1.1.1 Geographical distribution

Rhipicephalus microplus prevails in tropical and sub-tropical regions of the world in comparison to the more temperate zones of the globe (Jongejan, Uilenberg 2004; Estrada-Peña *et al.* 2006). *Rhipicephalus decoloratus* predominates within Africa, but previous

studies showed that *R. microplus* tends to displace *R. decoloratus* when the two species co-exist in the same environment. This phenomenon has been recorded in Limpopo and Eastern Cape province, South Africa (TØnnensen *et al.* 2004; Horak *et al.* 2009), the Ivory Coast, West Africa (Madder *et al.* 2011), Maputo, Mozambique (Horak *et al.* 2009) and Tanzania (Lynen *et al.* 2008). Figure 1.2 illustrates the geographical distribution of *R. microplus* (A) and *R. decoloratus* (B) within Africa. The data represented on the maps include incidences of *R. microplus* found for the first time in Ivory Coast in 2007 (Madder *et al.* 2007). A distribution map for *R. microplus* and *R. decoloratus* within South Africa was published in 2011 (Terkawi *et al.* 2011), however, these results only include data from 2003.

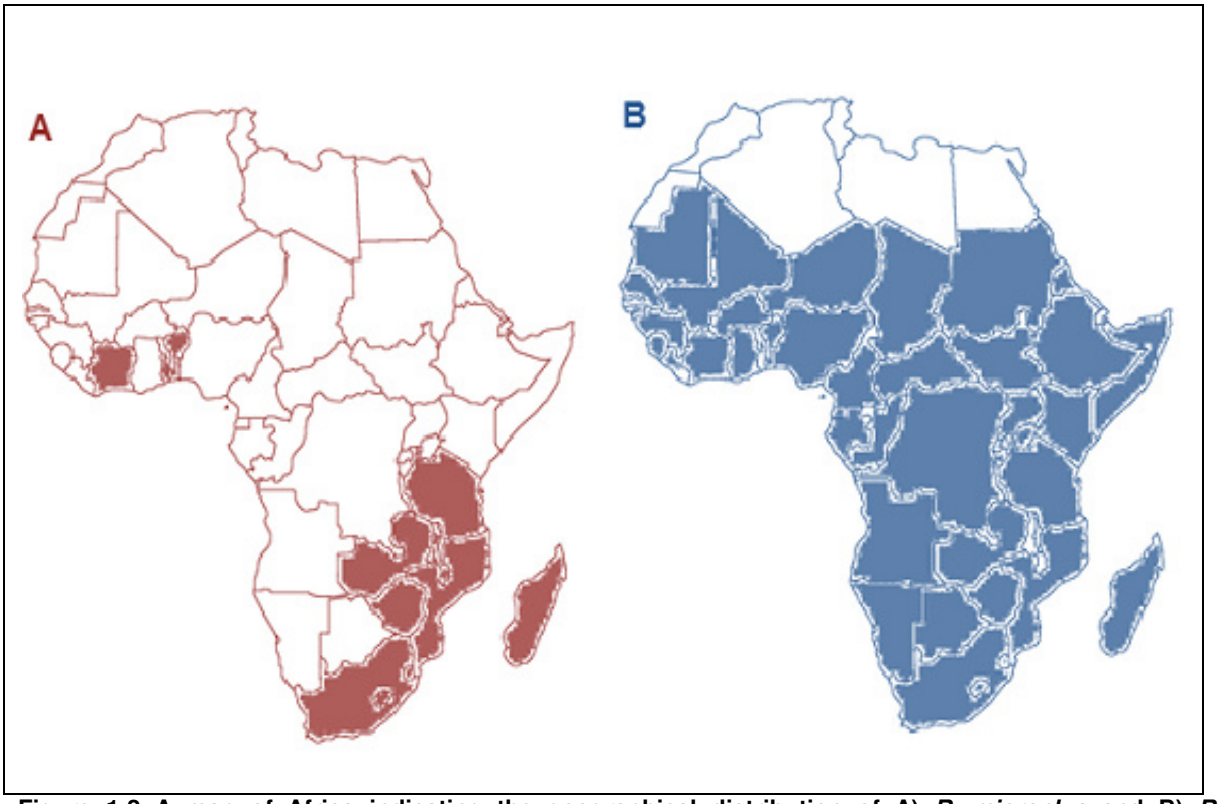


Figure 1:2 A map of Africa indicating the geographical distribution of A) *R. microplus* and B) *R. decoloratus* (Madder, Horak 2010).

Reports of *R. microplus* displacing ticks from the same sub-genus and occurring in regions that were previously unoccupied by this tick are quite alarming. The introduction of *R. microplus* in the Ivory Coast is just one example. Recently, *R. microplus* was found in Burkina Faso, Mali and Togo, with a general spread northwards within Benin (Adakal *et al.* 2013). Additionally, *R. microplus* was reported for the first time in Namibia indicating the tick's swift adaptation to varying climatic zones (Nyangiwe *et al.* 2013). This introduction into Namibia is unusual, because previous studies have shown that temperature and humidity severely influence tick population dynamics (Corson, Teel, Grant 2004). Due to tick

migration being mainly dependant on host movement, and climate to a certain degree, these factors become essential in the future prediction of tick spread as well as the spread of acaricide resistance (Chevillon *et al.* 2013).

1.1.2 Life cycle of *R. microplus*

Rhipicephalus microplus is a one-host obligatory parasite constituting of four life stages namely; egg, larvae, nymph and adult (Figure 1.3). The eggs are laid by engorged females and hatched in the surrounding environment after which the larvae quest for a host to feed on. Upon feeding, larvae moult into nymphs and continue feeding on the same host. Nymphs then subsequently moult into adults who may change positions on the same host for mating purposes. Engorged females drop to lay their eggs after which they perish (Walker *et al.* 2003).

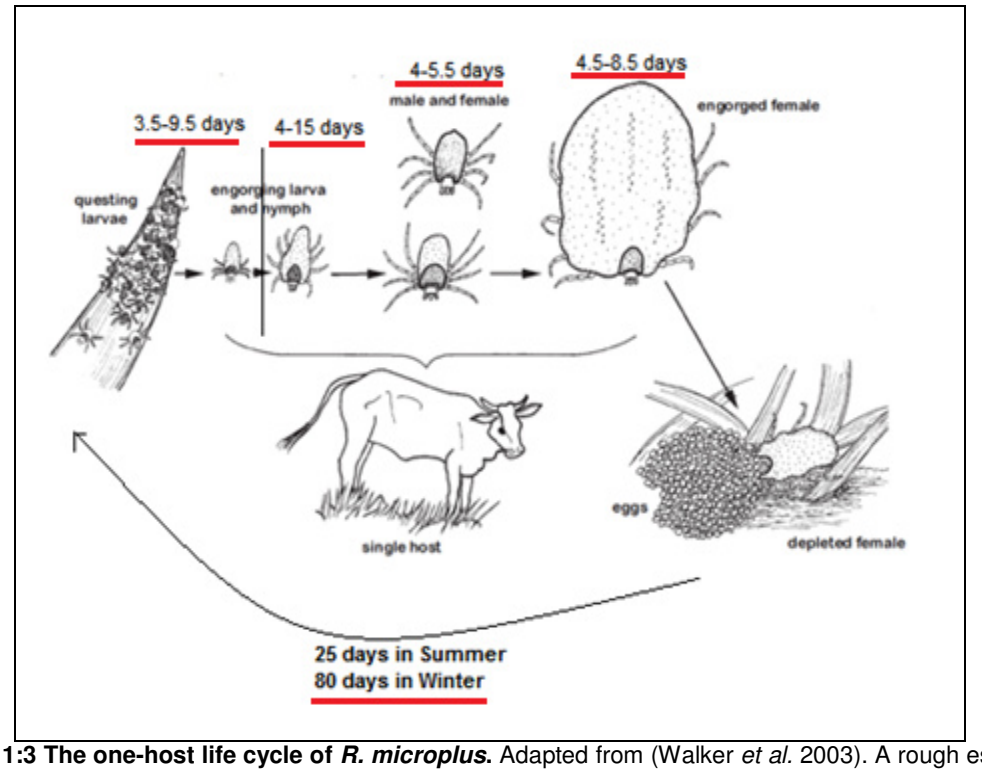


Figure 1:3 The one-host life cycle of *R. microplus*. Adapted from (Walker *et al.* 2003). A rough estimate of the time scale for the life cycle of *R. microplus* is indicated. The most time-consuming phase during development is from egg to neolarvae before they become engorged larvae. This development is sensitive to microclimate conditions such as temperature and humidity therefore developmental times differ between summer and winter.

Previous studies demonstrated that multiple paternities took place in *R. microplus* ticks. It was shown that progeny from a single female tick was not due to mating with a single male

but rather several males (Cutullè, Jonsson, Seddon 2010). It was also shown that there is a random association of male and female genotypes in the progeny (pangamous mating structure) which significantly contributes to the large genetic differentiation between different tick populations. Due to their short generation times and their ability to produce extreme genetic diversity, these ticks are able to evolve and adapt swiftly (Chevillon *et al.* 2007). This phenomenon can be suggested to largely contribute to the rapid development of resistance against acaricides.

1.2 Tick-borne diseases

Ixodid ticks, particularly *R. microplus*, are well documented vectors for various tick-borne diseases. Table 1.1 summarizes the different pathogens transmitted by common tick species.

Table 1-1 Tick species and the pathogens they transmit. Adapted from (Marufu 2008).

Tick Species	Pathogen
***Rhipicephalus* (*Boophilus*) spp.**	*Babesia bigemina*
	Babesia bovis
	Anaplasma marginale
	Borrelia theileri
Rhipicephalus appendiculatus	*Theileria parva*
Rhipicephalus evertsi evertsi	*Anaplasma marginale*
	Babesia bigemina
***Hyalomma* spp.**	*Anaplasma marginale*
	Theileria annulata
***Amblyomma* spp.**	*Ehrlichia ruminantium*

Rhipicephalus microplus constitutes the main vector for the intra-erythrocytic protozoan parasites *Babesia bovis* and *B. bigemina*, with the former being the major causative agent of Asiatic babesiosis which results in immense losses in cattle production. This tick can also transmit the bacteria *Anaplasma marginale* causing anaplasmosis (gallsickness), as well as *Borrelia theileri* which causes spirochaetosis in cattle (Walker *et al.* 2003; Marufu 2008).

Babesia bovis undergoes its development within the host's erythrocytes. Transmission of the pathogen within *R. microplus* can be transovarial; where the pathogen moves vertically from the maternal body to the eggs in the ovaries, or transstadial; where the pathogen progresses from one developmental stage to the next within the host. Interactions between the *Babesia* parasite and the tick vector results in the initiation of two reservoirs. The first reservoir being the presence of the pathogen within the vertebrate host from the onset of parasitemia, and the second one occurring throughout the life cycle of the tick due to the previously mentioned transovarial and transstadial transmission (Chauvin *et al.* 2009). The presence of *B. bovis* can be closely correlated to the presence of *R. microplus* because *R. decoloratus* only transmits *B. bigemina* (Potgieter 1977).

The life cycle and transmission of *B. bovis* and *B. bigemina* are much the same, and differ only with regard to developmental stages when transmission occurs (Radunz 2008). In the instance of *B. bovis*, only larval stages of the tick are able to carry out the transmission as infection is not retained within the tick after the first molting stage. *Babesia bigemina* on the other hand is evident in both nymphal and adult stages of the tick, with nymph being the primary preference for transmission (Radunz 2008).

Anaplasma marginale is similar to babesia in the sense that it also attacks the red blood cells of the cattle. Larvae, nymph and adult ticks are able to transmit *A. marginale* however very little is known about its development cycles within the tick itself (Radunz 2008).

1.3 Economic impact of *R. microplus* ticks in South Africa

Tick infestation alone as well as tick-borne diseases have a severe impact on the agricultural and livestock industry in terms of revenue streams. Cattle condition is diminished due to granuloma formation in the vicinity of the tick bite, resulting in further dilapidation of hide and skin products. Severe cattle loss is also brought about by the propensity of *R. microplus* to transmit *B. bovis* and *B. bigemina*, resulting in bovine babesiosis, as well as *A. marginale* resulting in anaplasmosis in cattle (Walker *et al.* 2003).

It is estimated that the global economic loss within these industries are US$18 billion per annum (deCastro 1997). The capital loss within South Africa due to cattle loss during the 1980's was estimated at 200 million rands per year (Bigalke 1980). However, this is exceedingly outdated with recent calculations suggesting the true losses to fall within the

range of 1.3 to 3.7 billion rands per year (Oberholster Unpublished data). This estimation only takes into account direct cattle losses and excludes costs incurred by regular cattle dipping for tick control, treatment of diseased animals and damages to hides and skin. These estimations further exemplify the dire need for improvement in current tick control procedures.

1.4 Control strategies

Tick control can be plainly defined as the treatment used to reduce the amount of tick exposure on livestock within a certain geographical area and time frame (Walker 2011). The control strategies implemented for tick control are much like other pest control strategies formed on the basis of biological, chemical and vaccination approaches. Chemical control is the most common form of tick control as no vaccine is currently available and few other options are feasible.

1.4.1 Biological control

Biological control aims to diminish the effects of any potentially harsh chemicals on both humans and the environment. However, biological pesticides are generally not used due to their dependency on environmental factors, as well as the potential of harming non-target invertebrates despite their beneficial attributes (Domingos *et al.* 2013). Examples of different biological control agents that can be implemented for tick control are discussed below.

Pathogens:

Entomopathogenic fungi have been considered as biological control agents against ticks and mites, with *Metarhizium anisopliae* and *Beauveria bassiana* demonstrating the most potential (Hajek, Delalibera 2012). These fungi are natural enemies of arthropods (Chandler *et al.* 2000; Samish, Ginsberg, Glazer 2004) and are able to penetrate the integument, after which they produce toxins within the tick. Histological studies have shown that ticks of all life stages are naturally plagued by these entomopathogenic fungi (Kalsbeek, Frandsen, Steenberg 1995). There are several advantages to using fungi as biological control agents for ticks and mites, the foremost being species-specific treatments. Secondly, biological control agents pose less of a threat to the surrounding environment by drastically diminishing toxicity, which is generally of great concern when using chemical control methods. Furthermore, implementing the use of fungi for tick control could possibly circumvent the

problematic occurrence of acaricide resistance (Chandler *et al.* 2000; Angelo *et al.* 2010). Disadvantages surrounding the use of fungi as a control agent are the dependency it displays for its hosts behavior, its reliance on the host preferences of the tick, as well as the overall susceptibility of the tick to the fungal infection. Additionally, they also display slow killing rates and require high humidity for germination and sporulation (Chandler *et al.* 2000; Samish, Ginsberg, Glazer 2004).

Bacteria are commonly found in field strain ticks and generally don't display any pathogenicity towards the tick. However, research has shown that certain bacteria can be pathogenic to the tick and shows potential for biological control. The bacteria *Cedecea lapagei* has been shown to be pathogenic towards *R. microplus*. *Cedecea lapagei* initiates infection upon entry into the genital tract of the tick. Laboratory controlled experiments demonstrated 100% mortality using *C. lapagei* (Brum, Teixeira 1992).

Obligatory parasites:

Entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) are well known for being obligatory parasites of a variety of insects (Samish, Ginsberg, Glazer 2004). The third infective juvenile (IJ) stage of the parasitic nematode enters the tick via natural cavities or openings. The nematode then releases symbiotic bacteria which is the eventual cause for fatality of the tick (Samish, Ginsberg, Glazer 2004).

An alternative biological approach is the use of Hymenoptera parasitoids for tick control with the most common being the parasitic wasp, *Ixodiphagous hookeri* (Mwangi *et al.* 1997). Female wasps oviposit their eggs into internal tick cavities and upon the nymphs first blood meal, egg development is initiated (Hu, Hyland 1998). *Ixodiphagous* species appear to be restricted to ticks thus eliminating any non-target effects (Samish, Ginsberg, Glazer 2004).

Predators:

Natural enemies of ticks may also be exploited for tick control. Avian tick predators include some 50 bird species that are known to prey on ticks. One such example is the yellow-billed oxpecker (*Buphagus africanus*) and the red-billed oxpecker (*B. erythrorhynchus*). These birds are known for their preference for feeding on ectoparasites (Samish, Ginsberg, Glazer 2004). Chickens (*Gallus gallus*) are not tick specific predators but they have been known to be opportunistic predators. This largely depends on the level of tick infestation as well as alternative food availability (Samish, Ginsberg, Glazer 2004).

Genetic control:

An attractive alternative to tick control is the introduction of naturally resistant hosts. Cattle have undergone adaptation, rendering certain breeds less susceptible to tick infestation. For example, Zebu cattle (*Bos indicus*) are generally more resistant to tick infestations by *R. microplus* in comparison to European cattle (*Bos taurus*) (O'Kelly, Spiers 1976). Due to the importation of European cattle breeds into Africa some 300 years ago, there are currently cross-breeds of *B. indicus* and *B. taurus*. Cross-breeding of these cattle has recently been exploited in an attempt to improve their natural resistance to *R. microplus* infestations (Brossard 1998). However, immunological control by inducing protection against *R. microplus* by means of immunization is the preferable route for eventual tick control. This can be brought about by the development of a vaccine.

1.4.2 Vaccines

A commercially available vaccine containing the recombinant Bm86 antigen from *R. microplus* has shown great potential in reducing the fecundity of female ticks, as well as their ability to become fully engorged. This ultimately affects their overall ability to reproduce effectively (Willadsen, Riding 1989; Cobon *et al.* 1995). However, despite the immense potential of Bm86 as a vaccine, it displayed geographical variance among tick strains. This variation could be as a result of sequence differences of Bm86 from various tick populations (Parizi *et al.* 2009). Currently, researchers are trying to find novel antigens or targets for the development of new vaccines that can eradicate the use of acaricides as the primary control agent. Potential candidates for new vaccines have been suggested (Parizi *et al.* 2009). Furthermore, certain research groups endeavor to improve current antigens used for vaccines by the implementation of adjuvants and formulations (Imamura 2007).

An advantage of developing a tick vaccine includes the limited use of conventional acaricides, prolonging the overall effective time of the acaricides. The use of vaccines will serve to be more ecologically conscientious of the surroundings with less toxicity that is displayed by acaricidal usage. A vaccine will undoubtedly be more cost effective for farmers to the more expensive acaricide counterparts. A potential downfall of vaccines could be their species specificity limiting its use as a multi-tick species vaccine, however, this still has to be determined (De la Fuente *et al.* 2007; Parizi *et al.* 2009).

1.4.3 Chemical control

Chemical control includes phytotherapeutic extracts that exhibit acaricidal properties. A recent review describes all the plant extracts displaying acaricidal properties (Borges, Dias de Sousa, da Silva Barbosa 2011). Plant extracts are attractive for tick control because they can be implemented on organic farms and drastically lower contamination levels when compared to acaricides. Plant extracts could possibly replace acaricides once certain limitations have been dealt with. These limitations include complexity with regard to formulation preparation, variation in chemical compositions among individuals of the same plant species, and the overall lack of information of active acaricide compounds (Borges, Dias de Sousa, da Silva Barbosa 2011). Therefore, acaricides remain the main form of tick control.

1.5 Acaricides and their targets

In spite of the disadvantages associated with acaricide usage, it remains the foremost control strategy implemented by farmers for tick control. The main concern at this point is the declining longevity of current acaricides due to the adaptive mechanisms a tick utilizes to develop resistance. Resistance to organophosphates (OPs) first came about in the 1980's (Aguirre *et al.* 1986) after which synthetic pyrethroids and the formamidine (amitraz) was introduced to circumvent OP-resistant ticks (Aguirre *et al.* 1986; Soberanes *et al.* 2002). Initial over-exploitation of amitraz treatment was limited due to higher cost margins, which enforced the extensive use of synthetic pyrethroids instead. However, after the prevalent eruption of pyrethroid resistance in the 1990's, amitraz usage became more intensive (Parrodi, Fragoso, Nunez 1995), which lead to the inception of amitraz resistance with the first case being reported in 2001 in Mexico (Soberanes *et al.* 2002). With increased selection pressure promoting the development of acaricide resistance, the progression towards novel control strategies is more imperative than ever before.

1.5.1 Formamidines

The most common formamidine used for tick control in South Africa is amitraz (Figures 1.4A and 2.4). The target site for amitraz in *R. microplus* has yet to be defined, which ultimately delays any further development with regard to screening assays for diagnostics. It was proposed that monoamine oxidase, alpha-2-adrenoceptors and the octopamine receptor

could be candidates for the target site of amitraz, with the latter being the most probable in ticks (Jonsson, Hope 2007). It is thought that amitraz is a potential agonist of the octopaminergic system located in the tick synganglion. In the presence of amitraz, the octopamine receptor is activated and this overstimulation at synapses has lethal effects on the tick (Booth 1989; Lees, Bowman 2007). The octopamine receptor is a G-protein coupled receptor (GPCR). Baxter and Barker (1999) discovered that the *R. microplus* octopamine receptor sequence contained eight hydrophobic domains in contrast to the seven found in most GPCRs. The role of this additional transmembrane loop still needs to be elucidated (Baxter, Barker 1999). The octopamine receptor plays a role in signal transduction pathways via intracellular calcium (Ca^{2+}) and cyclic adenosine monophosphate (cAMP) second messenger pathways (Gilman 1987; Lees, Bowman 2007). Previous studies have also shown that this receptor regulates egg production in female ticks as well as the production of a wax-like layer along their oral cavities (Lees, Bowman 2007).

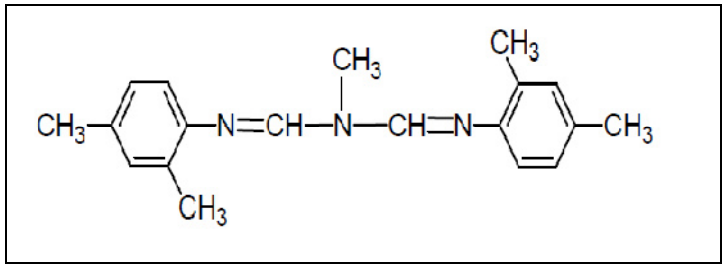


Figure 1:4 Chemical structure of the formamidine acaricide, amitraz (Marčić, Perić 2011).

1.5.2 Pyrethroids

Pyrethroids are synthetic composites encompassing similar attributes to extracts from the *Chrysanthemum cinerariaefolium* flowers. Pyrethrins represent the easily oxidized building blocks of pyrethroids (Anadon, MArtinez-Larranaga, Martinez 2009). There are two classes of pyrethroids (Figure 1.5), with a cyano-group present in class II but not class I (Anadon, MArtinez-Larranaga, Martinez 2009). Additional substitutions such as chlorine or bromine ions can also be found within the different classes however, they don't differentiate the classes from one another.

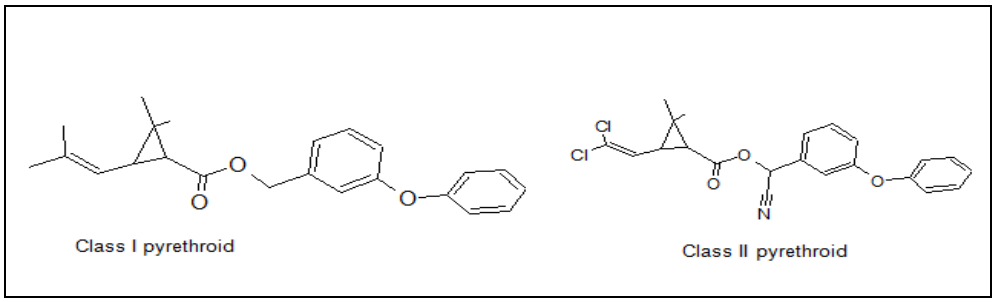


Figure 1:5 Chemical structure of class I and II pyrethroids. A cyano-group can be seen in class II distinguishing it from the class I pyrethroids (Anadon, MArtinez-Larranaga, Martinez 2009).

The voltage-gated sodium channel is the well established target of synthetic pyrethroids (SPs). In arthropods, the voltage-gated sodium channel occurs in nerve membranes at chemical synapses allowing sodium ions to flow into the cell (Narahashi 1971; Dong 2007). The sodium channel constitutes four domains each of which have six trans-membrane spanning segments (1-6) (Figure 1.6). Trans-membrane spanning segments number 5 and 6 form the ion-conducting pore. The 4th segment is comprised of a highly repetitive motif of positively charged amino acids, after which two hydrophobic residues trail to provide this segment with the ability to serve as the primary voltage sensor. The inactivation gate is also shown in Figure 1.6 with a motif of isoleucine-phenylalanine-methionine-threonine (IFMT) which plays an essential role in the activation of the voltage-gated sodium channel (Yu *et al.* 2005).

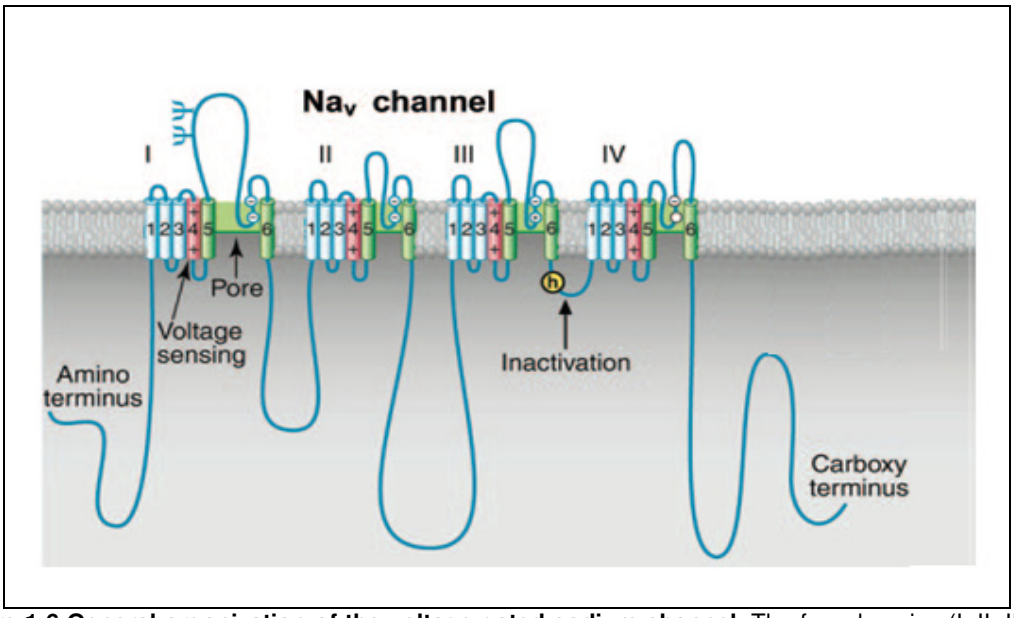


Figure 1:6 General organization of the voltage-gated sodium channel. The four domains (I, II, III, and IV) are indicated with each comprising of six trans-membrane spanning segments (labeled 1-6). Segments 5 and 6 represent the pore forming segments, while segment 4 acts as the voltage sensor (Yu *et al.* 2005).

Upon exposure to pyrethroids, the sodium channel undergoes hyperpolarization, causing neurons to swiftly liberate resulting in convulsions and eventual death of the arthropod. This effect is brought about by the prevention of the IFMT deactivation mechanism (Yu *et al.* 2005; Lees, Bowman 2007).

1.5.3 Organophosphates

Organophosphates (OPs) and carbamates have been shown to target acetylcholinesterase (AChE, E 3.1.1.7) as a quasi-irreversible inhibitor in *R. microplus* ticks (Pruett 2002; Pruett, Guerrero, Hernandez 2002). AChE, a serine hydrolase, is responsible for the degradation of the neurotransmitter acetylcholine (ACh) into acetate and choline at post-synaptic membranes (Figure 1.7). In the presence of an OP, the serine residue is phosphorylated in the enzyme active site, thereby disabling catalytic function (Pang *et al.* 2012). This prevents the breakdown of ACh into acetate and choline, leading to the build-up of ACh at the post-synaptic membrane which results in neuromuscular paralysis (Lees, Bowman 2007).

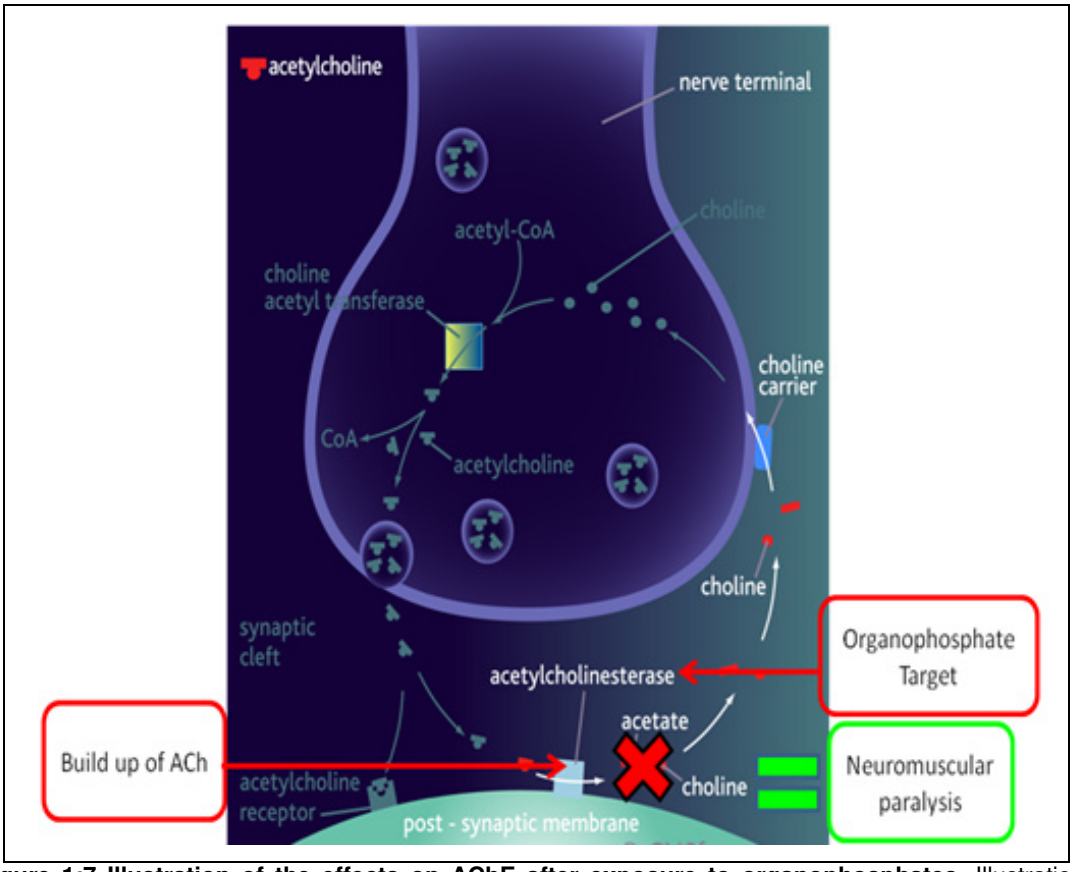


Figure 1:7 Illustration of the effects on AChE after exposure to organophosphates. Illustration is representative of nerve synapses where there is a build-up of ACh when exposed to organophosphates resulting in neuromuscular paralysis. Adapted from (Katzung 2001).

In comparison to other insects, pests or arthropods, *R. microplus* seems to have several genes that may encode AChE with three *BmAChEs* identified to date (Baxter, Barker 1998; Hernandez *et al.* 1999; Temeyer, Davey, Chen 2004).

1.5.4 Cyclodienes

Cyclodienes, also known as organochlorides, are known to target the gamma-aminobutyric acid (GABA) gated chloride channel (Lees, Bowman 2007). Dieldrin is a common cyclodiene acaricide (Figure 1.8), but its use has been prohibited due to its excessive toxicity. The GABA-gated chloride channel plays an essential role at the neuromuscular junction and synapses in the central nervous system (CNS) of the tick (Lees, Bowman 2007). The arrangement of the channel includes five protein chains which assemble to exhibit a pentameric like structure with exoplasmic receptor and membrane spanning domains (Hope, Menzies, Kemp 2010).

Upon exposure to an acaricide such as dieldrin, the GABA neurotransmitter cannot bind to the receptor, and this prevents chloride ions from passing through the channel. The result is hyperexcitation at the nerve synapses inevitably leading to convulsion within the arthropod (Bloomquist 2003).

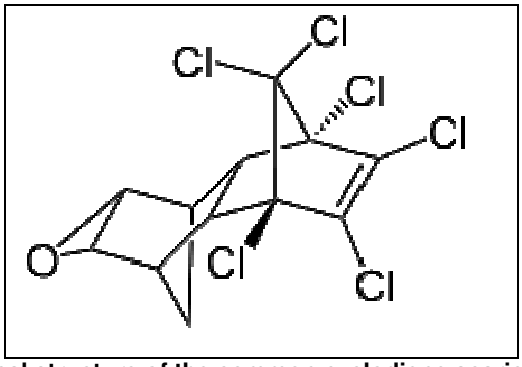


Figure 1:8 Chemical structure of the common cyclodiene acaricide, dieldrin.

Although the use of dieldrin was banned from most countries in the 1970's, cyclodiene resistance still remains a model for target site resistance studies. Furthermore, the target site of these cyclodienes has also shown to be the target site for novel insecticides coming onto the market, such as macrocyclic lactones and fipronil (ffrench-Constant *et al.* 2000).

1.5.5 Macrocyclic lactones

The use of macrocyclic lactones (MLs) is becoming increasingly more common for tick control due to the development of resistance against other classes of acaricides. The target site of MLs is believed to be the GABA- and/or glutamate-gated chloride channels (Guerrero, Lovis, Martins 2012). The most common ML implemented for tick control is avermectins and its derivatives (Figure 1.9A). Avermectin is produced by *Streptomyces avermitilis*, a common soil bacterium (Shoop, Mrozik 1995). By binding to the channel it elicits a large chloride influx into the nerve cells of the insect, thereby disturbing effective action potential (nerve impulses) and ultimately decreasing nerve signal transmission (Martin, Robertson, Wolstenholme 2002). Therefore, MLs are agonists of these channels, in contrast to the antagonistic mechanism seen with cyclodienes. Upon exposure to avermectins, there is irreversible activation of the GABA/glutamate-gated chloride channels resulting in the overall deficiency of the ability of voluntary muscle coordination (Bloomquist 2003).

Fipronil, a phenylpyrazole acaricide, is also thought to target the GABA/glutamate channels (Figure 1.9B). As with cyclodienes, fipronil is an antagonist of these channels blocking the neurotransmitters from essentially binding to the channel receptors. Whether cross-resistance between fipronil and avermectin can occur still has to be elucidated. However, recent studies have suggested that these two compounds have different sites of action within the channels (Castro-Janer *et al.* 2011).

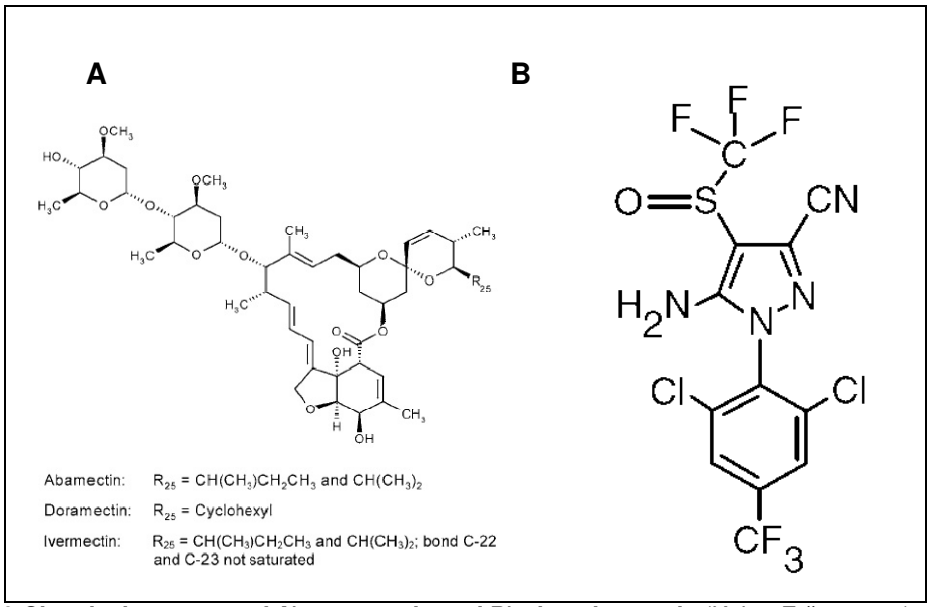


Figure 1:9 Chemical structure of A) avermectin and B) phenylpyrazole (Kolar, Eržen 2006).

1.6 Acaricide resistance

Ticks have acquired the ability to evade the toxic effects of chemical acaricides by developing different resistance mechanisms (Figure 1.10). The cuticle surrounding the tick represents the first line of defense against any chemical attack. The ability of the tick to diminish the means of access of the acaricide into its body is termed penetration resistance. Further investigations into this type of resistance occurring in *R. microplus* has not been reported since 1983 (Schnitzerling, Nolan, Hughes 1983). A common resistance mechanism in arthropods is target site insensitivity. This adaptive mechanism involves the alteration of the drug target site on a DNA level by alteration of the wild-type allele to a mutant form. This mutation renders the acaricide treatment ineffective. Metabolic resistance to acaricide treatment involves the increased ability in an entity to detoxify or sequester the acaricide. This involves the up-regulation of common detoxifying enzymes including cytochrome P450 oxidase, esterases and glutathione-S-transferases (Guerrero, Lovis, Martins 2012). All of these mechanisms have shown to play a vital role in ticks developing resistance to chemical acaricides.

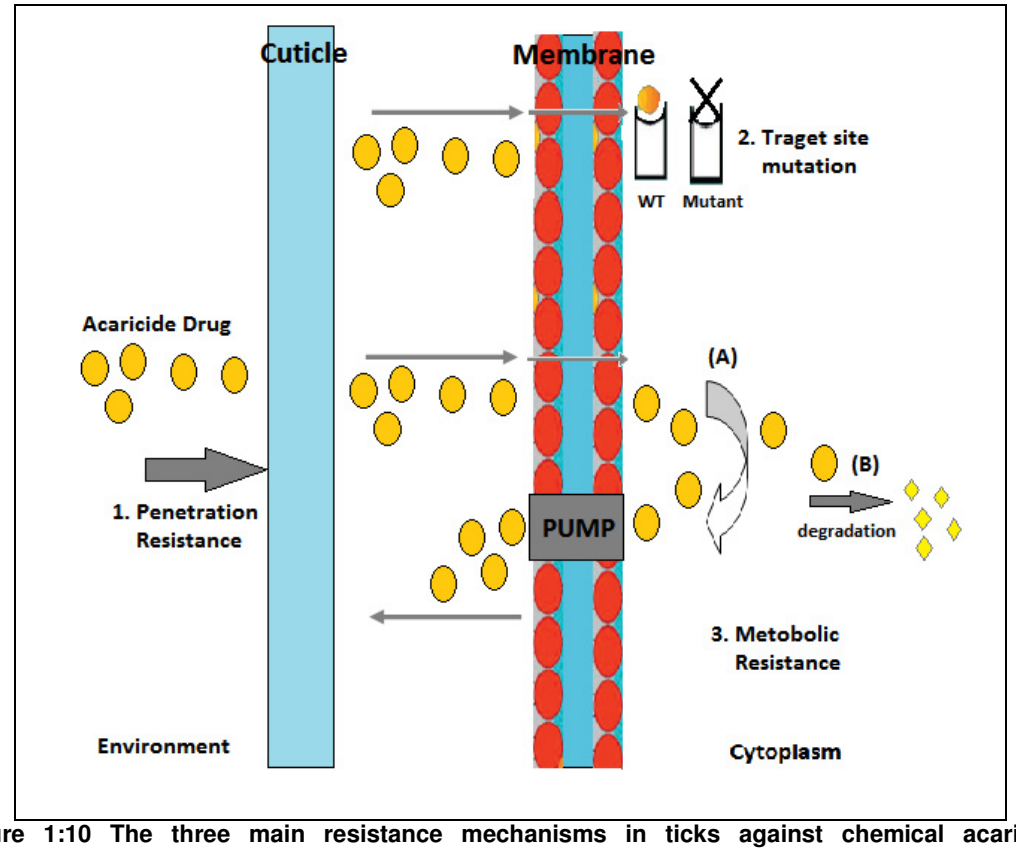


Figure 1:10 The three main resistance mechanisms in ticks against chemical acaricides. [1] Represents penetration resistance by the inability of an acaricide to cross the cuticle effectively, [2] represents target site mutation by the formation of a mutant allele rendering the drug ineffective and [3] represents metabolic resistance where detoxification of the acaricides are enhanced by means of (A) pumps or (B) degradation.

There are various assays available for detecting acaricide resistance in ticks, and some have been developed to detect resistance in both adults and larvae. A brief description of the most common assays available is given below.

Larval Packet Test (LPT)

The LPT assay was established to detect acaricide resistance in *R. microplus* (Stone, Haydock 1962). Briefly, acaricidal solutions are placed onto filter paper, and allowed to dry for 24 hrs at room temperature. Larvae are then transferred to the filter paper which is sealed, and further incubated for another 24 hrs. The number of live and dead larvae is then counted. It takes approximately 6 weeks to obtain results from this assay due to the laborious collection of ovipositing female ticks, after which newly hatched larvae can be utilized for the assay. Recent modifications to the protocol have allowed for a broader range of acaricides to be tested. Acarine growth regulators such as fluazuron cannot be used in these LPT bioassays.

Shaw Larval Immersion Test (SLIT)

The SLIT bioassay was established to detect susceptibility towards a range of acaricides in *R. microplus* ticks (Shaw 1966). Briefly, larvae are placed into fabric bags which are then immersed in the acaricidal solution for five minutes. After the bags are dry, they are incubated for 48 hrs and subsequent mortality quantified. This method only provides results within 6 weeks for the same reasons mentioned with LPT, and results are comparable with those of LPT. Fluazuron cannot be used with the SLIT bioassay either.

Adult Immersion Test (AIT)

The AIT assay was initially developed for testing the efficacy of new acaricides on the market against several tick species (Drummond *et al.* 1973). The bioassay requires engorged female ticks which are subsequently dipped in an acaricidal solution for 10 minutes. Once dry, they are incubated for three weeks, after which total egg mass is weighed and hatching visually estimated. The evaluation of acarine growth regulators is possible with this bioassay.

Larval Tarsal Test (LTT)

The LTT assay was established in 2011 to determine the resistance level that *R. microplus* displayed against acaricides (Lovis *et al.* 2011). Acaricidal solutions are distributed in a 96-well plate; a calculated number of eggs are then placed in each well. After incubation in an

environmental chamber, egg hatching and larvae mortality are quantified. Results from the LTT bioassay are comparable with those from the LPT.

Synergistic assays

Synergistic assays generally accompany LPT bioassays to determine the role of metabolic enzymes in acaricide resistance. The same protocol is followed, with the addition of one of three synergists to the acaricidal solution. The synergists include triphenylphosphate (TPP) which inhibits esterase activity, piperonyl butoxide (PBO) which inhibits cytochrome P450 oxidase and diethylmaleate (DEM) which inhibits glutathione S-transferase. There have been several studies using these synergistic assays to define metabolic detoxification of acaricides in resistant tick strains (Li *et al.* 2004; Li *et al.* 2007; Li *et al.* 2008; Vatsya, Yadav 2011).

1.6.1 Formamidines

Resistance to amitraz is complex and multigenic in nature, and involves recessive inheritance of resistant alleles (Li *et al.* 2004; Li *et al.* 2005; Fragoso-Sanchez *et al.* 2011). To date no adequate resistance mechanisms against amitraz have been shown for *R. microplus*, however, several have been suggested. Li *et al.* (2004) illustrated that metabolic detoxification plays a role in amitraz resistance in Mexican tick strains by means of synergistic studies with enzyme inhibitors. These results were, however, quite variable across different *R. microplus* strains with the overall contribution of detoxifying enzymes being difficult to evaluate (Li *et al.* 2004). The Mexican Pesqueria strain was confirmed to convey metabolic resistance to amitraz by up-regulation of glutathione-S-transferase which was established by synergistic assays using diethylmaleate (DEM) (Saldivar *et al.* 2008). It was further suggested that target site insensitivity could perhaps be the main mechanism of amitraz resistance. Unfortunately, conclusive studies to illustrate this mechanism have been unsuccessful to date (Fragoso-Sanchez *et al.* 2011; Guerrero, Lovis, Martins 2012).

Two single nucleotide polymorphisms (SNPs) in the octopamine receptor were proposed to be linked to amitraz resistance. These SNPs were however not confirmed but only inferred based on sequence alignments between a susceptible Australian strain, the susceptible American Gonzalez strain and the resistant Brazilian Santa Luiza strain (Chen, He, Davey 2007). Sequencing of the octopamine receptor from these strains revealed 37 SNPs of which nine were non-synonymous substitutions. Seven of these were attributed to geographical differences between the strains while the remaining two SNPs were potentially

linked to amitraz resistance. These two SNPs occur at amino acid position 8 (threonine to proline) and 22 (leucine to serine) (Chen, He, Davey 2007).

1.6.2 Pyrethroids

Metabolic resistance towards pyrethroids has been documented in the Coatzacoalcos Mexican *R. microplus* tick strain based on synergistic assays with piperonyl butoxide (PBO) and triphenyl phosphate (TPP) (Miller, Davey, George 1999). It was also found within this particular strain that there was an up-regulation in the expression of an esterase (CzEst9) which seemed to be involved in the rapid hydrolysis of permethrin (Pruett, Guerrero, Hernandez 2002). Further studies confirmed that this carboxylesterase enzyme played a major role in pyrethroid resistance in the Mato Grosso Brazilian strain (Baffi, De Souza, Vieira 2007).

It appears that target site resistance is the major resistance mechanism to pyrethroids in *R. microplus* ticks. Three SNPs in the voltage-gated sodium channel have been reported to be associated with pyrethroid resistance in ticks. The first SNP was discovered in domain III of the sodium channel in tick populations from Mexico, resulting in a phenylalanine to isoleucine mutation (He *et al.* 1999). This particular mutation seems to be confined to North America and has not been found in other regions of the world (Guerrero, Lovis, Martins 2012).

Target site mutations resulting in pyrethroid resistance were predominantly found to occur in domain II of the voltage-gated sodium channel in other arthropods that were investigated (Soderlund, Knipple 2003). This subsequently led to the discovery of two SNPs in domain II segments 4-5 of the linker region of the sodium channel. Morgan *et al.* (2009) reported an amino acid change from leucine to isoleucine, while Jonsson *et al.* (2010) showed a glycine to valine substitution (Morgan *et al.* 2009; Jonsson *et al.* 2010). Of the three mutations that have been reported, it has been suggested that those in domain III phenotypically convey a higher level of resistance while those reported by Morgan *et al.* (2009) confer moderate resistance (100-400 fold). The domain II mutation reported by Jonsson *et al.* (2010) only conferred resistance to flumethrin (Guerrero, Lovis, Martins 2012). A comparison of the occurrence of these mutations from different populations of *R. microplus* ticks was conducted by Guerrero *et al.* (2012) (Table 1.2).

Table 1-2 The prevalence of sodium channel mutations resulting in pyrethroid resistance in *R. microplus* ticks. Adapted from (Guerrero, Lovis, Martins 2012).

		Percentage of mutant larvae (%)		
Country	**Nr. of larvae tested**	**Morgan *et al.* (2009)**	**Jonsson *et al.* (2010)**	**Guerrero *et al.* (2001)**
Brazil	27	96	0	0
Argentina	133	76	0	0
Mexico	36	0	0	100
South Africa	69	26	0	0
Australia	17	100	0	0

From the results of the comparative study (Table 1.2) it is clear that the most common mutation is the one reported by Morgan *et al.* (2009). Guerrero *et al.* (2001) represents the mutation published by He *et al.* (1999) in domain III, which appears to be persistent throughout Mexico. The mutation published by Jonsson *et al.* (2010) was not found in any of the tick populations that were screened, including Australian tick populations in which the mutation was originally described. In the presence of both metabolic and target site resistance within a tick, target site resistance has shown to be the major contributor to pyrethroid resistance. However, this is largely strain dependant (Guerrero, Lovis, Martins 2012) as was seen with the Mexican Coatzacoalcos *R. microplus* strain which indicated that metabolic detoxification was the major resistance mechanism (Miller, Davey, George 1999).

Apart from the mutations occurring in the voltage-gated sodium channel, an additional mutation has been reported in the carboxylesterase gene that has been associated with pyrethroid resistance in Mexican tick populations (Hernandez *et al.* 2000; Hernandez *et al.* 2002). This mutation results in a G to A substitution at nucleotide position 1120 which creates an *Eco*RI restriction enzyme cut site for rapid diagnosis. Baffi *et al.* (2007) suggested that this mutation was also involved in organophosphate resistance; however, others in the field have not recognized this opinion.

1.6.3 Organophosphates

Jamroz *et al.* (2000) showed that a carboxylesterase (Est10) was significantly up-regulated in organophosphate resistant Mexican strains of *R. microplus*, suggesting its involvement in the metabolic detoxification of the acaricide (Jamroz *et al.* 2000). Four coumaphos resistant Mexican strains were shown to confer resistance via an elevated cytochrome P450 mediated

response, but the same was not obtained for diazinon resistant strains (Li *et al.* 2003). Glutathione-S-transferase has also been associated with coumaphos resistance in the San Alfonso Mexican tick strain (Saldivar *et al.* 2008).

Target site resistant mechanisms remain unclear within AChEs largely due to the uncertainty surrounding the true identity of the transcript which is functionally important to organophosphate resistance. As previously mentioned (Section 1.5.3) there are currently three *BmAChE*s that have been identified in *R. microplus*. Researchers have extensively investigated these *BmAChE*s in the hope of discovering a mutation which confers resistance to organophosphates (Baxter, Barker 1998; Hernandez *et al.* 1999; Temeyer, Davey, Chen 2004). Temeyer *et al.* (2010) expressed several *BmAChE* constructs in baculovirus infected sf21 cell cultures (Temeyer *et al.* 2006), and used cell culture supernatants to confirm their identity as AChEs through a series of biochemical characterization steps. Recombinant clones were sequenced and gave an indication of target site resistance in organophosphate resistant *R. microplus* tick strains (Temeyer, Pruett, Olafson 2010) which is summarized in Table 1.3. The susceptible Deutch 5 strain comprised of 13 amino acid substitutions which seemed to be prevalent among susceptible strains. Two organophosphate resistant San Roman strains (SR4 and SR11) revealed 13 amino acid substitutions with nine being common between the two resistant constructs (Table 1.3). The resistant Tuxpan strain (Tx11) displayed four amino acid substitutions which were unique to this particular construct and generated an expressed product which was insensitive to paraoxon (an organophosphate) (Temeyer, Pruett, Olafson 2010). These results strongly suggest that organophosphate resistance is complex and perhaps multigenic in nature with the involvement of more than one AChE in the resistance phenotypic profile.

Table 1-3 Amino acid substitutions found in BmAChE1 expressed in baculovirus. Adapted from (Temeyer, Pruett, Olafson 2010). (S) indicates that the strain is susceptible while (R) indicates the strain is resistant to organophosphates. SR4/11 represents the organophosphate resistant San Roman strain. Tx11 represents the resistant Tuxpan strain. The amino acid substitution arrangement is as follows; E55G refers to glutamic acid (E) at amino acid position 55 has been replaced with glycine (G). The blocked mutations represent those which are unique to an organophosphate resistant Tuxpan (Tx11) strain.

Strain Country R/S	Deutch 5 USA (S)	SR4 Mexico (R)	SR11 Mexico (R)	Tx11 Mexico (R)
Amino Acid Substitions found in *BmAChE1*		E55G		
	E60K	E60K	E60K	E60K
	P78T	P78T	P78T	P78T
		P157S	P157S	
				D188G
				E196G
	T219A	T219A	T219A	T219A
	E238G			E238G
	A260T	A260T	A260T	A260T
				V331A
		N333S		
	A349V	A349V	A349V	A349V
			T362A	
	K364R			K364R
				F390S
	M426V			M426V
	T437A			T437A
		Q488R	Q488R	
			I493T	
	R549H	R549H	R549H	R549H
	E552Q			E552Q
		W571F	W571F	
	T576A			T576A
	N583D			N583D

1.6.4 Cyclodienes

Target site resistance has been reported against dieldrin in the GABA-gated chloride channel for *R. microplus* tick populations from Australia. This resistance was conferred by a two base pair mutation that causes an amino acid change from threonine to leucine (Hope, Menzies, Kemp 2010). The mutation was present in 20% of tested samples, even though the chemical dieldrin was withdrawn from use in Australia approximately 20 years ago. This suggests that the mutation caused by exposure to dieldrin could be irreversible, or in the absence of selection pressure could take prolonged periods of time to disappear from the gene pool (Hope, Menzies, Kemp 2010). It has been suggested that the development of cross-resistance may occur due to the presence of this mutation with other acaricides that share the same target site (*i.e.* fipronil and MLs). Whether this actually takes place still has to be clarified. This particular mutation has the potential to shed some light onto the

resistance mechanisms for other acaricides such as fipronil and MLs (Guerrero, Lovis, Martins 2012).

It is clear that further investigation is required to clarify and characterize resistance mechanisms in *R. microplus* ticks, with regards to both metabolic and target site resistance. A comprehensive explanation of these mechanisms will provide much needed insight for the future development of improved acaricides and their overall longevity in the field. Without this information, adequate tick control programs will remain elusive.

1.7 Adaptation through natural selection

Adaption through natural selection is a key mechanism in evolution and is responsible for the vast genetic diversity of life today (Williams 1966). Mutation causes differences in DNA sequences between individuals, the eventual fates of which are determined by various evolutionary forces such as natural selection and genetic drift. If these differences increase the fitness of some individuals in the population, selection will act to increase the frequency of the mutant allele relative to the native alleles (Fisher 1930). In other words, mutations result in genetic variants within a population, and these are known as polymorphisms. These genetic polymorphisms will either be eradicated from the population, maintained as polymorphisms, or become fixed by replacing the original 'wild-type' allele in the population. Whether a mutation is eradicated or fixed in a population depends on a number of deterministic (*e.g.* positive or purifying selection) and stochastic (such as genetic drift) factors (Lemey, Salemi, Vandamme 2009).

Several different forms of natural selection are generally recognized. If a mutation increases in frequency, is beneficial to the organism and becomes permanent within the population, then positive directional selection has taken place. Directional selection is generally associated with a loss of variation and heterozygous deficiency (Kaplan, Hudson., Langley 1989). Positive balancing selection can also take place where polymorphisms within the target gene are maintained. This type of selection is generally associated with excess heterozygotes (Nordburg, Innan 2002).

If a mutation decreases in frequency and is eradicated from the population then purifying selection has taken place (Brodie, Moore 1995). An additional evolutionary force, namely genetic drift, may also increase the frequency of a mutation. Genetic drift allows mutant frequencies to change arbitrarily with no positive or negative selection pressure. Eventually

these mutations become fixed or are eradicated from the population. The probability of fixation is usually dependant on the initial frequency of the mutant allele. Genetic drift is most apparent in smaller populations, but takes much longer than natural selection to significantly change allele frequencies (Hartl, Clarke 1989).

In nature, when considering real populations rather than hypothetical ones, many forces are at play such as natural selection, genetic drift, migration and inbreeding. All of these forces may contribute to the observed allele frequencies. For example, the evolutionary state of a mutation is determined by the product 2N*s*, where N refers to the effective size of the population being studied, and *s* is the selection coefficient against or for a particular allele. When $2Ns >> 1$ or $2Ns << 1$, then the mutant allele is under positive or negative selection pressure, respectively (Lemey, Salemi, Vandamme 2009). This is based on the nearly neutral theory (Ohta 1992) which states that the absolute selection coefficient (*s*) should be ten times less than 1/2N for the allele to behave as "effectively" neutral. This theory takes into account both slightly advantageous and deleterious mutations. It constitutes a relationship between population size and the rate of molecular evolution in larger populations and genetic drift in smaller populations (Ohta 1992). However, if $-1 < 2Ns > 1$ then the fate of the mutant allele will be governed by genetic drift rather than selection forces (Lemey, Salemi, Vandamme 2009). It is possible that selection pressure can still play a role but genetic drift will dominate when both N and *s* are small. This is due to the neutral theory which states that *s* should be less than 1/2N for the allele to be effectively neutral, rather than ten times less as with the nearly neutral theory. The neutral theory states that evolutionary changes that occur are as a result of random drift of mutant alleles (Kimura 1983).

Hardy-Weinberg equilibrium (HWE)

The Hardy-Weinberg equilibrium (HWE) states that allele and genotypic frequencies within a population should remain constant from one generation to the next. In natural populations this is not possible; however, it provides a baseline for further analysis (Guo, Thompson 1992). When testing deviations from the HWE, observed and expected genotypic frequencies are implemented. The results obtained will indicate a relative excess or deficit of heterozygosity within the samples being tested. Generally, an excess of heterozygosity will lead towards stabilizing selection pressure acting on the population, or population contraction. In contrast, a deficiency of heterozygosity could indicate positive selection pressure acting on the population, or population expansion. It could also indicate the possibility of inbreeding (Stern 1943; Nachman 2006).

F-statistics:

Wright (1965) developed the F-statistic, which describes the relationship between alleles that were sampled randomly within the same sub-population in comparison to the entire population (Wright 1965). It provides insight into the genetic diversity between and among populations. Genetic differentiation has a close correlation to the rate of general evolutionary processes such as selection and genetic drift (Holsinger, Weir 2009). In large populations where migration is very common, genetic differentiation is very small. However, in smaller populations where there is minimal migration there is a higher degree of genetic differentiation. The F_{ST} statistic is a widely accepted measure of this genetic differentiation (Wright 1965; Holsinger, Weir 2009). The subscript '$_{ST}$ refers to a sub-population within the total population. This equates to the genetic diversity that arises due to the allele frequency differences in a population (Holsinger, Weir 2009).

The genetic structure of a population can also be described by additional parameters; F_{IT} and F_{IS}. F_{IT} represents the correlation of alleles within individuals relative to the entire population. F_{IS} represents the correlation of alleles within individuals relative to the sub-population in which they occur (Holsinger, Weir 2009).

Cockerham (1969) took a different approach to define these F-statistics by introducing new coefficients, namely *f*, θ, and F. By his definition it could be summarized that *f* and F_{IS} are related, θ and F_{ST} are related, and F and F_{IT} are related. For the purpose of this review we will focus on the correlation between θ and $F_{ST,}$ which can be defined as the genetic diversity that arises due to allele frequency differences among populations or within a population (Cockerham 1969; Holsinger, Weir 2009). To calculate the variance in allele frequency the following equation is applied:

$$\theta = \frac{\sigma_\pi^2}{\pi(1-\pi)} \qquad [1.1]$$

where σ_π^2 represents the variance in allele frequency among populations and $\pi(1-\pi)$ elucidates the variance of the allelic state of an allele chosen randomly from the entire population. Therefore, this calculation is characteristic of the fraction of genetic diversity that results due to differences in allele frequencies within populations (Cockerham 1969). From this calculation it is clear that θ is closely related to F_{ST}, and thus the same general conclusions can be formulated using both coefficients.

Gametic disequilibrium and recombination:

Gametic disequilibrium (GD) is the association between a polymorphism at two different loci and can arise for multiple reasons including hybridization, mutation, genetic drift and to an extent, natural selection. In the presence of recombination, disequilibrium will decay when forces acting to maintain the associations are not present (Halliburton 2003). Natural selection is capable of slowing down the decay of disequilibrium, and thus natural selection is often the explanation for tightly linked loci. This can be seen when the recombination rate between two loci is extremely high, therefore requiring intense natural selection to overcome this recombination (Halliburton 2003).

A common measure for GD is the index of association (I_A). This particular measure calculates the number of loci at which individuals are different from one another (Brown, Feldman, Nevo. 1980). A comparison is then made between these calculated 'distances' and those predicted if there was no GD present, and a determination of any discrepancy between the two is made (Smith *et al.* 1993; Haubold *et al.* 1998) . The derivation of I_A will not be shown but rather the equation implemented by the commonly used program Multilocus (V_D) (Agapow, Burt 2001).

$$V_D = \frac{\sum D^2 - \frac{\left(\sum D\right)^2}{n_p}}{n_p} \qquad [1.2]$$

D in the equation is the distance calculated between two individuals over all possible loci. This particular measure of GD is quite effective, but the number of included loci in the analysis restricts its application. This generally prevents the possibility of comparing results from different populations. Therefore, an improved measure has been derived to overcome this limitation, namely $\bar{r}_d$ which is a normalized measure of gametic disequilibrium in a population of arbitrary sample size.

As mentioned previously, recombination will result in the breakdown of associations between loci. Recombination results in the production of new combinations of alleles (Alberts 2002). Therefore, when determining the occurrence of LD between loci it would be beneficial to determine the level of recombination as well. SNAP workbench, an integrative program, provides the necessary tools for determining recombination events from molecular data (Price, Carbone 2005).

1.8 Hypothesis

A. Genotypic analysis of acaricide resistance genes in South African field populations of *Rhipicephalus microplus* illustrates the effects of positive selection pressure on mutant alleles within the population.
B. Gametic disequilibrium studies demonstrate that polymorphic loci between resistance genes are associated, and display little or no recombination throughout the gene.

1.9 Objectives

1. To sequence four genes proposed to be involved in acaricide resistance from *R. microplus* ticks across South Africa
2. To determine the genotypic status of acaricide resistance based on SNP analysis and allele frequencies.
3. To determine the linkage between polymorphic sites within and between resistance genes as well as their evolutionary histories within the genes by tracking recombination events that have occurred.
4. To develop a rapid diagnostic test for amitraz resistance based on the data that has been collected.

1.10 Outputs

The results within this dissertation will be published as follows:

1. *Reinecke, S., *Oberholster, T., Maritz-Olivier, C. "A survey of *Rhipicephalus microplus* and *Rhipicephalus decoloratus* ticks on cattle farms in South Africa and their current tick control procedures." (Manuscript to be submitted to Veterinary Parasitology) *Authors contributed equally to this work.
2. Reinecke, S., van der Merwe, N.A., Maritz-Olivier, C. "Identification and analysis of SNPs correlated to amitraz resistance in field populations of *Rhipicephalus microplus* in South Africa." (Manuscript to be submitted to Molecular Biology and Evolution)
3. Reinecke, S., van Wyk, R.D.J., Maritz-Olivier, C. "*Rhipicephalus microplus* field populations across South Africa present target site insensitivity to pyrethroids." (Manuscript to be submitted to the International Journal of Parasitology)
4. *Reinecke, S., *van Wyk, R.D.J., Maritz-Olivier, C. "Acaricide resistance in South Africa." (Manuscript to be submitted to Quest) *Authors contributed equally to this work.

Results have been presented at the following conference proceedings:

1. Reinecke, S., van Wyk, R.D.J., Maritz-Olivier, C. "Evaluation of acaricide resistance in field populations of *Rhipicephalus microplus* ticks." Oral presentations to the Gauteng Department of Agriculture and Rural Development (GDARD), University of Pretoria, South Africa during 2012-2013.
2. Reinecke, S., van der Merwe, N.A., Maritz-Olivier, C. "Allele frequencies and evolutionary genetics of acaricide resistance-associated SNPs in *Rhipicephalus microplus* ticks form South Africa". Invited poster presentation at the 24th International conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP) in Perth, Australia in August 2013.
3. Reinecke, S., van Wyk, R.D.J., van der Merwe, N.A., Maritz-Olivier, C. "Genotypic analysis of acaricide resistance in South Africa." Oral presentation presented on my behalf by Mr. RDJ van Wyk at the tick SU-ARC symposium, Leriba lodge, Pretoria, South Africa in September 2013.
4. Reinecke, S., van der Merwe, N.A., Maritz-Olivier, C. "Genetics of acaricide resistance in *Rhipicephalus microplus* ticks across South Africa." Invited oral presentation for the 11th Annual Genetics Postgraduate Symposium at the University of Pretoria, South Africa in November 2013.

Chapter 2: Genotypic analysis of acaricide resistance in South Africa.

2.1 Abstract

Rhipicephalus microplus is a largely invasive ectoparasite of great economic importance due to the negative effect it has on agricultural livestock on a global scale. The major method of tick control in South Africa, and globally, is the implementation of acaricides to prevent tick infestations. However, ticks have developed resistance to these acaricides and their effectiveness is drastically decreasing. It would be of great benefit for farmers to know if the ticks infesting their cattle are resistant to the particular acaricide they are using. If the ticks are resistant, then a different formulation of acaricide must be used, or perhaps even a combination.

Current detection methods for acaricide resistance involve bioassays which are both time-consuming and not particularly cost effective. Apart from that, these assays cannot detect the actual resistance mechanism. Therefore, an alternative approach is required for detecting acaricide resistance which circumvents these limitations. Sequence-based screening of resistance, with the potential of developing a rapid diagnostic test, is proposed in this study. This detection method will provide insight into whether target site insensitivity is involved in acaricide resistance at a fraction of the time required for LPTs. This screening method is well established for pyrethroid resistance, but has never been reported for amitraz resistance. Results obtained from this study suggest that more than half of the tick population studied is resistant to pyrethroids, and that 57.8% of the population contains the resistant and susceptible allele (heterozygous) giving rise to amitraz resistance.

2.2 Introduction

Rhipicephalus microplus is an ectoparasite that lives in hematophagy with its host. It is commonly known as the cattle tick or the southern cattle tick (CFSPH 2007). *Rhipicephalus microplus* is a hard tick belonging to the family Ixodidae constituting the largest group of ticks, and are primarily found in areas with a warmer climate having a higher prevalence in tropical and subtropical regions of the world (Estrada-Peña *et al.* 2006).

The pervasiveness of tick infestations is of great concern due to the large negative impact it has on agricultural livestock. Tick-borne diseases (babesiosis and anaplasmosis) transmitted by *R. microplus* are the main concern in this respect, as it decreases the quality of livestock (CFSPH 2007). There are several methods that are employed in an attempt to control tick infestations, acaricides being predominant. Dipping the cattle in acaricides is very effective, but with the increased development of resistance, acaricide efficacy is rapidly decreasing (Martins, Corrêa 1995). Other alternatives are available (Chapter 1, section 1.4) but due to several limitations they constitute small-scale solutions at present.

The development of acaricide resistance in *R. microplus* is due to one of three mechanisms namely penetration resistance, enhanced metabolic detoxification and target site insensitivity (Chapter 1, Figure 1.10), with the latter being the main subject in this study. Acaricide resistance achieved by a point mutation or frame shift within the relevant gene has been reported for multiple acaricide classes (Hernandez *et al.* 2000; Hernandez *et al.* 2002; Chen, He, Davey 2007; Morgan *et al.* 2009; Hope, Menzies, Kemp 2010). The most frequently reported mutation responsible for pyrethroid resistance in *R. microplus* is the one discovered by Morgan *et al.* (2009) in the voltage-gated sodium channel gene (Guerrero, Lovis, Martins 2012) (Chapter 1, Table 1.2). The mutation in the GABA-gated chloride channel gene giving rise to dieldrin resistance has not been reported often due to the banishment of dieldrin from the market for the use of tick control. The two SNPs published by Chen *et al.* (2007) were proposed to be responsible for amitraz resistance; however this remained elusive at the initiation of this study for the South African *R. microplus* tick population.

Once resistance develops within a population, it accumulates at a rapid pace due to the fact that pangamy takes place between the mating couples (Chevillon *et al.* 2007). Another contribution to the swift spread of resistance within a population is the capacity of the tick to generate several generations within a single season (Li *et al.* 2007; Budeli *et al.* 2009). Therefore it is vital to understand and diagnose acaricide resistance to potentially lessen the selection of resistant ticks within a given population.

The current study aims to resolve whether sequence-based resistance screening is a practical method for screening tick populations for acaricide resistance. It also aims at simplifying this screening procedure by developing rapid diagnostic screening methods. Results indicate an alarming level of resistance to pyrethroids on South African farms which is of great concern. Novel SNPs in the octopamine receptor gene were analyzed and their association with the resistant alleles determined by defining the level of gametic disequilibrium. Several significant allelic associations were discovered within the gene.

Furthermore, each acaricide resistant gene was analyzed for recombination events that may have occurred. Only one recombination event was evident in the octopamine receptor which corroborated with the gametic disequilibrium results. Based on the information obtained, a rapid diagnostic test was developed for detection of amitraz resistance. This study provides the first comprehensive analysis of genotypic resistance in South Africa, the first confirmation of amitraz resistance associated SNPs in field populations of *R. microplus*, as well as the first diagnostic approach for amitraz resistance. The information provided from this study will aid in the improvement of tick control procedures within South Africa, allowing farmers to select acaricides based on resistance-profiling of ticks on their farms.

2.3 Methods

2.3.1 Tick collection and database construction

Ticks were collected by Zoetis (Pty) Ltd. representatives from 108 different farms across South Africa. During planning of the sampling strategy, several factors were considered that could influence the outcome. *Rhipicephalus microplus* is an obligatory parasite, implying that there should be a correlation between the presence of the tick and its host distribution. For that reason, cattle density within South Africa was the major contributing factor in the tick sampling process thus a proportional number of sampling points were chosen relative to cattle densities (Figure 2.1 and Figure 2.2).

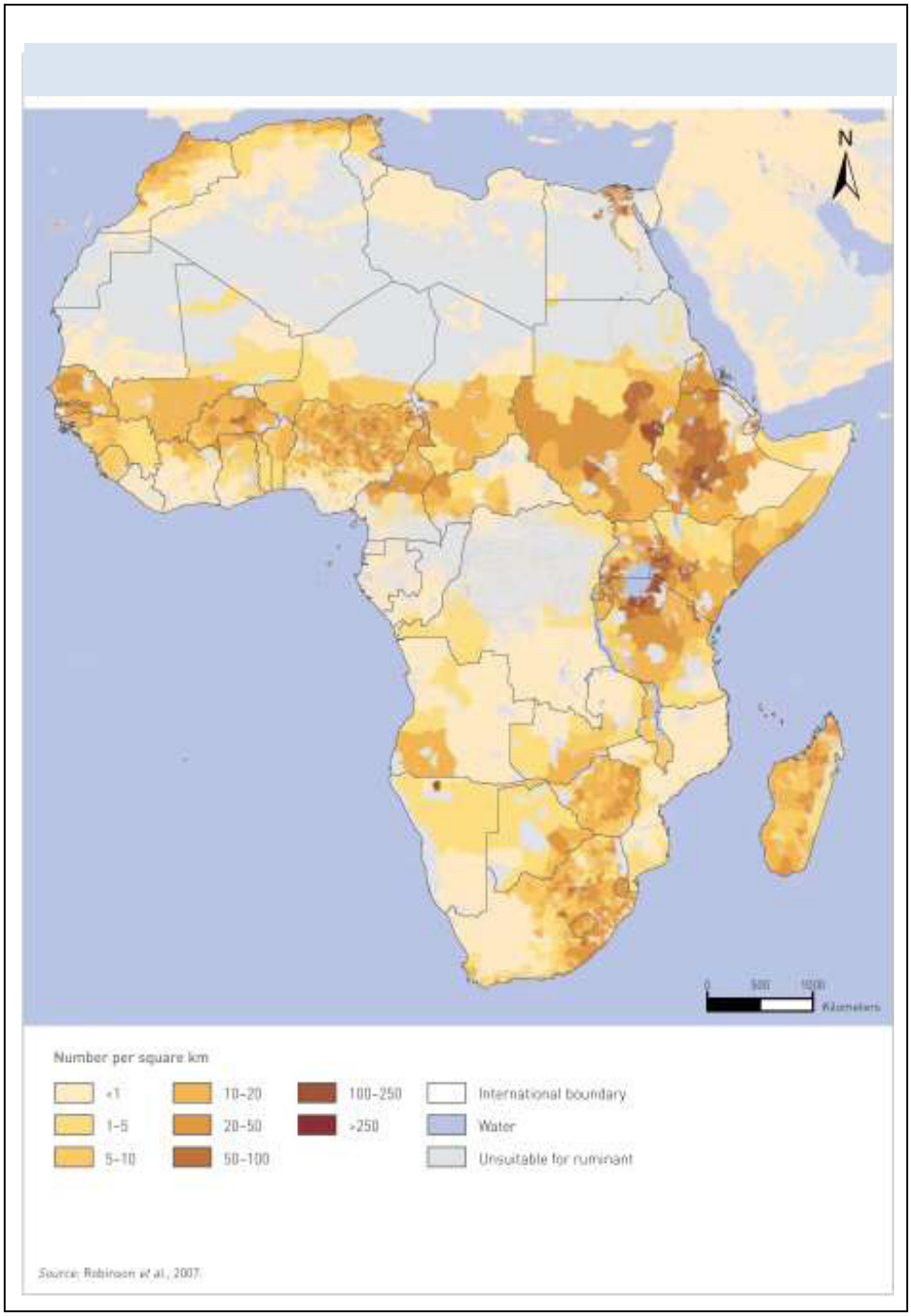


Figure 2:1 Cattle density in Africa represented as numbers of cattle per square kilometre (Wint, Robinson 2007). This map was used as a reference to sample ticks in various provinces of South Africa with medium to high cattle density (high = 20-100 cattle/km^2, medium = 1-20 cattle/km^2).

Secondly, the distance between sampling points was taken into account to provide a more realistic overview of the sampling area (Figure 2.2). In addition to this, Zoetis (Pty) Ltd. representatives also sampled from several communal dip tanks. These dip tanks are generally regulated by the government with scheduled dipping programs which are available to the surrounding farms. This results in the increased probability of cattle interaction

between different farms consequently leading to the further distribution of *R. microplus* ticks, in contrast to the more isolated commercial or private farms.

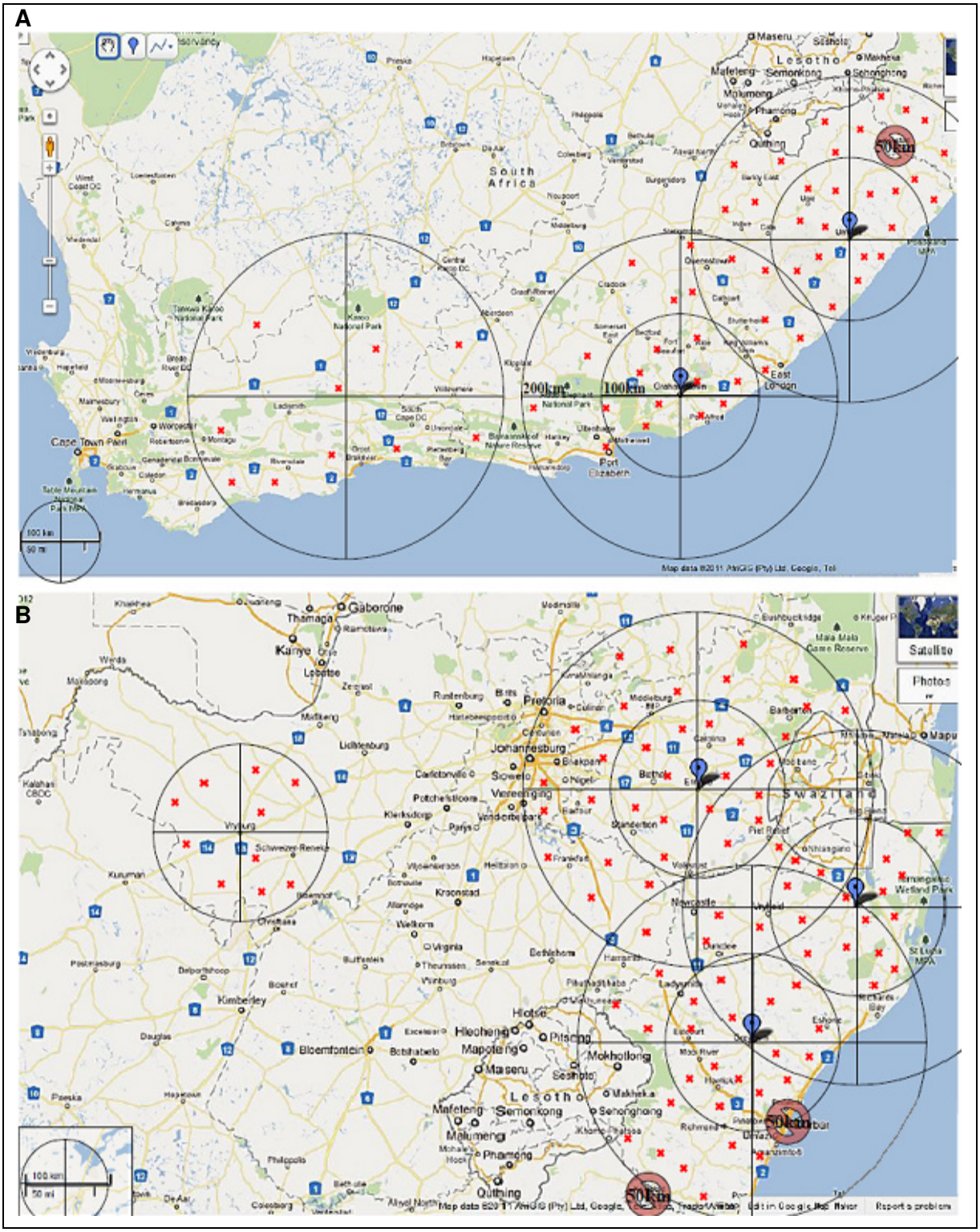


Figure 2:2 Tick sampling map based on cattle densities in South Africa. A) Tick sampling areas along the coast where (x) indicates the 85 sampling areas. B) Tick sampling areas inland of South Africa, with (x) indicating the 55 sampling areas.

In summation, ticks were collected from 108 different farms across South Africa, with approximately 50 ticks collected per farm. These farms showed a vast display in terms of the acaricides they implemented for tick control, the cattle breeds on the farm, as well as tick species on the farm. The detailed account from each individual farm can be found in Appendix B.

Upon collection the ticks were placed in 50 ml tubes containing 70% ethanol as a preservative and sent to the University of Pretoria for further analysis. Farmers were also asked to complete a questionnaire that contained some vital information for this study (Appendix A). Upon arrival of all the tick samples with accompanying questionnaires, the information was logged onto a database (Appendix B).

Apart from the adult tick collection carried out by Zoetis (Pty) Ltd., amitraz resistant larvae were also obtained from the Mnisi area in the Kruger National Park from Dr Rosalind Malan. Twelve different strains were provided of which three were classified as resistant based on their resistance factors (determined by conventional larval packet assays). These larvae were placed in 70% ethanol preservative and delivered to the University of Pretoria for further analysis.

2.3.2 Population determination

To effectively represent the final data without skewing the statistical significance, sampling areas needed to be represented as sub-populations. These sub-populations were determined by constructing grid-blocks across South Africa of 300 x 300 km (Fig. 2.3). Each sampling unit (farm) resides within a sampling frame (grid block) which directly coincides with the target population (South Africa) (Albert *et al.* 2010). The GPS coordinates for each farm determined which grid block the farm resided in.

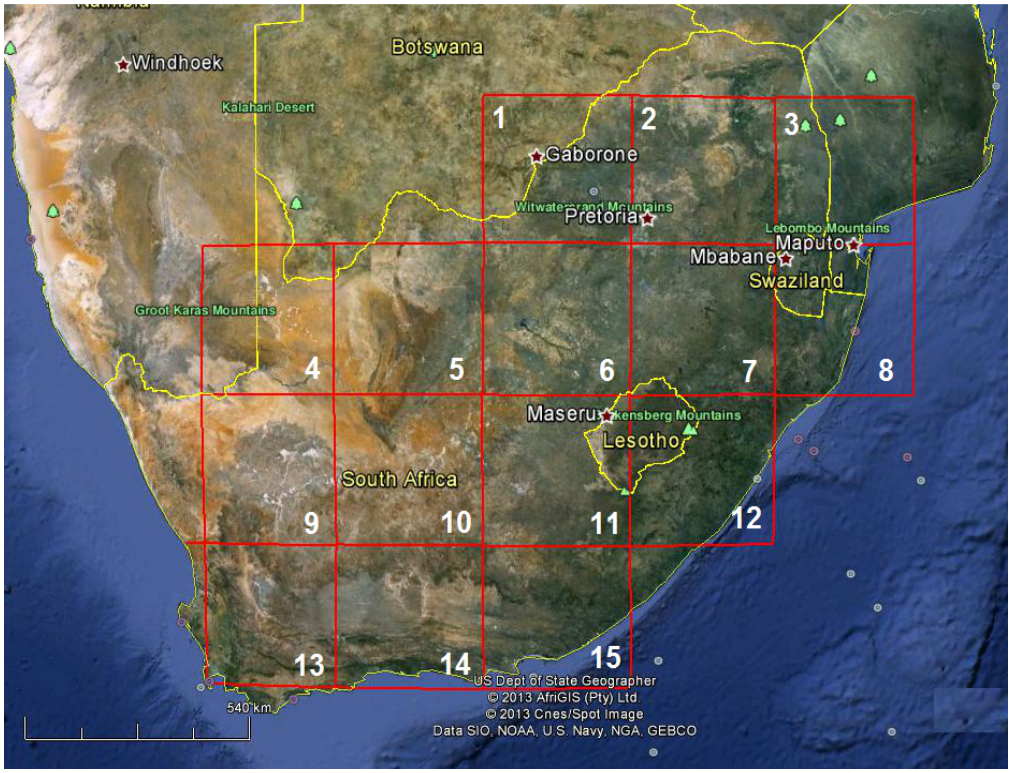


Figure 2:3 Grid blocks representing different populations of *R. microplus* across South Africa. Grid blocks of 300 x 300 km with no overlapping regions were designed. Each Grid block (labeled 1 to 15) represents a sub-population of the overall population. The map was constructed using Google Earth.

2.3.3 Tick identification

Ticks collected from the different regions of South Africa were identified to species level. Classification of collected ticks into their respective genera was performed according to previously described guidelines (Walker *et al.* 2003; Madder, Horak 2010). Distinguishing features were easily detectable for the three most common tick genera found in South Africa namely; *Amblyomma, Hyalomma* and *Rhipicephalus*. In contrast, further differentiation between *Rhipicephalus (Boophilus)* species was done using microscopy. To distinguish between *R. microplus* and *R. decoloratus* females, the hypostome dentition was examined along with the adanal spurs for the male ticks (Walker *et al.* 2003; Madder, Horak 2010). Due to the fact that morphology may result in erroneous identifications, molecular identification of ticks was also performed. This was based on restriction fragment length polymorphism (RFLP) analysis (Section 2.3.5) (Lempereur, Geysen, Madder 2010).

2.3.4 Genomic DNA extraction

A modified salt based extraction method published by Aljanabi, Martinez (1997) was used for genomic DNA isolation from whole adult ticks (predominantly female ticks). Whole ticks were homogenized in 200 µl lysis buffer (0.5 M EDTA, 0.5% (w/v) Sodium lauroyl sarcosinate) using a mortar and pestle. The homogenized suspensions were placed into sterile 2 ml microcentrifuge tubes and an additional 400 µl of DNA extraction solution (0.4 M NaCl, 60 mM Tris-HCl, 12 mM EDTA, 0.25% SDS, pH 8.0) was added to the samples along with 2 µl proteinase K (15 mg/ml). Samples were briefly vortexed and incubated overnight at 55°C. The following day samples were incubated for 20 min at 65°C to inactivate the proteinase K, after which 1 µl of RNase A (10 mg/ml) was added. Samples were briefly vortexed and further incubated at 37°C for 15 min. Protein precipitation was performed by adding 360 µl of 5 M NaCl, vortexing for 10 sec, and incubation on ice for 5 min, followed by centrifugation at 25 500*xg* for 20 min at room temperature. The supernatants were transferred into sterile 2 ml microcentrifuge tubes while avoiding the white flocculant that was present. An equal volume of isopropanol was added to the samples, briefly vortexed, followed by an incubation step of 1 hour at -20°C. Samples were then centrifuged for 20 min at 10 000*xg* and the supernatants discarded. DNA pellets were washed with 500 µl of 70% ethanol, centrifuged for 5 min at 10 000*xg*, and the supernatant discarded, followed by two more wash steps. The final DNA pellets were air dried in a flow cabinet for 20 min (or until ethanol was no longer visible at the bottom of the tube) and then re-suspended in 50 µl of 1 x TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.0).

Genomic DNA of whole adult ticks was visualized with 2% (w/v) agarose (Whitehead Scientific, SA) electrophoresis in TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide (1 µg/ml). Electrophoresis was performed in a Mini-Sub® Cell GT System (Bio-Rad Laboratories, SA) at a speed of 8 V/cm. DNA was visualized using the Gel Doc™ XR+ System (Biorad, USA).

Genomic DNA was extracted from individual larvae using a modification of the protocol by Hernandez et al. (2002). Briefly, individual larvae were crushed in a 1.5 ml microcentrifuge tube containing 25 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6). The suspension was then boiled for 5 min and centrifuged at 4000*xg* for 30 sec at room temperature. The supernatant was directly used for PCR.

2.3.5 PCR-RFLP

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) was performed according to the method published by Lempereur, Geysen, Madder (2010). The internal transcribed spacer (ITS2) gene was amplified using the published primers Boophits2F (5'-GCC GTC GAC TCG TTT TGA-3') and Boophits2R (5'-TCC GAA CAG TTG CGT GAT AAA-3'). Amplification was performed in a 25 µl reaction containing 200 ng of template, 10 pmol of each primer and Econo*Taq*® PLUS GREEN 2X Master Mix (Lucigen, USA) which contained; 1.25 U of Econo*Taq* DNA polymerase (0.1 units/µl), 200 µM dNTPs, 1.5 mM $MgCl_2$ and a proprietary PCR enhancer/stabilizer. The temperature cycles were as follows; 94°C for 4 min, 40 cycles of 92°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, with a final extension at 72°C for 8 min. Amplified products were analyzed as described in section 2.3.4.

The amplified ITS2 PCR products were subsequently treated with 6 U of *Msp*I in a 50 µl reaction containing 5 µl of 10 x NEB buffer (50 mM Potassium acetate, 20 mM Tris acetate, 10 mM Magnesium acetate and 100 µg/ml BSA, pH 7.9). All digested products were visualized with 3% (w/v) agarose. Samples were classified into their respective species based on the restriction pattern observed. Only samples which were confirmed by this technique to be *R. microplus* were used for amplification of resistance genes (Section 2.3.7).

2.3.6 Optimization of PCR reactions

The Taguchi method was implemented for the initial optimization of PCR conditions (Cobb, Clarkson 1994).This method enables the optimization of a large number of parameters using the least amount of reactions.

Table 2-1 Representation of a typical Taguchi reaction for optimization of the template: primer ratio.

Reaction Number	Template (ng/ul)	Primer (pmol)
1	50	5
2		10
3		15
4	100	5
5		10
6		15
7	150	5
8		10
9		15

To establish at which annealing temperature the primer would anneal best to the template, gradient PCR was performed, which simultaneously explores several different annealing temperatures within a single reaction. The melting temperature (T_m) for the forward and reverse primer for each gene was determined based on the product information received from the primer synthesis. Temperature intervals were established on the gradient PCR machine to incorporate the calculated T_m, T_a (annealing temperature) as well as temperatures above and below these values. These optimizations were essential for the eventual efficient amplifications of the target genes (Section 2.3.7).

2.3.7 PCR amplification of resistance genes

Octopamine receptor gene:

Published primers were used for PCR amplification of a 417 bp fragment of the octopamine receptor; OAR-F171 (5'-GGT TCA CCC AAC CTC ATC TCT GAA-3') and OAR-R587 (5'-GCA GAT GAC CAG CAC GTT ACC G-3') (Chen, He, Davey 2007). Amplification was performed in a 25 µl reaction containing 200 ng of template DNA, 10 pmol of each primer and Econo*Taq*® PLUS GREEN 2X Master Mix (Lucigen, USA) which contained the following: 1.25 U of Econo*Taq* DNA polymerase (0.1 units/µl), 200 µM dNTPs, 1.5 mM $MgCl_2$ and a proprietary PCR enhancer/stabilizer. Temperature cycles were as follows; 94°C for 4 min, 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min with a final extension at 72°C for 7 min. This method worked sufficiently for a few samples; however, a new forward primer had to be designed due to tick strain differences. The new forward

primer, OAR-F172 (5'-AGC ATT CTG CGG TTT TCT AC-3') was designed based on sequence data from the few samples that amplified successfully. This forward primer was used for the majority of tick samples with the same reaction set-up and cycling parameters as above. When larval DNA was amplified, 2-5 µl of the crude homogenate was added to the 25 µl reaction, and the same cycling parameters were performed.

Voltage-gated sodium channel gene:

Published primers were used to amplify a 167 bp fragment of the sodium channel (domain II segment 4-5 region); BmNaF5 (5'-TAC GTG TGT TCA AGC TAG C-3') and BmNaR5 (5'-ACT TTC TTC GTA GTT CTT GC-3') (Jonsson *et al.* 2010). Amplification was carried out in a 25 µl reaction containing 200 ng of the template DNA, 10 pmol of each primer and KAPA2G™ Robust HotStart ReadyMix which contained 200 µM dNTPs, 2.0 mM $MgCl_2$ and the HotStart DNA polymerase in a propriety buffer,. The cycling parameters were as follows; 94°C for 4 min, followed by 40 cycles of 94°C for 30 sec, 51°C for 30 sec and 72°C for 1 min with a final extension at 72°C for 7 min.

Gamma-aminobutyric acid (GABA) gated chloride channel gene:

Published primers were used to amplify a 284 bp fragment from the GABA-gated channel; GABA 12 (5'-AAC TAC TCC CGC CTG GTA TGT G-3') and GABA 13 (5'-TCC AGG AGC GCG GTA AAC AC-3') (Hope, Menzies, Kemp 2010). Amplification was performed in a 25 µl reaction as described with the octopamine receptor gene. Temperature cycles were as follows; 94°C for 4 min, followed by 37 cycles of 94°C for 30 sec, 58°C for 30 sec and 68°C for 1 min with a final extension at 68°C for 5 min.

Carboxylesterase gene:

Published primers were used to amplify a 372 bp fragment of the carboxylesterase gene; GS138B (5'-AGC ATC GAC CTC TCG TCC AAC-3') and GS139R, (5'-GTC GGC ATA CTT GTC TTC GAT G-3') (Hernandez *et al.* 2002). The amplification reaction was set up as described for the octopamine receptor gene with the subsequent cycling parameters; 95°C for 3 min, followed by 40 cycles of 94°C for 30 sec, 54°C for 30 sec and 68°C for 1 min with a final extension at 68°C for 5 min.

All PCR reactions were performed in a GeneAmp 9700 thermocycler (PE Applied Biosystems, USA). PCR products were analyzed with 2% (w/v) agarose (Section 2.3.4). All amplified PCR products were purified and sent for sequencing (Section 2.3.8).

2.3.8 PCR amplicon purification and gene sequencing

Amplified gene products of the correct sizes were purified in a 96-well plate using the PureLink® *Pro* 96 PCR Purification Kit (Invitrogen™, Life Technologies, USA). Purified PCR products were analyzed with DNA gel electrophoresis to determine if the concentration was sufficient for DNA sequencing. Concentrations were determined by comparing the known concentrations of the molecular standard (1 kb DNA marker, Fermentas, Canada) with that of the unknown sample. All gene products were sequenced by Macrogen Inc. (Netherlands) in a 96-well plate according to the standard dye terminator sequencing strategy. Both forward and reverse primers were used for sequencing. Plate preparation required 5 µl of template (~ 50 ng/µl) along with 5 µl of primer (5 pmol/µl) per well.

2.3.9 Sequence analysis

Sequences received from Macrogen were analyzed using BioEdit sequence alignment editor version 7.2.0 (Hall 2007). Samples with any inconsistencies were re-sequenced. Multiple alignments were performed using the online MAFFT program which is available at (http://mafft.cbrc.jp/alignment/software/) (Katoh, Standley 2013). Sequences were aligned with published NCBI sequences to determine if any SNPs were present in the gene. When novel SNPs were found, it was determined if they were synonymous or non-synonymous mutations. This was done by comparing the amino acid sequences using BioEdit.

2.3.10 Gametic disequilibrium and ancestral recombination

Gametic disequilibrium studies were performed to determine associations (linkage) between alleles within the same gene (intragenic), as well as linkage between genes (intergenic). Analysis was carried out using the Multilocus 1.2b1 beta version program (Agapow, Burt 2001). All sequences were converted into a binary format. If the base pair was the same as that of the NCBI sequence (Accession: AJ010743.1) it was designated as 0/0, if a mutation occurred at that position resulting in a nucleotide substitution it was designated as 1/1, heterozygotes containing both alleles at a particular locus were designated 0/1. For the analysis 100 000 randomizations were performed. If the observed dataset displayed increased gametic disequilibrium compared to the randomized datasets, it was assumed that there is association between the linkage groups being analyzed. This was further supported by P-values, therefore, if $P<0.05$ then alleles are non-randomly associated and there is gametic disequilibrium. Functions carried out using Multilocus included analysis of genotypic diversity versus the number of loci, linkage disequilibrium and population differentiation analysis.

Genotypic diversity is calculated as follows:

$$\frac{n}{n-1}\left(1-\sum_i p_i^2\right) \qquad [2.1]$$

The *Pi* signifies the frequency of the *i* genotype in equation [2.1] while *n* is the number of individuals that have been sampled (Agapow, Burt 2001).

A statistical measure $\bar{r}_d$ is calculated by the Multilocus program to represent the linkage between loci of interest [equation 2.2]

$$\bar{r}_d = \frac{\sum\sum cov_{j,k}}{\sum\sum\sqrt{var_j \cdot var_k}} \qquad [2.2]$$

The program calculates the covariance between the distance of two loci (*j* and *k*), with the double summation being the distance over all possible pairs of loci. Therefore, if the observed data suggests that there is no association between the loci being analyzed, then the covariances will equal zero.

Population differentiation analysis is calculated by the Multilocus program as follows;

$$\theta = \frac{\sum Q_2 - \sum Q_3}{\sum(1-Q_3)} \qquad [2.3]$$

where θ is Weir's equivalent to Wrights F_{st} statistics (Chapter 1, section 1.7), Q_2 is the likelihood that two alleles from the same population are identical, and Q_3 the likelihood that two alleles from different populations are the same.

Ancestral recombination graphs were constructed using the SNAP workbench (Price, Carbone 2005) to determine the evolutionary histories within resistance genes. Sequence alignments were converted into haplotypes by excluding indels and infinite site violations. Once haplotype identities were established, recombination was determined by using the Beagle and Kwarg function. The haplotype map was converted into FASTA format to

perform the analysis. The branch and bound beagle algorithm was then implemented to determine the minimal number of recombination events within the gene (Lyngsø, Song, Hein 2005). This would allow inference of the ancestral history within the gene. The program subsequently converts this data into an ancestral recombination graph.

2.3.11 Rapid diagnostic test for amitraz resistance

The rapid diagnostic test for amitraz resistance was evaluated in the form of a RFLP analysis. Susceptible and resistant *R. decoloratus* larvae (confirmed through larval packet tests) were obtained from the University of the Free State (Ms. Ellie van Dalen) for the purpose of a blind study. Larvae were labeled from one through 14, with the status of their resistance unknown until after the experiment. Larvae were pooled together and their genomic DNA isolated (Section 2.3.4) and the octopamine receptor gene amplified (Section 2.3.7). Amplified fragments of the octopamine receptor were subsequently treated with 4 U of *Eci*I restriction enzyme in a 50 µl reaction containing 5 µl of the CutSmart™ buffer (50 mM Potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium acetate and 100 µg/ml BSA, pH 7.9). Digested samples were visualized with 3% (w/v) agarose (Section 2.3.4). Depending on the particular pattern that was observed after digestion, samples were classified as susceptible or resistant to amitraz treatment. These results were then compared to the known resistance status to confirm the accuracy of the diagnostic test.

2.4 Results and discussion

2.4.1 Farmer questionnaire results

Acaricides implemented by farmers for tick control:

Farmers were asked which acaricides they used for tick control, how often they applied these acaricides, as well as how they applied it. This provides valuable information in terms of which genes are currently being targeted by selection pressure due to exposure to these acaricides. The most common commercial acaricides used by South African farmers is shown in Appendix C1 and further elaborated in Table 2.2. It is clear that the commercial product Pouracide®NF is the most common acaricidal treatment used by South African farmers, followed by Amipor and Triatix. Amitraz is also very popular for tick control among farmers (9%); however, stating that they just use amitraz seemed ambiguous as it could have been the active ingredient in the treatment they were using rather than the commercial name of a product. This was the case for several treatments constituting the pyrethroids;

cypermethrin, deltamethrin, alphamethrin, and flumethrin. Nonetheless, these were included in the data to prevent skewing the current distribution of acaricidal usage. It was also taken into account the fact that some farmers would alternate chemical acaricide applications several times throughout the year. These commercial acaricides are supplied by various companies across South Africa, with each farmer having their own specific preference. Therefore, an impartial view needs to be considered when looking at the frequency with which these acaricides are being used. Even though Pouracide®NF is the most common acaricide being used among farmers, it does not indicate that it is the most effective treatment. The frequency with which they are applied may depend on several factors including the accessibility of the particular acaricides to the farmers, cost, marketing strategy implemented by companies, as well as the level of acaricide resistance on the farms.

There are several methods in which farmers can apply these commercial acaricides namely by means of plunge, spray, pour-on or injection. Appendix C2 shows the relation between the different forms of acaricide applications on farms in South Africa. It is clear that the pour on method is most common among farmers. Even though pour-on acaricides are quite costly, they are generally easier to use, because they can simply be poured along the back of the cattle and it will dispense evenly over the hide due to its enhanced formulations. This relation between acaricidal applications is, however, dependent on the farmer's preference and the method of application offered by their particular acaricide treatment.

When considering the method of application and the relative cost of each application, the frequency of acaricide applications should also be considered. Appendix C3 illustrates the frequency with which famers are treating their cattle with these commercial acaricides. It appears as though two acaricidal treatments in the past two years is most common, followed by one acaricidal treatment in two years. Whether one or two acaricide applications in a two year period is sufficient for tick control will depend on the level of tick infestation, the development of resistance and the possible spread of these ticks into and around these areas. From the data it seems as though farmers are either performing two or less acaricidal treatments, or more than five every two years. Possible reasons for farmers having to apply more than five treatments in a two year period could be related to the level of acaricide resistance within that area.

Classes of acaricidal dips:

Commercial acaricides consist of active ingredients that target certain genes, and can be grouped into different classes based on these active ingredients. Table 2.2 summarizes the

commercial acaricide being used, its active ingredient, the class of acaricides it falls under, its primary site of action, as well as its method of application.

Table 2-2 A summary of the commercial acaricides used by South African farmers. This includes their active ingredients, method of application, class of acaricide they fall into, as well as their primary site of action. Classes of acaricides are represented as letters (A-F). A is organophosphates and carbamates, B is pyrethroids/pyrethrins, C is formamidines, D is chitin synthesis inhibitors, E is macrocyclic lactones and F is combinational treatments (more than one class of acaricide). The method of acaricidal application is designated as numbers (1-4) where 1 is plunge, 2 is spray, 3 is pour-on and 4 is injection.

Name of Dip	Active Ingredient	Application	Class	Description	Primary Site of Action
Amipor	Amitraz 1% m/v			Formamidine	Octopamine receptor
	Cypermethrin 1% m/v	3	F	Pyrethroids	Para-sodium channel
	Piperonyl butoxide 5% m/v			Pesticide Synergist	Pesticide Synergist
Pouracide-NF	Alphamethrin 0.5% m/m			Pyrethroids	Para-sodium channel
	tetrachlorvinphos 2% m/m	3	F	Organophosphates and carbamates	Acetylcholine esterase inhibitor
	Cypermethrin 1% m/m			Pyrethroids	Para-sodium channel
	piperonyl butoxide 7.5% m/m			Pesticide Synergist	Pesticide Synergist
Zero Par	Alphamethrin 3% m/v	2	F	Pyrethroids	Para-sodium channel
	Chlorfenvinphos 30% m/v			Organophosphates and carbamates	Acetylcholine esterase inhibitor
Amitraz	Amitraz 12.5% m/v	1,2,3	C	Formamidine	Octopamine receptor
Ektoban	Cypermethrin 25g/L	1,2	F	Pyrethroids	Para-sodium channel
	Cymiazol 175g			Benzenamine	
Triatix 125/250	Amitraz 12.5 - 25 % m/v	2	C	Formamidine	Octopamine receptor
Triatix Cattle pour-on	Amitraz 2% m/v	3	C	Formamidine	Octopamine receptor
Paracide	Alphamethrin 7% m/v	2	B	Pyrethroids	Para-sodium channel
Cyperdip 20%	Cypermethrin 20% m/v	1	B	Pyrethroids	Para-sodium channel
Deltamethrin	Deltamethrin	2	B	Pyrethroids	Para-sodium channel
Taktic Cattle Spray	Amitraz 12.5% m/v	2	C	Formamidine	Octopamine receptor
Taktic Pour-on	Amitraz 2% m/v	3	C	Formamidine	Octopamine receptor
Amidip Max	Amitraz 25% m/v	1,2	C	Formamidine	Octopamine receptor
Ecobash	Cypermethrin 2.5% m/v	1	F	Pyrethroids	Para-sodium channel
	Cymiazol 17.5% m/v			Benzenamine	N/A
Drastic Deadline Extreme	Flumethrin 1% m/v	3	F	Pyrethroids	Para-sodium channel
	Fluazuron 2.5% m/v			Mite growth regulator	Mite growth regulator
TickGuard	Vaccine				N/A
Bodygard	Flumethrin 1% m/v	3	B	Pyrethroids	Para-sodium channel
	Piperonyl butoxide 5% m/v			Pesticide Synergist	Pesticide Synergist
Cypertraz Pour-on	Cypermethrin 15.0 g / ℓ	3	F	Pyrethroids	Para-sodium channel
	Amitraz 17.5 g / ℓ			Formamidine	Octopamine receptor
Alphamethrin	Alphamethrin 10%	2,3	B	Pyrethroids	Para-sodium channel
Amitrac	Amitraz	2	C	Formamidine	Octopamine receptor
Amigard	Amitraz 12.5% m/v	2	C	Formamidine	Octopamine receptor
Pro-Dip Cyp 20%	Cypermethrin 20%	2	B	Pyrethroids	Para-sodium channel

	m/v				
Dectomax	Doramectin 1 % (w/v)	3	E	Macrocyclic Lactone	Chloride channel activator
Delete All	Amitraz 2.0% m/v			Formamidine	Octopamine receptor
	Deltamethrin 0.5% m/v	3	F	Pyrethroids	Para-sodium channel
	Piperonyl Butoxide 2.0% m/v			Pesticide Synergist	Pesticide Synergist
Cypermethrin	Cypermethrin 15% m/v	2	B	Pyrethroids	Para-sodium channel
Decaspot	Deltamethrin 0.5% m/v	3	B	Pyrethroids	Para-sodium channel
	Piperonyl Butoxide 2.5% m/v			Pesticide Synergist	Pesticide Synergist
Sovereign	Ivermectin 15g/l	3	E	Macrocyclic Lactone	Chloride channel activator
Ivotan	Ivermectin 1% m/v	3	E	Macrocyclic lactone	Chloride channel activator
Acatak	Fluazuron 15g/l	3	E	Mite growth regulator	Mite growth regulator
	Ivermectin 5g/l			Macrocyclic lactone	Chloride channel activator
Ectoshield	Cypermethrin 15 g /l	3	F	Pyrethroids	Para-sodium channel
	Amitraz 17.5 g /l			Formamidine	Octopamine receptor
Supatraz 25%	Amitraz 25% m/v	1	C	Formamidine	Octopamine receptor
Supatraz Cattle Pour-on	Amitraz 2% m/v	3	C	Formamidine	Octopamine receptor
Bantik Cattle Dip	Cymiazol 17.5% m/v	1,2	F	Benzenamine	
	Cypermethrin 2.5% m/v			Pyrethroids	Para-sodium channel
Blitzdip Pour-on for Cattle	Cypermethrin 1% m/v	3	B	Pyrethroids	Para-sodium channel
Supona 30 Cattle Dip	Chlorfenvinphos 30% m/v	2	A	Organophosphates and carbamates	Acetylcholine esterase inhibitor
Ivomec	Ivermectin 1% m/v	4	E	Macrocyclic Lactone	Chloride channel activator
Amitraz/Pyrethroid	Amitraz	3	F	Formamidine	Octopamine receptor
	Cypermethrin			Pyrethroids	Para-sodium channel
Virbamec L	Ivermectin 1% m/v	4	F	Macrocyclic Lactone	Chloride channel activator
	clorsulon 10% m/v			?	?
Ivermax Injectable Solution	Ivermectin 1% m/v	4	E	Macrocyclic Lactone	Chloride channel activator
Flumethrin	Flumethrin	1,2,3	B	Pyrethroids	Para-sodium channel
Ivermectin	Ivermectin 1% m/v	3	E	Macrocyclic lactone	Glutamate-gated chloride channels
Supadip	Chlorfenviphos 30% m/v	1,2	A	Organophosphates and carbamates	Acetylcholine esterase inhibitor
Redline	Flumethrin 1% m/v	2	B	Pyrethroids	Para-sodium channel
Maxipour	Flumethrin 1% m/v	3	B	Pyrethroids	Para-sodium channel
Decatix 3	Deltamethrin 2.5% m/v	1,2	B	Pyrethroids	Para-sodium channel

Figure 2.4 shows which acaricide classes are used most often in South Africa. In terms of singular acaricidal treatments, formamidines (C) seem to be most commonly used, followed by pyrethroids (B). Combination treatments (F), however, seem to be used most frequently (43.4%). It was also noted that the majority of the combination treatments were formamidines and pyrethroids. As previously mentioned, the hypothesized primary site of action for formamidines is the octopamine receptor and the confirmed site of action of

pyrethroids is the voltage-gated sodium channel. Based on these results it can be speculated that the octopamine receptor and voltage-gated sodium channel are under high selection pressure. These results confirm the importance of analyzing these target sites for SNPs. No chitin synthesis inhibitors were used as singular treatments by farmers but were used in combinational treatments instead.

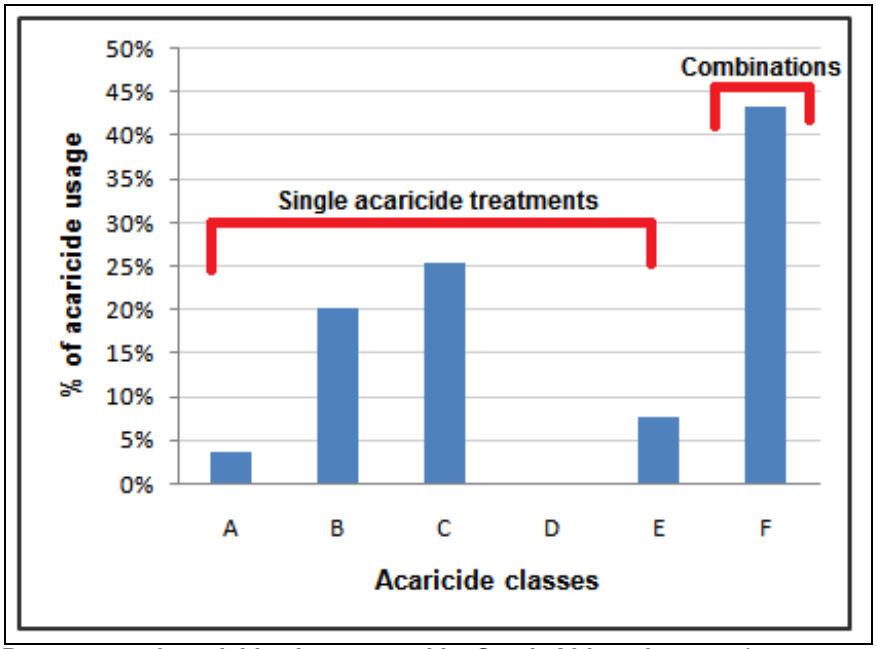


Figure 2:4 Percentage of acaricide classes used by South African farmers. Letters correspond to (A) Organophosphates/Carbamates, (B) Pyrethroids/Pyrethrins, (C) Formamidines, (D) Chitin synthesis inhibitors, (E) Macrocyclic lactones and (F) Combinations of acaricide classes.

2.4.2 Genomic DNA extraction and PCR-RFLP

Genomic DNA of whole adult ticks was visualized using DNA gel electrophoresis (Figure 2.5). Large smears were seen with almost all extractions, especially towards the bottom of the gel. Initially it was thought to be RNA contamination; however, after modifying the extraction protocol to contain RNAse, these smears were still evident. It is therefore hypothesized that tick DNA starts degrading after detachment from its host and continues to degrade for some time even in the presence of 70% ethanol. This would explain why any modification to the extraction protocol did not improve the resolution or integrity of the genomic DNA.

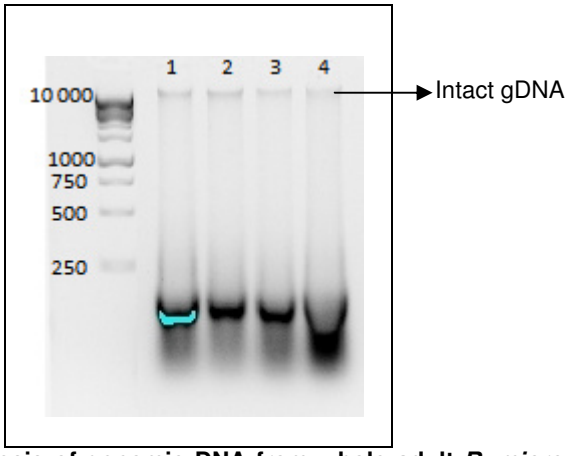


Figure 2:5 DNA gel electrophoresis of genomic DNA from whole adult *R. microplus* female ticks. DNA degradation is evident from the large smears towards the bottom of the gel, however, intact genomic DNA can be seen at the 10 000 bp mark at the top of the gel. Lanes 1 through 4 represent samples 1 to 4 from farm 67 found in the database (Appendix B).

Genomic DNA concentrations were determined using the Nanodrop spectrophotometer (Thermo Scientific, USA). DNA concentrations were measured at 260 nm and purities analyzed by the A260/280 ratio. The optimal value of A260/280 for genomic DNA is between 1.8 and 2. The concentrations for DNA samples and purities were recorded in a laboratory catalogue. The naming of samples was as follows; if we consider sample 67:1MF for example, 67 is the farm's number correlating to the database, 1 is the number of the tick where genomic DNA has been extracted, and MF indicates that the tick is a *R. microplus* female (Appendix D). Samples thought to be *R. microplus* based on microscopy were subjected to PCR-RFLP analysis. Amplified ITS2 PCR products were visualized using gel electrophoresis (Figure 2.6).

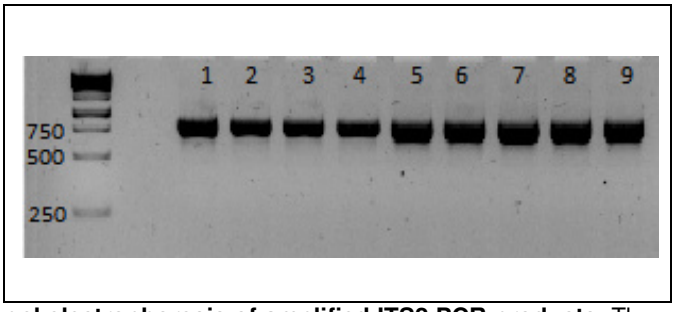


Figure 2:6 Agarose gel electrophoresis of amplified ITS2 PCR products. The amplified PCR products are expected to be around 750-800 bp in size (Lempereur, Geysen, Madder 2010). Lanes 1 through 9 represent tick samples 2 to 5 from farm 66 and 1 to 5 from farm 67, respectively.

PCR products of the correct size were subsequently digested with *Msp*I to generate a unique restriction profile for each *Rhipicephalus* (*Boophilus*) species. Figure 2.7 illustrates the profile obtained for differentiation between *R. microplus* and *R. decoloratus*. These results were

also recorded in the laboratory catalog (Appendix D), where MF/MM indicated that samples were *R. microplus* females or males, and DF/DM indicated that they were *R. decoloratus* females or males. When digested with *MspI* enzyme, *R. microplus* generates the following band sizes; 400, 250, 100 and 70 bps. In contrast, *R. decoloratus* produces band sizes of approximately 300, 150, 100, 80 and 70 bps (Figure 2.7A).

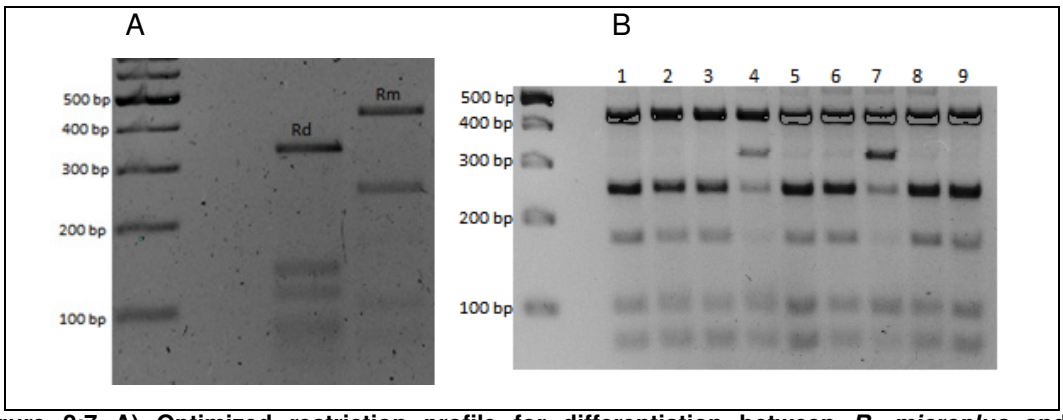


Figure 2:7 A) Optimized restriction profile for differentiation between *R. microplus* and *R. decoloratus* and B) Restriction profiles obtained for field samples. A) Abbreviations Rd and Rm represent *R. decoloratus* and *R. microplus*, respectively. B) Lanes 1 through 9 represents the same samples in Figure 2.6.

RFLP analysis was optimized on colony strains of *R. microplus* and *R. decoloratus* ticks (Clinvet (Pty) Ltd. Figure 2.7A). When applying it to field strains collected from the farms, the profile seen in Figure 2.7B was obtained. All the samples in Figure 2.7B displayed the band sizes consistent with those of *R. microplus*, however, in lanes 4 and 7 an additional band (300 bp) was discovered which corresponds to that of *R. decoloratus*. Initially it was thought to have been cross-contamination, but this theory was rejected after repeating the experiment multiple times, and encountering this phenomenon through-out the sample range from different farms. It was then hypothesized that perhaps these few samples could be hybrids, a combination between *R. microplus* and *R. decoloratus*. Whether this is the true still needs to be determined and studies to fully elucidate this mystery are currently underway. Samples which generated this unique profile were recorded as 'H' in the laboratory catalogue.

2.4.3 Population density for each grid block

Upon completion of PCR-RFLP analysis, *R. microplus* ticks were placed into a sub-population group according to the grid block structure (Figure 2.3). As suspected, certain areas contained a higher density of *R. microplus* ticks in comparison to others. The *R.*

microplus samples within each grid block are shown in Appendix E. To sufficiently gain enough information from each grid block, an upper limit of 100 alleles (50 ticks) and a lower limit of 10 alleles (5 ticks) were sampled from each block for each gene. Some grid blocks did not have enough *R. microplus* ticks, or only contained *R. decoloratus* ticks and were therefore not validated in this study.

The initial grid blocks that seemed to contain enough *R. microplus* ticks for analysis were 2, 3, 7, 8, 11, 12, 13 and 15. However, after many attempts at trying to gain enough sequence information, blocks 3, 13 and 15 had to be excluded from further analysis as 10 alleles could not be sampled from these areas. However, their sequence information was still included when considering the entire South African population. Additional sampling is currently underway.

2.4.4 PCR amplification of resistance genes

Octopamine receptor gene:

Amplification of the 417 bp fragment of the octopamine receptor using the published forward and reverse primers was visualized using gel electrophoresis (Figure 2.8).

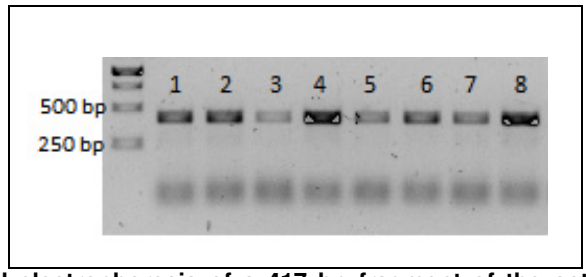


Figure 2:8 Agarose gel electrophoresis of a 417 bp fragment of the octopamine receptor for *R. microplus* field strains. Lanes 1 through 8 represent the following samples; 20.2MF, 20.3MF, 62.3MF, 65.1MF, 65.2MF, 65.3MF, 66.1MF and 66.2MF, respectively.

Amplification of the 417 bp fragment of the octopamine receptor resulted in severe primer dimer formation, regardless of attempts to optimize the reaction conditions. In Figure 2.8 it is also clear that the efficiency of the PCR amplification is largely template dependant (*i.e.* the same conditions won't work as efficiently for all samples being tested).

The published forward and reverse primers seemed to be effective for the minority of the samples, with the remaining samples being difficult to amplify with these primers. After sequencing some samples, it was found that a SNP occurred in the forward primer binding region. This was suspected to be due to strain differences between South African *R.*

microplus, and the Mexican strain that was used to design the forward primer (Chen, He, Davey 2007). Therefore, a new forward primer was designed to circumvent this problem using sequence data from samples which did amplify with the previous forward primer. The new forward primer annealed slightly downstream from the original forward primer, thus amplifying a 394 bp fragment (Figure 2.9).

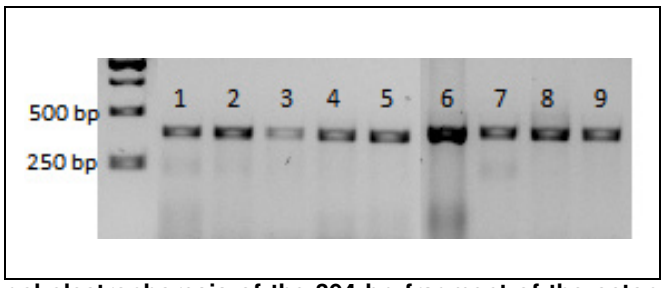


Figure 2:9 Agarose gel electrophoresis of the 394 bp fragment of the octopamine receptor for *R. microplus* field strains. Lanes 1 through 9 represent the following samples; 9.6MF, 9.8MF, 12.1MF, 12.4MF, 17.1MF, 17.3MF, 17.7MF, 42.2MF and 42.4MF, respectively.

The remaining samples were amplified with the new forward primer. The level of primer dimer formation was significantly diminished when using this primer. Regardless of all the optimization steps that were pursued as well as the implementation of the new forward primer, there were still numerous samples for which amplicons could not be obtained. This substantiated the complexity of the template being amplified, as well as corroborated the intricacy involved when amplifying from degraded genomic DNA template.

Additionally, the 394 bp fragment of the octopamine receptor gene was amplified from both susceptible and resistant larvae obtained from Mnisi in the Kruger National Park (Figure 2.10).

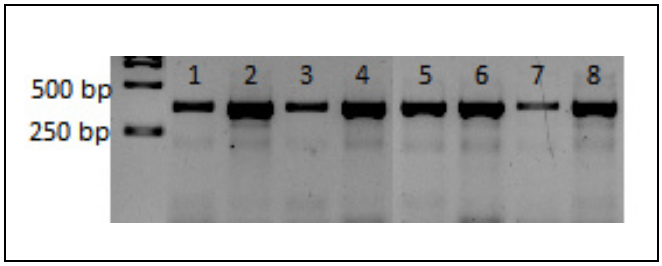


Figure 2:10 Agarose gel electrophoresis of the 394 bp fragment of amitraz resistant and susceptible larvae of *R. microplus* field strains. Lanes 1 through 4 represent resistant larvae, while lanes 5 through 8 are those of susceptible larvae.

Amplification from larvae DNA was challenging, mainly due to the low genomic DNA concentrations obtained from individual larvae. Pooling the larvae in this instance to obtain

higher DNA concentrations was not an option as individual genotypes were essential for confirming SNPs.

Voltage-gated sodium channel gene:

The amplification of the 167 bp fragment of the sodium channel was immensely challenging and only a robust *Taq* enzyme under stringent cycling conditions lead to the amplification of the fragment (Figure 2.11).

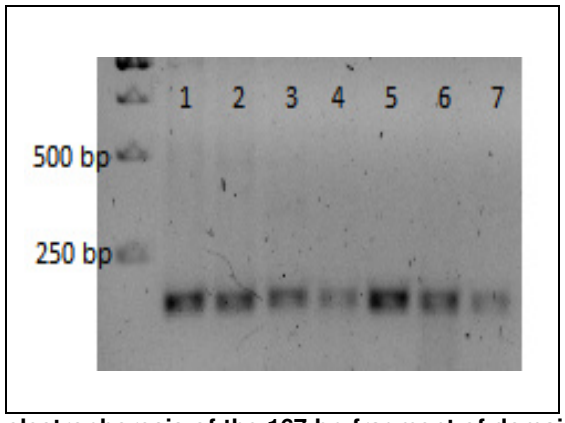


Figure 2:11 Agarose gel electrophoresis of the 167 bp fragment of domain II segment 4-5 region of the voltage-gated sodium channel for *R. microplus* field strains. Lanes 1 through 7 represent the following samples; 45.1MM, 45.4MM, 50.4MF, 50.5MF, 50.6MF, 66.1MF and 66.3MF, respectively.

To achieve amplification (Figure 2.11), the KAPA2G™ Robust *Taq* polymerase was used. According to the suppliers of the polymerase, it was specifically designed for increased processivity as well as enhanced tolerance to PCR inhibitors. This polymerase has shown robust amplification of a wide-range of amplicons with a minimal error rate of 1 error per 1.7 x 10^5 of incorporated nucleotides. Amplification of the sodium channel gene fragment was not consistent with every sample, thus displaying the same template complexity as was seen with the octopamine receptor gene.

GABA-gated chloride channel gene:

Amplification of the 284 bp fragment of the gamma-aminobutyric acid gated chloride channel was performed using the published primers as per section 2.3.7 (Figure 2.12).

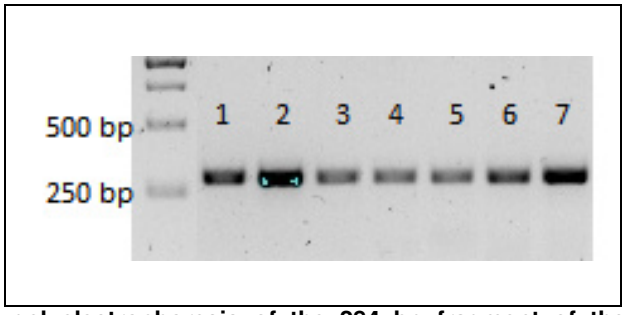


Figure 2:12 Agarose gel electrophoresis of the 284 bp fragment of the GABA-gated chloride channel of *R. microplus* field strains. Lanes 1 through 7 represent the following samples; 50.2MF, 50.3MF, 50.4MF, 50.5MF, 55.1MF, 69.1MF and 69.2MF, respectively.

Amplification of the GABA-gated chloride channel gene was easily achieved. Even though the efficiency of the reaction was template dependant, a PCR amplicon could be obtained for most samples.

Carboxylesterase gene:

Amplification of the 372 bp fragment of the carboxylesterase gene was undoubtedly the most challenging of all the genes (Figure 2.13).

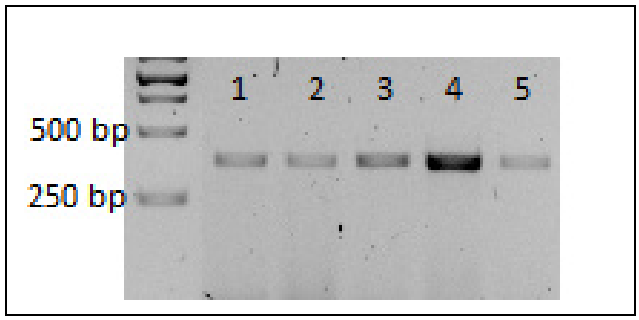


Figure 2:13 Agarose gel electrophoresis of the 372 bp fragment of the carboxylesterase gene of *R. microplus* field strains. Lanes 1 through 5 represent the following samples; 50.2MF, 50.4MF, 51.1MF, 51.2MF and 51.3MF, respectively.

Applying the conditions for amplification (Section 2.3.7) allowed for the amplification of only a few samples. Several different polymerases were tested in an attempt to improve the reaction conditions. A new forward primer, G8-New (5'-ACG AGC TAG TGA AGG CCG CCG C-3'), was designed based on obtained sequences from field strains but this too resulted in no amplification of the template DNA. For this reason, results for the carboxylesterase gene are limited in comparison to the others, with only two grid blocks (7 and 8) meeting the requirements of the lower limit for allele sampling.

2.4.5 Sequence analysis

Sequence chromatograms obtained from Macrogen were analyzed using BioEdit version 7.2.0 (Hall 2007). After completing the base calling for each sample, all sequences for a particular gene were aligned using the online program MAFFT (Katoh, Standley 2013).

Octopamine receptor gene:

Sequencing of the 417 bp or 394 bp portion of the octopamine receptor revealed the abundance of heterozygosity within the gene. The presence of two alleles at multiple positions was evident throughout the gene when analyzing the chromatograms (Figure 2.14). This was also seen at the two positions that are thought to give rise to amitraz resistance (position 157 and 200 bp in the multiple alignment file, Appendix F). The sequenced fragments of the octopamine receptor were then aligned with the NCBI sequence of *Boophilus microplus* mRNA for the relevant G-protein coupled receptor (Accession: AJ010743.1) as well as the susceptible (Accession:EF490688.1) and resistant (Accession:EF490687.1) alleles published by Chen *et al.* 2007.

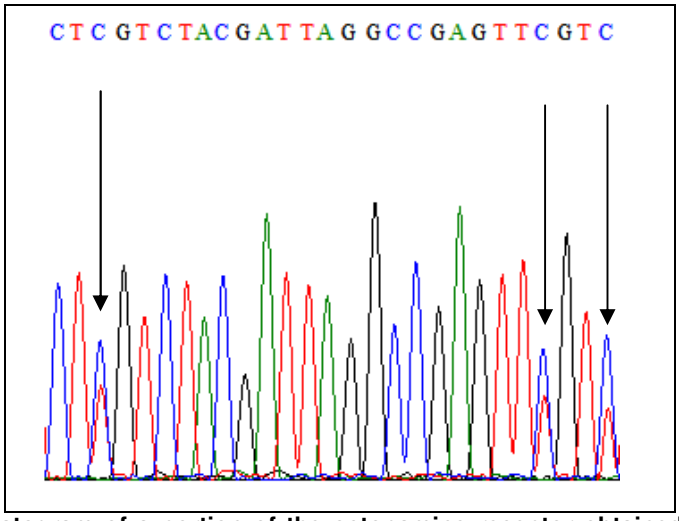


Figure 2:14 Chromatogram of a portion of the octopamine receptor obtained for sample 46.3MF. Three positions where two alleles are clearly visible is indicated by the arrows.

Several nucleotide substitutions were found when the published sequences and the sequences obtained from the South African *R. microplus* tick population were compared. Of the 27 nucleotide substitutions, two were the published resistant SNPs, and seven were ascribed to be due to strain differences between the South African, USA and Brazilian strains as they were largely conserved throughout. The first three of these substitutions occurred in the non-coding region of the gene (11, 13 and 67 bp). Of the remaining four substitutions, two resulted in amino acid changes at nucleotide position 178 (isoleucine to

valine) and position 193 (threonine to alanine). Isoleucine and valine are both hydrophobic amino acids, and a change from the latter to valine is due to the loss of a methyl group. A change from threonine to alanine is a change from a polar to a hydrophobic residue due to the loss of a hydroxymethyl group. The full effect of these amino acid changes cannot be determined by sequence information alone, and needs to be further analyzed through techniques such as *in silico* predictive structural analysis.

Apart from the seven nucleotide substitutions, the additional 18 variable positions found in the gene were further characterized. It was of interest whether or not these SNPs were related to the two published resistant SNPs, and how closely these alleles were associated. Therefore, allelic association (gametic disequilibrium) studies were done (Section 2.4.6).

In addition to this, amitraz resistant larvae that was obtained from the Mnisi area in the Kruger National Park was sequenced for the confirmation of the two resistant SNPs published by Chen *et al.* (2007). By confirming the presence of these SNPs in the amitraz resistant larvae, a correlation could be made between the two (Figure 2.15).

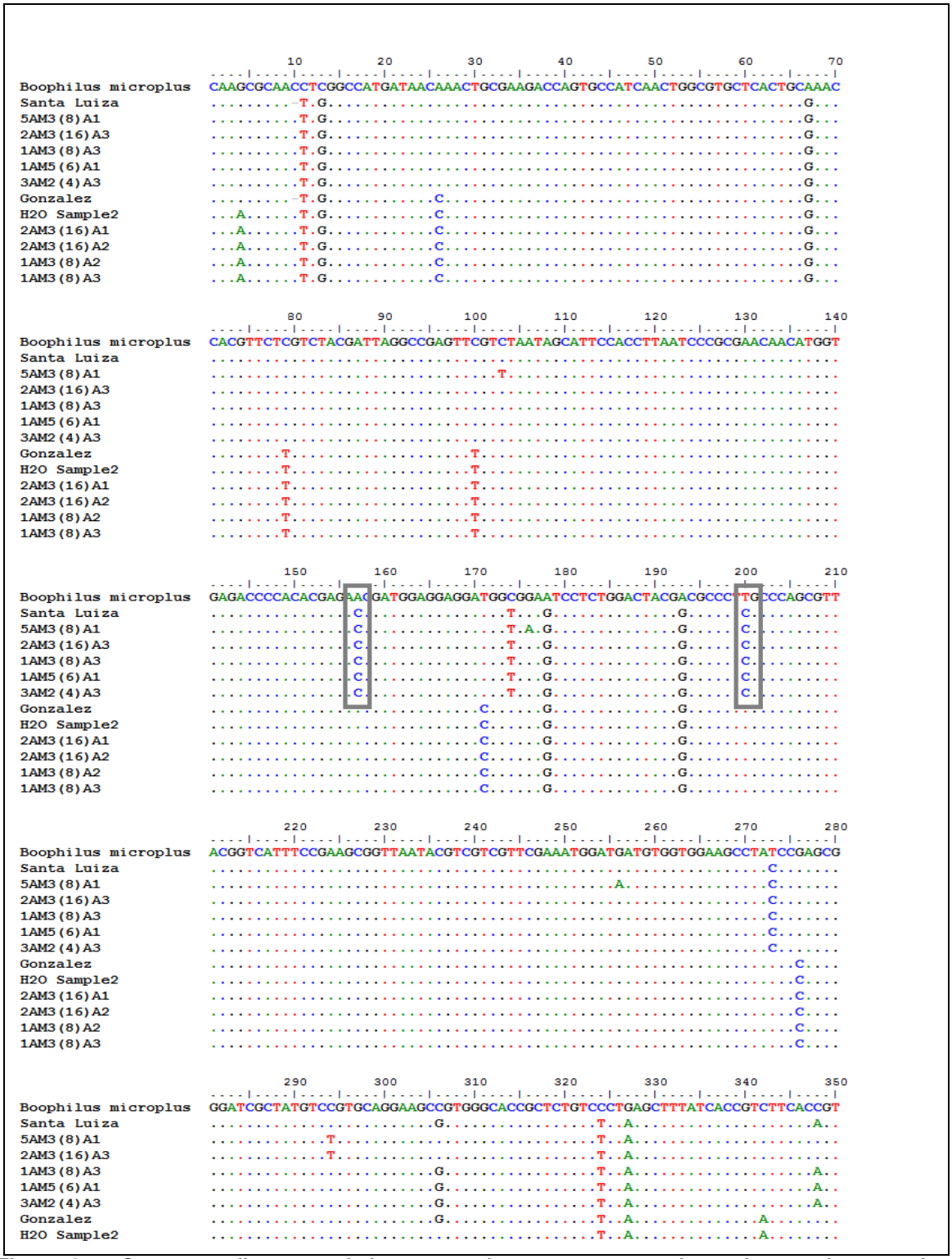


Figure 2:15 Sequence alignment of the octopamine receptor gene for amitraz resistant and susceptible *R. microplus* larvae. *Rhipicephalus (Boophilus) microplus* was the reference sequence used for the alignments (Accession AJ010743.1) along with the Santa Luiza resistant strain (Accession:EF490688.1) and the Gonzalez susceptible strain (Accession:EF490687.1). The five samples aligned below the Santa Luiza strain are the resistant samples, and those below the Gonzalez strain are the susceptible ones. Blocked residues indicate the two SNPs that were published by Chen *et al.* 2007 and are only present in amitraz resistant larvae sequences.

Firstly, it must be noted that the two SNPs published by Chen *et al.* (2007) are indeed present in the amitraz resistant *R. microplus* larvae (indicated with blocks in Figure 2.15), and were absent in the susceptible larvae samples. For the purpose of this study, samples displaying the two SNPs published by Chen *et al.* (2007) will be referred to as resistant. Certain patterns can be seen in the above alignment with certain nucleotide substitutions only occurring in either the resistant or the susceptible strains of the larvae. The seven conserved nucleotide substitutions thought to be due to strain differences can also be seen throughout the samples for both resistant and susceptible larvae. It can therefore be hypothesized that these additional SNPs may be necessary for the tick depending on whether or not the tick is under selection pressure by means of exposure to amitraz treatment. To determine whether or not these SNPs increase the fitness of the tick will require further investigation.

Voltage-gated sodium channel gene:

As previously mentioned (Chapter 1, section 1.6.2) it has been confirmed that there are three SNPs that can result in resistance to pyrethroids. Two of these SNPs occur in domain II S4-5 linker region of the sodium channel, while the other is found in domain III S6 (He *et al.* 1999; Morgan *et al.* 2009; Jonsson *et al.* 2010). Sequencing of the 167 bp region of domain II from the voltage-gated sodium channel revealed the presence of the published SNP (Morgan *et al.* 2009) at nucleotide position 23 in the multiple alignment file, resulting in a C to A mutation (L64I) (Appendix G). This amino acid change replaced one hydrophobic residue with another. No other SNPs were found in domain II S4-5 linker region of the sodium channel from *R. microplus* field strains. It was calculated that 58.8% of the samples were homozygous resistant at this position (RR). It is therefore suggested that this particular nucleotide substitution constitutes the main resistance mechanism against pyrethroids in the South African strain of *R. microplus*. Very few samples (8.8%) displayed heterozygosity at this position, suggesting that the resistant allele is very responsive to selection pressure by exposure to pyrethroids. Heterozygous ticks are considered to be susceptible due to the inheritance of pyrethroid resistance being recessive (Morgan *et al.* 2009).

An alignment between susceptible female and male *R. microplus* ticks, as well as a few resistant samples is shown in Figure 2.16. Only variable positions were indicated in the following alignment.

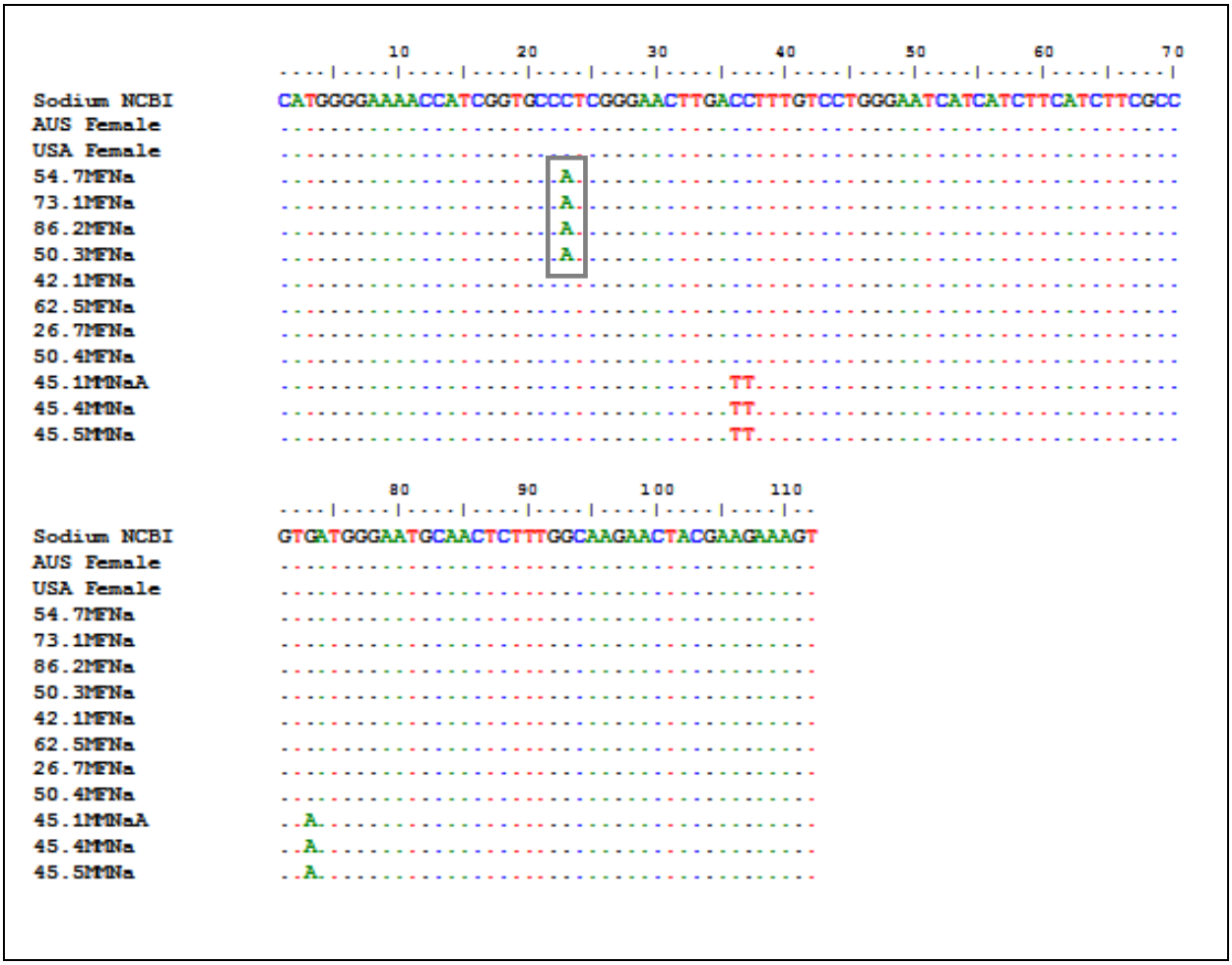


Figure 2:16 Sequence alignment of a portion of the voltage-gated sodium channel (domain II S4-5) between female and male *R. microplus* ticks. Sodium NCBI sequence was used as the reference sequence (Accession: AF134216.2). Included in the alignment were samples from Australia and the USA which were sequenced alongside the samples from South Africa. The resistant SNP is indicated by the block (C to A mutation). All samples denoted as MF are females, while those displayed as MM are the male *R. microplus* ticks.

Four samples were included in the above alignment containing the resistant SNP which is indicated by the block. This occurs at position 23 in the above alignment. It is clear that there are some nucleotide differences between male (MM) and female (MF) ticks. At nucleotide position 36 and 37 there is a substitution from a C to a T resulting in the amino acid change from threonine (polar) to isoleucine (hydrophobic). The advantage of this difference between males and females is unknown and further studies need to be conducted. Furthermore, another nucleotide substitution occurs at position 73. However, this change does not affect the sequence on an amino acid level due to the degeneracy of the third codon position.

GABA-gated chloride channel gene:

Sequencing of the portion of the GABA-gated chloride channel revealed no SNPs within the gene (Appendix H). A total of 78 ticks (156 alleles) were screened across South Africa with no polymorphisms arising in the nucleotide sequences of the GABA-gated chloride channel. Any further screening was discontinued, because the probability of discovering a mutation would not be significant enough for further consideration.

Carboxylesterase:

In section 2.4.4 it was mentioned that amplification of the 372 bp fragment of the carboxylesterase gene was problematic. It later appeared that sequencing this gene also had its difficulties. Initially, when cleaning amplified PCR products using the same protocol as with all the other genes, all the PCR products were lost during the clean-up. Therefore, different concentrations of the PureLink™ Pro 96 Binding Buffer had to be tested along with different volumes of PureLink™ Pro 96 Elution Buffer. Once the binding buffer was established to be 2:1 with the PCR products, and the volume of elution buffer determined to be 75 µl, it became possible to clean samples without losing the product. In addition to this, when preparing the plates for Macrogen sequencing, double the recommended template concentration was needed for sufficient sequencing of the carboxylesterase gene. Therefore, several factors contributed to the results being limited for this gene. Once the genome information for this gene is available, new and improved primers can be developed.

Eventual successful sequencing of the 372 bp fragment of the carboxylesterase gene revealed heterozygosity within the gene similar to what was observed for the octopamine receptor. The published SNP (Hernandez *et al.* 2000; Hernandez *et al.* 2002) at nucleotide position 1120 in the multiple alignment file (Appendix I) was present in two field strains which displayed heterozygosity, thus having the resistant and susceptible alleles. A total of 27 variable positions were found throughout the gene of which 17 appear to be due to strain differences. Of the 17 nucleotide substitutions, four resulted in amino acid changes. The first and second (at nucleotide position 1048 and 1049) results in the amino acid change from alanine to methionine. This change is from a small hydrophobic residue to a larger hydrophobic residue. The third (at nucleotide position 1070) resulted in the amino acid change from a leucine (hydrophobic) residue to a proline (cyclic hydrophobic) residue. And finally, the fourth (at nucleotide position 1132) resulted in the drastic change from a polar threonine residue to a hydrophobic alanine residue. The full effect of these amino acid changes on the overall functioning of the carboxylesterase enzyme is unknown and is currently being investigated using a recombinant carboxylesterase enzyme (Mr RDJ van Wyk, MSc student, University of Pretoria).

Allele frequencies:

Upon completion of sequence analysis, the frequencies of the susceptible (S) and resistant (R) alleles were calculated for each grid block for each gene. Only the SNPs suggested to be responsible for resistance were included in these results. The combined allelic frequency for each gene is shown in Table 2.3 (Appendix E).

Table 2-3 Allele frequencies for resistance associated SNPs. SS, susceptible homozygote; RS, heterozygote; RR, resistant homozygote. * Indicate positive directional selection, # indicates positive balancing selection. SNP location is represented as an amino acid substitution where *L64I* is a change from leucine to isoleucine at position 64.

Genes	Acaricides	SNP Location	SS	RS	RR	HWE
Voltage-gated sodium channel	Pyrethroids	*L64I*	0.324	0.088	0.588*	Heterozygous deficient
Octopamine receptor	Amitraz	*T8P* and *L22S*	0.229	0.578#	0.193	Heterozygous excess
Carboxylesterase	Pyrethroids	*D374N*	0.924*	0.076	0	Heterozygous deficient
GABA-gated chloride channel	Dieldrin/Macro lactones	*T289L*	1	0	0	Heterozygous deficient

The frequency of the homozygous resistant (RR) genotype for pyrethroids is 0.588 and can therefore be interpreted as 58.8% of the population being resistant to pyrethroids, while 32.4% is susceptible. Only 8.8% of the sampled population was heterozygous indicating that directional selection was taking place driving the conversion from the SS genotype to RR. For amitraz, majority of the population (57.8%) displayed the heterozygous (RS) genotype indicating that balancing selection could have been acting on the population at the time of sampling. There was no significant presence of the R allele in the carboxylesterase gene or the GABA-gated chloride channel.

In Figure 2.4 it was noted that the most common acaricides used by South African farmers were those targeting the voltage-gated sodium channel and the octopamine receptor. Therefore, it is not surprising to see such a high frequency of the R allele within these genes. This provides strong evidence towards the hypothesis that the target site mutations seen in this study are largely responsible for the development of resistance towards the respective acaricides.

2.4.6 Gametic disequilibrium studies

Gametic disequilibrium studies were performed to determine if there is any association (linkage) between the loci of interest. This can be done for loci within a single gene (intragenic) or loci from different genes (intergenic).

In this study all SNPs that were present in the octopamine receptor were analyzed to determine if there was any association between them. A total of 22 different loci were analyzed for 105 isolates (sequences). The first function that was performed using the Multilocus program was that of genotypic diversity versus the number of loci (Fig. 2.17). This is essential to determine if there are sufficient samples for the proposed analysis (Figure 2.17 and Appendix J). The plot of mean diversity versus the number of loci should eventually reach a plateau. This signifies that there are sufficient samples for further analysis, and any additional samples or loci will not change the diversity that is being observed in the sample set. In all cases it was observed that the sample size was sufficient for further analysis.

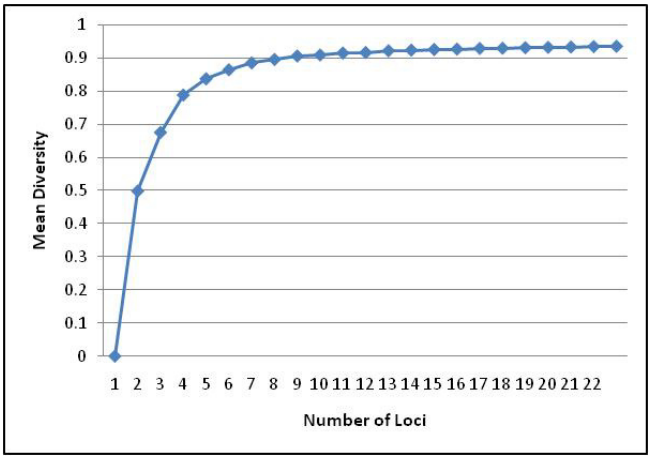


Figure 2:17 Distribution of the mean diversity versus the number of loci for the octopamine receptor.

The next function that was performed was that of genotypic diversity and linkage disequilibrium (Appendix K). The Multilocus program computes three measures of genotypic diversity and four measures of linkage disequilibrium (Agapow, Burt 2001). For genotypic diversity it was calculated that; i) the observed number of different genotypes was 48, ii) the observed frequency of the most frequent genotype was 22/48 = 0.46 and iii) the observed genotypic diversity was calculated to be 0.935. Genotypic diversity can be defined as the likelihood that any two individuals, who are taken at random, will display different genotypes.

A value of 0 would indicate that all individuals in the sample set are the same, while a value of 1 would denote that they are all different. This was calculated using equation [2.1 – section 2.3.10]. Therefore, based on the genotypic diversity value obtained in the octopamine receptor dataset (0.935), it suggests that there is an abundance of genotypic diversity.

In terms of the four measures of linkage disequilibrium, only one is of interest for this study. In previous studies it was shown that linkage disequilibrium measures (such as Index of Association, I_A) were usually dependant on the number of loci that were being analyzed. This largely influenced any attempts in trying to compare results from different studies (Smith *et al.* 1993). To circumvent this problematic encounter a tailored statistical measure ($\bar{r}_d$) was developed which eliminated any relevancy that the number of loci might have had. This was calculated using equation [2.2 – section 2.3.10]. The closer $\bar{r}_d$ is to one, the higher the association between the loci of interest. The distribution of $\bar{r}_d$ (Fig. 2.18) was constructed by sorting all $\bar{r}_d$ values from smallest to largest followed by placing them into categories (Appendix K). The relative count of each time a particular $\bar{r}_d$ value within a certain category occurred was then plotted against the category number. Included in this distribution was the observed $\bar{r}_d$ value that was generated from the non-randomized dataset. The graph shows a bell shaped curve, and the observed $\bar{r}_d$ value falls outside of the distribution. With the observed $\bar{r}_d$ value being significantly higher than that generated by the randomized dataset, gametic disequilibrium is quite evident which is supported by a P-value of < 0.00001.

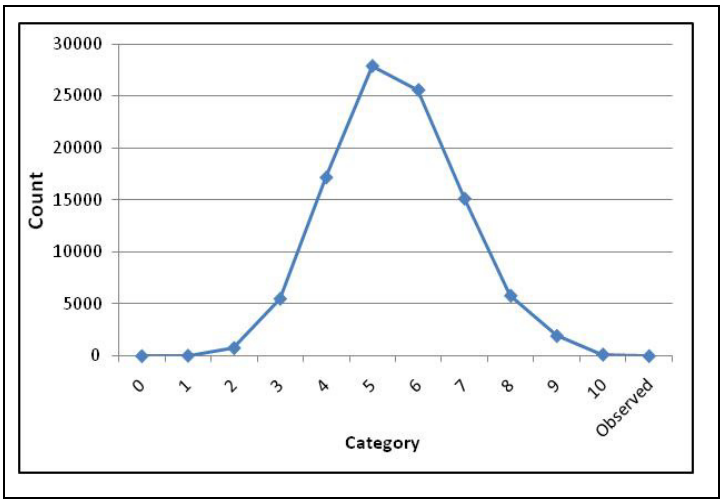


Figure 2:18 The $\bar{r}_d$ distribution for the octopamine receptor. The category in which the $\bar{r}_d$ values were placed is represented on the x-axis while the relative count that each time these values occurred is displayed on the y-axis.

In addition to this, the program also calculates a pairwise comparison for every pair of loci being analyzed, thus generating a $\bar{r}_d$ value for every pairwise comparison. The output file that was generated can be seen in Appendix L. The closer the value is to 1, the more associated/linked the loci are and *vice versa* as previously discussed.

Loci comparisons of initial interest were that of the two loci suggested to be involved in amitraz resistance (loci 11 and 17 in the binary input file). Locus 11 corresponds to the nucleotide position 157 in the octopamine multiple alignment file (Appendix F), and locus 17 with that of 200. A pairwise comparison of these two loci gave a $\bar{r}_d$ value of 0.804 (P-value < 0.00001) indicating that these two sites are in gametic disequilibrium. Therefore, if it is proposed that these two SNPs are related to amitraz resistance, it would then require that both loci contain nucleotide substitutions for the tick to be amitraz resistant. A possible explanation as to why this $\bar{r}_d$ value is not as high as 1 could be due to the heterozygous nature of the gene itself when exposed to amitraz selection pressure. It is suggested that some samples that were analyzed were still acquiring resistance and therefore at that point in time only displayed a nucleotide substitution at locus 11 and not 17. Homozygous samples shown in section 2.4.5 also illustrate that both substitutions are present in the resistant samples. The next consideration would be whether there are any other loci significantly associated with loci 11 and 17. Additional significant associations that were found are summarized in Table 2.4.

Table 2-4 Loci significantly associated with resistant loci 11 and 17.

Locus 1	Locus 2	r_d value	Aggregate	P-value
11	12	0.834	0.877	< 0.00001
17	12	0.921		< 0.00001
11	13	0.872	0.878	< 0.00001
17	13	0.884		< 0.00001
17	19	0.803	0.803	< 0.00001

Locus 12 corresponds to the nucleotide position 171 in the multiple alignment file, locus 13 to that of 174, and locus 19 to that of 273. Both loci (11 and 17) appear to be strongly associated with loci 12 and 13. The aggregate value of $\bar{r}_d$ was calculated to illustrate which loci, *i.e.* 12 or 13, the two resistant loci (11 and 17) were more strongly associated with. The results show that the resistant loci are equally associated with both 12 and 13. When comparing sequences obtained in section 2.4.5, it is evident that a nucleotide substitution at

locus 12 only takes place when the tick is suspected to be susceptible. A substitution at locus 13, however, occurs every time the tick is suspected to be resistant. Therefore, perhaps one could consider this particular association as a vital marker for resistance in the gene. This association becomes imperative in the development of a rapid diagnostic test for amitraz resistance (Section 2.4.8).

Population differentiation analysis generated a θ value of 0.0253643. This θ value is the equivalent to Wright's F_{ST} measure of differentiation within a population (Cockerham 1969; Holsinger, Weir 2009). A value close to zero implies that all populations are interbreeding freely, while a value of one indicates no interbreeding. From the θ value calculated by the Multilocus program it is suggested that inbreeding is taking place between different tick populations in South Africa. Incorporating θ into the following equation (Halliburton 2003) gives an estimation of the migration rate between populations:

$$\widehat{M} = \frac{1}{2}\left(\frac{1}{\theta} - 1\right) \qquad [2.4]$$

The migration rate is estimated to be ~ 19 alleles per generation. This is not an idealized model, and the data analyzed was largely polymorphic, therefore interpreting migration rate is relatively difficult. However, it does provide an estimation based on the calculated genetic differentiation.

The gametic disequilibrium analysis was repeated, but this time with the addition of the resistant loci for the voltage-gated sodium channel as locus 23. The plot for mean diversity versus the number of loci can be found in Appendix M, which indicates that sample size is sufficient as expected. Pairwise comparisons that were made between the loci from the sodium channel and those from the octopamine receptor can be seen in Appendix N. No significant associations were found between the loci from the sodium channel and the octopamine receptor generating $\bar{r}_d$ values of almost zero. Therefore, it can be hypothesized that since these loci are not significantly associated with one another, the evolution of resistance within each individual gene occurs independently of the other.

Gametic disequilibrium analysis was also performed for the SNPs found in the carboxylesterase gene, and included in this analysis was the resistant loci for the voltage-gated sodium channel. This analysis seemed valuable because both result in pyrethroid resistance in *R. microplus* ticks. The sequence data was first converted into binary format as previously mentioned. The genotypic diversity versus the number of loci illustrated that the

sample set was large enough and sufficient for further analysis (Figure 2.19A). Genotypic diversity and linkage disequilibrium analysis showed that the observed genotypic diversity within this sample set was 0.982 therefore most individuals were different from one another. The distribution of $\bar{r}_d$ values was constructed as mentioned previously (Figure 2.19B).

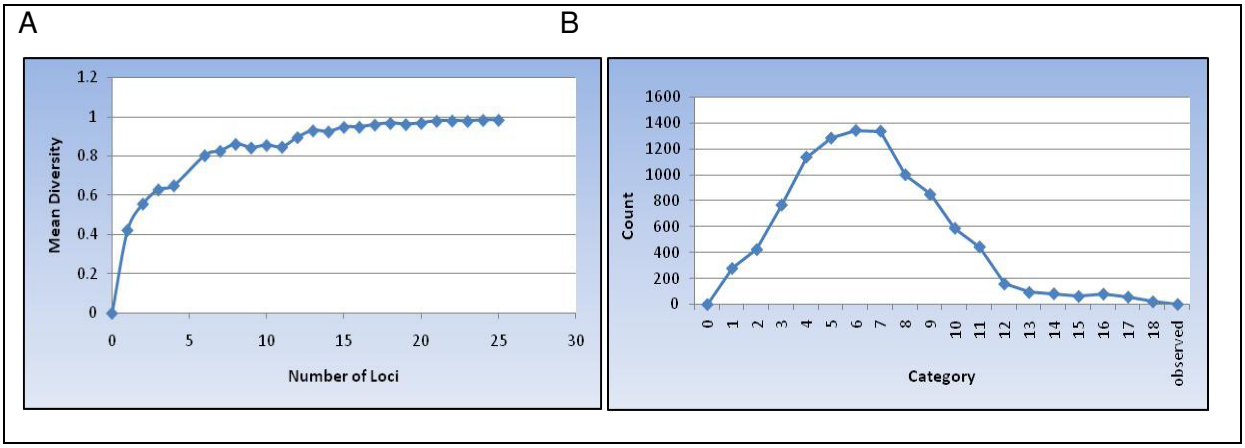


Figure 2:19 Linkage disequilibrium analysis for carboxylesterase gene and voltage-gated sodium channel. A) The plot of mean diversity versus the number of loci for the carboxylesterase gene and the voltage-gated sodium channel. B) The $\bar{r}_d$ distribution for carboxylesterase and the voltage-gated sodium channel.

Figure 2.19B illustrates that there is gametic disequilibrium between the loci that were analyzed and they are therefore non-randomly associated. This association is confirmed to be significant with a P-value < 0.0001. In addition to this, pairwise comparisons were also done (Appendix O) for all loci involved. Locus number 20 corresponded to the resistance position in the carboxylesterase gene while locus number 25 was the resistant locus from the voltage-gated sodium channel. There seem to be no significant associations between the resistant loci from the two genes. Furthermore, there was also no significant association between the resistant loci in carboxylesterase with any of the other loci that were found in the gene itself. In Appendix O it can be seen that there are several SNPs in the carboxylesterase gene that are strongly associated with another, however, none of these seemed to be linked to the resistant loci. No significant association was found between the resistant loci in the voltage-gated sodium channel and the remaining loci in carboxylesterase.

Population differentiation analysis was not done for the sodium channel gene because it only displayed one variable site, nor was it done for carboxylesterase due to the lack of association between the resistant loci with other loci. No association studies were conducted

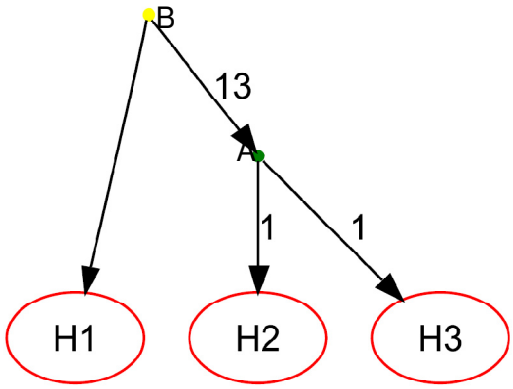
for the GABA-gated chloride channel because there were no variable positions in the sequences obtained from the *R. microplus* field strains in South Africa.

2.4.7 Ancestral recombination studies

Ancestral recombination graphs were constructed to determine the evolutionary histories within each gene. When haplotypes were constructed, infinite site violations needed to be excluded. The infinite sites model of mutation states that only a single mutation can occur at a particular site. Therefore, the polymorphism seen among sequences was only brought about by a single mutation event (Kimura 1969). Any violation of this theory was excluded from the haplotype construction. Implementation of the Beagle and Kwarg function was due to the fact that this algorithm was less computationally intensive and allowed for the analysis of a larger set of sequences. It also directly displays the data in the most parsimonious ancestral recombination graph through calculating the minimum number of recombination events R_{min} *(M)* (Lyngsø, Song, Hein 2005). Ancestral recombination graphs were only constructed for the octopamine receptor and the voltage-gated sodium channel because no significant resistance was found in the other two genes (carboxylesterase and GABA-gated chloride channel).

Octopamine receptor gene:

Only homozygous samples were used in this analysis because multiple alleles at a particular position in the heterozygotes would skew the representation of the data (results not shown). The ancestral history of the homozygous susceptible *R. microplus* ticks is shown in Figure 2.20.



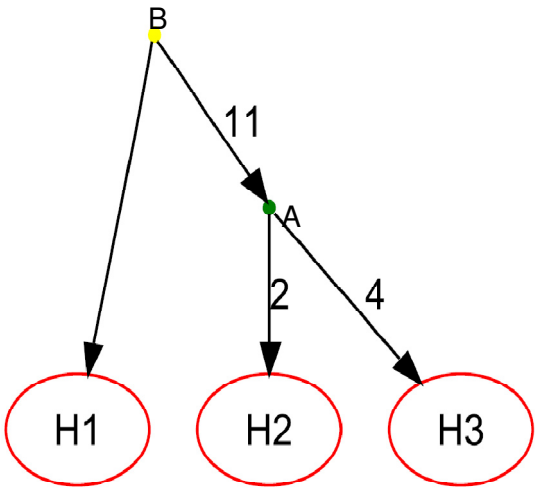
Haplotype	Sequence Samples
H1	*Boophilus microplus* NCBI
H2	20.2MF 20.3MF 44.2MF 62.4MF 66.2MF 73.8MF 86.2MF 86.7MF 18.2MF
H3	Gonzalez Susceptible

Figure 2:20 Ancestral recombination graph for homozygous amitraz susceptible *R. microplus* ticks in South Africa. H1, H2 and H3 represent the different haplotypes that were generated by the program. The peak of the graph represents the ancestral form of the gene with adjoining points illustrating coalescent haplotypes. The table indicates which samples belong to which haplotype in the graph.

There was no recombination in the octopamine receptor gene (Figure 2.20). The adjoining points represent coalescence between haplotype 2 and 3 (point A) and haplotype 1 and 2 (point B). A mutation occurred at site 1 in H2 and site 12 in H3 making them different haplotypes, the two sequences then coalesce at point A. Mutations occurred at sites 2 through 11, and 13 to 15 in H2, after which H2 and H1 then coalesce at point B. The different sites are the variable positions seen in the alignment (Appendix P).

From Figure 2.20 it is clear that homozygous susceptible *R. microplus* ticks tend to be grouped into their own haplotype differing from the American susceptible Gonzalez strain as well as the reference strain. This implies that over time, the South African ticks have acquired certain mutations absent in the other strains. The Clinvet (Pty) Ltd. reference strain was not included in the analysis as it displayed heterozygosity. There appears to be no geographic significance with the haplotype grouping seen in Figure 2.20.

An ancestral recombination graph was then constructed for the homozygous resistant *R. microplus* ticks (Figure 2.21).

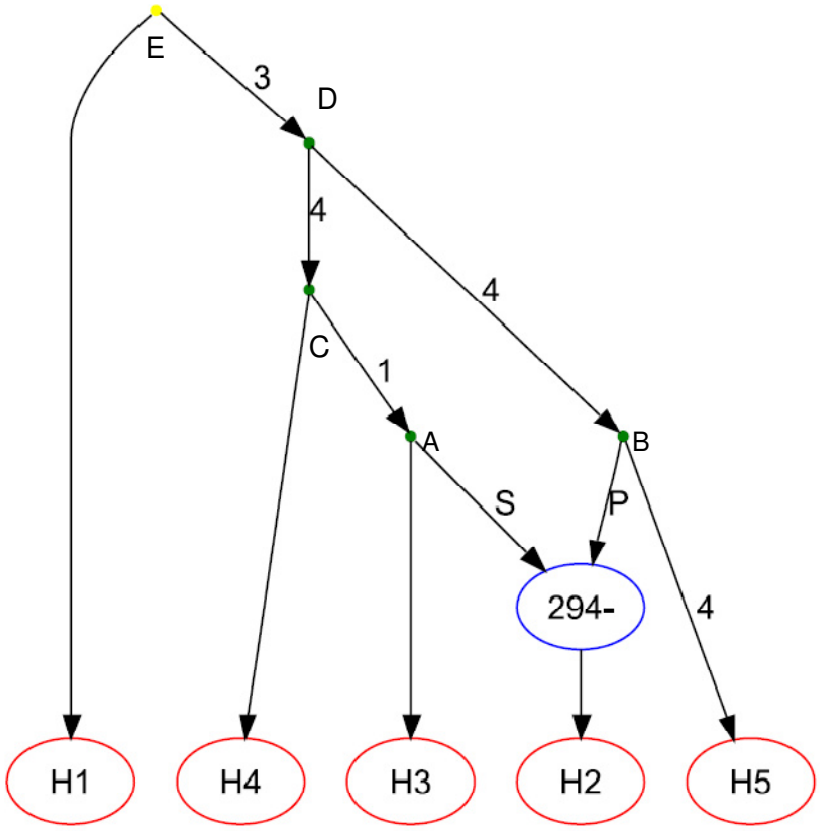


Haplotype	Sequence Samples
H1	*Boophilus microplus* NCBI
H2	SantaLuiza resistant strain, 65.1MF 67.2MF
H3	17.1MF 26.6MF 54.1MF 51.2MF 9.1MF 49.8MF

Figure 2:21 Ancestral recombination graph for homozygous amitraz resistant *R. microplus* ticks in South Africa. H1, H2 and H3 represent the different haplotypes that were generated by the program. The peak of the graph represents the ancestral form of the gene with adjoining points illustrating coalescent haplotypes. The table indicates which samples belong to which haplotype in the graph.

No recombination was evident in the homozygous resistant *R. microplus* tick samples. Four mutations occurred at sites 4, 7, 11 and 13 in H3. Two mutations occurred at site 14 and 17 in H2, after which H2 and H3 coalesce at point A. A further 11 mutations (sites 1-3, 5, 6, 8-10, 12, 15 and 16) occurred before H1 and H2 coalesced at position B. Segregation of samples into different haplotypes shows that majority of the homozygous resistant South African *R. microplus* ticks fall into their own haplotype (H3), with two exceptions (65.1MF and 67.2MF) which collapse into H2. The different sites are the variable positions that can be seen in the multiple alignments (Appendix Q). There was no geographical significance in terms of the way haplotypes grouped in Figure 2.21.

Ancestral recombination graphs were then constructed by incorporating both the resistant and susceptible sequence information (Figure 2.22).



Haplotype	Sequence Samples
H1	*Boophilus microplus* NCBI
H2	SantaLuiza resistant strain, 65.1MF 67.2MF
H3	Gonzalez
H4	20.2MF 20.3MF 44.2MF 62.4MF 66.2MF 73.8MF 86.2MF 86.7MF 18.2MF
H5	17.1MF 26.6MF 54.1MF 51.2MF 9.1MF 49.8MF

Figure 2:22 Ancestral recombination graph for homozygous amitraz resistant and susceptible *R. microplus* ticks in South Africa. H1, H2, H3, H4 and H5 represent the different haplotypes that were generated by the program. The peak of the graph represents the ancestral form of the gene with adjoining points illustrating coalescent haplotypes. The table indicates which samples belong to which haplotype in the graph. A recombination event is represented by the circle 294- indicating that the recombination took place at around the 294th nucleotide position.

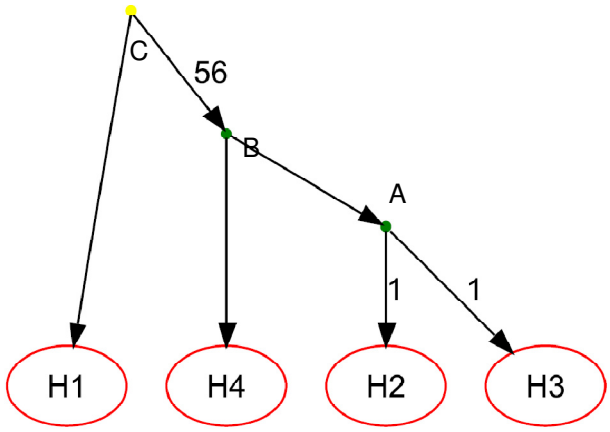
Four mutations occurred at sites 4, 8, 12 and 15 in H5. A recombination event then took place in H2 after site 15 (new designation as H6) after which H5 and H6 coalesce at point B. An additional four mutations occurred at sites 5, 7, 11 and 13 in H5 after point B. Point A indicates the coalescence between H2 and H3, and after a mutation at site 16 in H3, it then coalesces with H4 at point C. Four mutations occur at sites 2, 3, 6 and 14 in H3 after which H3 then coalesces with H5 at point D. A final three mutations occur at sites 1, 9 and 10 in H3 leading to the coalescence of H1 and H3 at point E.

It is known that when there is high gametic disequilibrium, there is minimal recombination. Recombination breaks down any association between loci (Halliburton 2003). Therefore, the results obtained from the ancestral recombination graph make theoretical sense because the octopamine receptor displayed a high level of allelic associations between the different variable loci in the gene. A recombination event took place just after nucleotide position 294

(site 15) which corresponds to the nucleotide substitution seen at position 306. This position (306 bp) was designated as locus 22 in the gametic disequilibrium studies, and results indicate very minimal linkage with this site. Therefore, recombination at this point makes sense because there is no linkage at this locus. Recombination at this locus should not affect the associations between the other loci. When this recombination event was determined, point S was chosen uniformly at random on the octopamine gene. Everything to the left of point S is then thought to follow one line of descent while that on the right follows another. Again no geographical significance was found with the haplotype groupings.

Voltage-gated sodium channel:

An ancestral recombination graph was constructed for the voltage-gated sodium channel (Figure 2.23). Majority of the samples were included in this analysis due to their homozygous genotypes.



Haplotype	Sequence Samples
H1	Sodium Channel NCBI (AF134216.2)
H2	AUS, USA, 9.3MFNa, 17.1MFNa, 20.1MFNa, 20.3MFNa, 42.1MFNa, 44.2MFN 46.2MFNa, 46.3MFNa, 54.2MFNa, 62.1MFNa, 62.2MFNa, 62.5MFNa, 65.1MFN 66.2MFNa, 73.3MFNa, 26.7MFNa, 26.8MFNa, 26.1MFNa, 26.6MFNa, 47.2MFN 54.7MFNa, 54.10MFNa, 73.1MFNa, 73.2MFNa, 73.4MFNa, 73.5MFNa, 73.7MFN 73.8MFNa, 86.2MFNa, 86.4MFNa, 86.6MFNa, 86.7MFNa, 50.3MFNa, 50.2MFN 66.1MFNa, 50.4MFNa, 66.5MFNa, 50.5MFNa, 67.1MFNa, 67.2MFNa, 67.3MFN 67.4MFNa, 51.1MFNa, 51.2MFNa, 51.3MFNa, 51.4MFNa, 51.5MFNa, 69.3MFN 70.1MFNa, 70.3MFNa, 70.2MFNa, 7.5MFNa, 37.1MFNa, 41.2MFNa, 37.2MFN 41.12MFNa, 41.13MFNa, 93.1MFNa, 95.1MFNa, 95.3MFNa, 18.3MFNa, 79.1MFNa
H3	45.1MMNa
H4	45.4MMNa, 45.5MMNa, 67.5MFNa

Figure 2:23 Ancestral recombination graph for pyrethroid susceptible and resistant *R. microplus* ticks from South Africa. H1, H2, H3 and H4 represent the different haplotypes that were generated by the program. The peak of the graph represents the ancestral form of the gene with adjoining points illustrating coalescent haplotypes. The table indicates which samples belong to which haplotype in the graph.

A mutation occurred at site 12 of H2, and site 25 of H3 at which point H2 and H3 coalesced at point A, and H2 and H4 coalesced at point B. Mutations then occurred at sites 1-11, 13-24, 26-58 in H2, after which H2 and H1 coalesce at point C. There was no evidence of recombination occurring in domain II S4-5 of the voltage-gated sodium channel.

From the haplotypes generated it is clear that almost all of the South African samples fall under the same haplotype (H2) with the exceptions of the male ticks (H3 and H4) and one female sample collapsing into H4. The Australian and American samples that were sequenced alongside the South African samples fall under the same H2 haplotype. Therefore, it can be hypothesized that very little differentiation has occurred between samples in the sodium channel, and that perhaps this section of the sodium channel is generally more conserved and therefore not prone to recombination events. It should also, however, be kept in mind that only a small portion of the gene was sequenced, and that recombination and heterogeneity could be abundant in other parts of this gene.

2.4.8 Rapid diagnostic test (RFLP)

Locus 13 corresponds to nucleotide position 174 in all octopamine alignment files and was significantly associated with the two resistant loci (locus 11 at position 157 and locus 17 at position 200) in the octopamine receptor. A nucleotide substitution at locus 13 from C to T occurs every time the tick displays the two resistant alleles. In heterozygotes, if both the susceptible and resistant alleles are present, then locus 13 also display's both the C and T alleles. This substitution does not result in an amino acid change due to the degeneracy of the third codon position. Such tight association between loci in the gene could potentially represent a vital marker for amitraz resistance, which until now can only be determined via time-consuming bioassays. A restriction enzyme mapper was used to determine different sites in the octopamine receptor that displayed restriction enzyme cut sites (Figure 2.24).

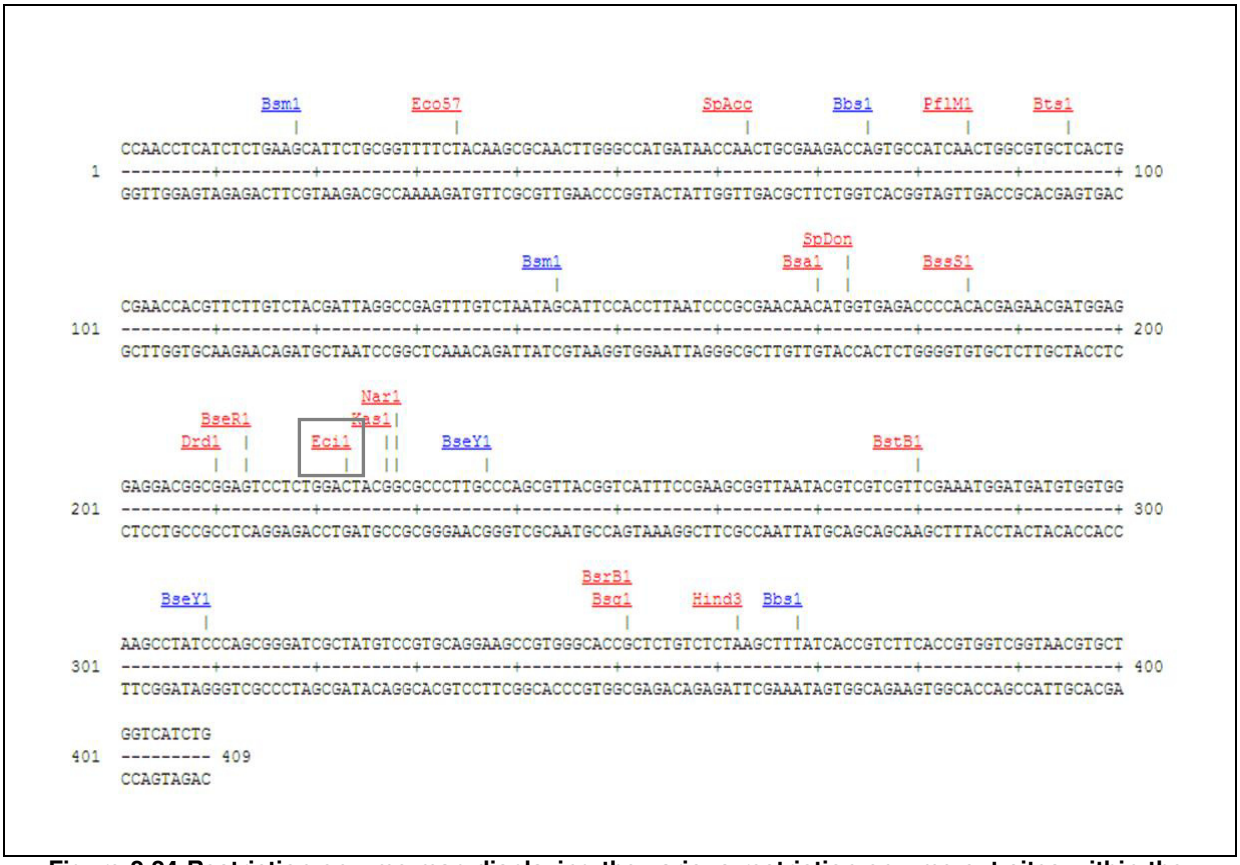


Figure 2:24 Restriction enzyme map displaying the various restriction enzyme cut sites within the octopamine receptor. The octopamine receptor contains multiple recognition sites for a variety of restriction enzymes, the one of interest in this study is *Eci*I indicated by the gray block.

Of all the potential restriction enzymes (Figure 2.24) only *Eci*I is of concern in this study. The recognition sequence of *Eci*I happens to be the region of locus 13, where the nucleotide substitution C to T always seems to be associated with the two resistant SNPs (Figure 2.25).

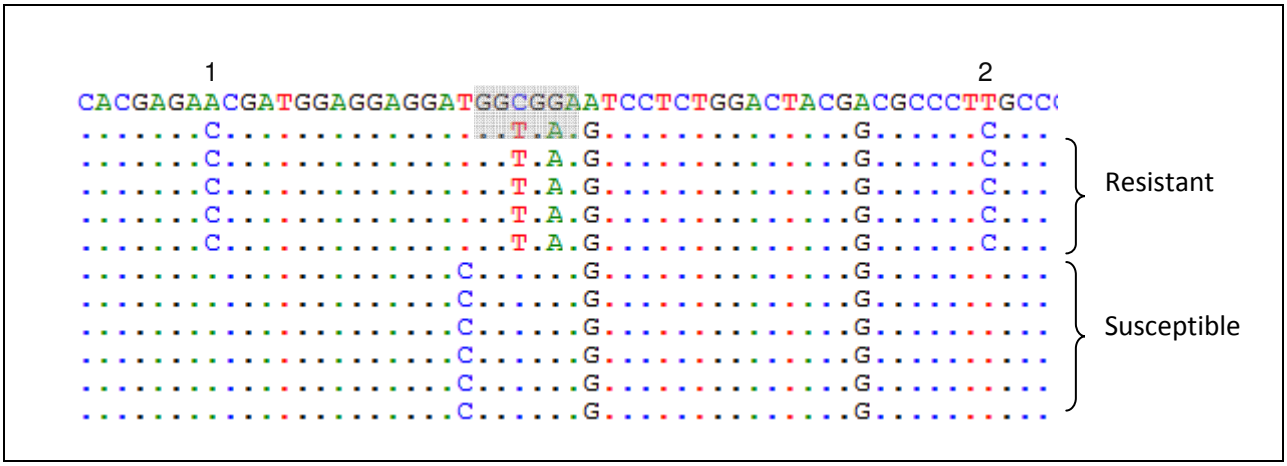


Figure 2:25 Snapshot image of the recognition site of *Eci*I restriction enzyme in the octopamine receptor. The top five sequences are the resistant samples containing the two mutations (1 and 2) while the bottom six samples are susceptible. The gray block indicates the recognition site for the *Eci*I restriction enzyme (5'….GGCGGA $(N)_{11}$↓….3').

Figure 2.25 characterizes the general trend in sequence information obtained near the *Eci*I restriction enzyme recognition site (gray block). The resistant samples display nucleotide substitutions within this recognition site, thus preventing the enzyme from cutting at this position. The susceptible samples lack the substitutions enabling the enzyme to recognize the site and cut the DNA 11 nucleotides downstream. Heterozygous samples on the other hand, displaying both characteristics of susceptible and resistant samples, will give a unique profile where some DNA will be digested and some not. Homozygous resistant sequences will not digest thus will produce a band of about 400 bp, susceptible sequences will digest generating bands of sizes 223 and 186 bp, whereas heterozygous samples will most likely produce all three band sizes after digestion.

Rhipicephalus decoloratus ticks with known amitraz resistance and susceptibility were used for testing the effectiveness of the above restriction enzyme digestion as a possible diagnostic tool. Fourteen samples were provided, with each sample containing multiple larvae. Some of the larvae were almost three years old, and of these 14 samples that were received, only five contained enough DNA for the RFLP analysis.

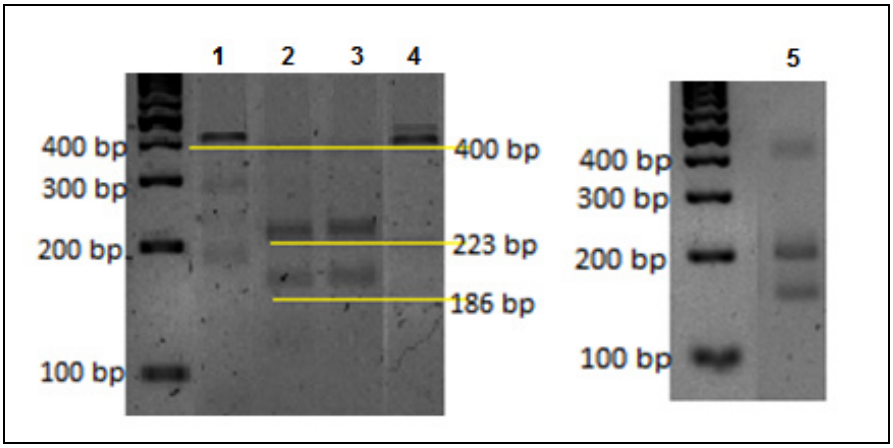


Figure 2:26 Agarose gel electrophoresis of the octopamine receptor from *R. decoloratus* larvae digested with *Eci*I restriction enzyme. The lane numbers represent samples from the original set of 14 that was received. They are indicated as follows; lane 1-sample 5, lane 2-sample 7, lane 3-sample 8, lane 4-sample 6 and lane 5-sample 9 (Appendix R).

Sample number 5 (lane 1) had a very prominent band at around 400 bp indicating a homozygous resistant status. When comparing this result to that from the LPT (Appendix R), we see that this sample is indeed resistant. This particular sample exhibited a 13.2% control at field concentration indicating that there was only a 13% mortality of ticks treated with a 250 ppm concentration of amitraz. Sample 7 (lane 2) and 8 (lane 3) produced very similar bands. A very faint band was seen at 400 bp, with prominent bands at 223 and 186 bp. This

would signify that the sample is heterozygous (more of a tendency towards susceptibility due to the lack of intensity at the 400 bp mark). Comparing this to the LPT results, samples 7 and 8 are susceptible with 100% effectiveness at field concentration. Sample 6 (lane 4) provided evidence for homozygous resistance due to the prominent band at 400 bps. Comparing this to the LPT results it is clear that sample 6 is indeed resistant with a 30% control at field concentration. Lastly, sample 9 (lane 5) displayed all three bands indicating that the sample is heterozygous; LPT results show that sample 9 is susceptible with 100% effectiveness at field concentrations. From these results it seems as though the diagnostic test is relatively accurate in diagnosing homozygous resistant ticks, while it seems as though all samples which exhibit heterozygosity are still susceptible to amitraz. It is therefore hypothesized that even though the tick may possess the resistant allele, they have to be homozygous resistant to display phenotypic resistance to amitraz.

Chapter 3: Integrative assessment of acaricide resistance in South Africa

3.1 Amitraz resistance and the octopamine receptor

Amitraz usage was implemented in the 1990's soon after resistance to organophosphates (OPs) developed, and its use became progressively more frequent after resistance to synthetic pyrethroids (SPs) began emerging (Rosado-Aguilar *et al.* 2008). The first reported case of amitraz resistance was in 2001 from a farm in Mexico (Soberanes *et al.* 2002). The emergence of resistance to amitraz is of great concern because this acaricide was implemented to circumvent the resistance to OPs and SPs. Presently; the only way to unequivocally validate whether amitraz resistance is present on a farm is by means of bioassays (Chapter 1, section 1.6). This process is time-consuming, arduous and not cost effective. Alternative assays to diagnose amitraz resistance have been explored, however, this becomes increasingly difficult when the target site of amitraz remains to be confirmed (Guerrero, Lovis, Martins 2012). Chen, He, Davey (2007) previously proposed that two SNPs in the octopamine receptor could be responsible for amitraz resistance, but this was not confirmed or validated by subsequent studies. Consequently, this study investigated the occurrence of these two SNPs in field populations of South African *R. microplus* ticks.

The octopamine receptor gene of *R. microplus* larvae was sequenced to determine whether or not the proposed resistance SNP's were present following confirmation of amitraz resistance via LPTs. Remarkably, the two SNPs were only present in resistant larvae, but absent in the susceptible ones. A country-wide survey was then conducted to determine the frequency of these SNPs in *R. microplus* tick populations in South Africa. Consequently, the homozygous resistant (RR) genotype was found in 19.3% of the tick population that was studied, with 57.8% displaying heterozygosity (Chapter 2, section 2.4.5). This implies that the alleles required for resistance is still being fixed within the overall population. Balancing selection pressure seemed to be present at the time of sampling and was evident in the abundance of heterozygotes and very few homozygotes. However, to conclusively validate the type of selection pressure driving amitraz resistance, extensive re-sampling will need to be conducted over a longer period of time. This will also give an indication of how swiftly amitraz resistance develops, allowing us to provide farmers with a time scale suggesting how long they have before homozygous resistance ticks predominate.

Gametic disequilibrium analysis indicated an association between several loci within the octopamine receptor and lead to the discovery of a potential resistance marker within the gene (Chapter 2, section 2.4.6). This association between loci was confirmed when no recombination was found to have occurred in these linkage sites since the two mechanisms oppose one another (Chapter 2, section 2.4.7). It could be suggested that these associations between loci are deliberately being conserved in this manner to support or maintain amitraz resistance within the population. If resistant loci become fixed in the population, they may passively carry these linked loci along with them to fixation. This is known as genetic hitchhiking which reduces the variation between tightly linked loci (Halliburton 2003).

Ancestral recombination graphs that were generated for the resultant haplotypes displayed no geographical significance in the grouping of isolates. This could be as a result of free haplotype movement between different geographical areas thus preventing the discriminate grouping of haplotypes.

Additional restriction enzyme mapping indicated that the enzyme *Eci*I recognized a site within locus 13 closely associated with the two published SNPs. Therefore, a mutation at this site would render the enzyme incapable of digesting the DNA fragment, indicating the tick to be resistant (Chapter 2, section 2.4.8). Analysis of five *R. decoloratus* larvae, previously analyzed via LPT, indicated that an RFLP diagnostic approach has great potential in diagnosing amitraz resistance. Consequently, two samples (5 and 6) revealed homozygous resistance by means of the RFLP analysis that was confirmed following comparison with the LPT results. The remaining samples displayed heterozygosity by the use of RFLP analysis and therefore their status was unknown. However, when comparing these results to that obtained from the LPTs, it appears that heterozygous samples are susceptible to amitraz treatment. The latter therefore substantiates, in part, the claims that amitraz resistance is recessively inherited (Fragoso-Sanchez *et al.* 2011). However, more samples need to be analyzed before this can be proved conclusively.

In conclusion, this study represents the first report of the occurrence of the two SNPs published by Chen *et al.* (2007) in South African field populations of ticks, confirming their presence in known resistant larvae and their absence in the susceptible. It is also the first report of any association studies and evolutionary histories being conducted for the octopamine receptor in ticks. Furthermore, this study proposes an alternative assay to diagnose amitraz resistance in *R. microplus* and *R. decoloratus* ticks which is less time-consuming and more cost effective. As a result, a patent application is currently in progress for this novel diagnostic tool. Lastly, this study is the first indication that heterozygous field

populations are susceptible to amitraz treatment by means of genotypic analysis using RFLPs.

Naturally, further studies are required to fully elucidate the outcomes from this study. However, it does provide a first proof of concept and an improved baseline to work from, which previous studies have been unable to do. Finally, the results presented in this study do not exclude the possibility of a multigenic approach to amitraz resistance, nor does it exclude metabolic detoxification from playing an important role in the complex resistance mechanism.

3.2 Pyrethroid resistance and the voltage-gated sodium channel

Morgan *et al.* 2009 discovered a mutation in domain II segment 4-5 linker region of the voltage-gated sodium channel in *R. microplus* ticks (Morgan *et al.* 2009). This C to A mutation was shown to be under high selection pressure upon exposure to pyrethroids. It was also suggested that the resistant alleles were inherited recessively because heterozygotes didn't convey the same level of resistance when compared to bioassay results (Morgan *et al.* 2009). An additional mutation was then discovered in the same region which seemed to be associated with flumethrin but not cypermethrin resistance (Jonsson *et al.* 2010). Of the two mutations only the one reported by Morgan *et al.* 2009 was found in *R. microplus* ticks within South Africa during the current study. The frequency of the homozygous resistant (RR) genotype within the population was estimated to be 58.8%. The relative abundance of homozygotes suggests that directional selection is taking place favoring the formation of resistant homozygotes in the presence of pyrethroids.

Novel nucleotide substitutions were found when comparing sequences from female and male ticks. It has been shown that allele frequencies may differ in males and females (Halliburton 2003). The overall effect of these substitutions cannot be determined by sequencing alone and require *in silico* docking for further analysis.

Additional linkage studies showed no significant associations between the resistant locus of the sodium channel with any of the other loci analyzed. Furthermore, no recombination was evident in the linker region of the gene either. Final ancestral recombination graphs also displayed no geographical significance in the groupings of the resultant haplotypes. Since only a very small portion of the gene encoding the voltage-gated sodium channel was analyzed, no other conclusions could be made other than the resistant status of the tick isolates. Therefore, it can be concluded that the main mechanism of pyrethroid resistance in

South African populations of *R. microplus* ticks is due to this single point mutation. Results from this study provide the very first genotypic assemblage of pyrethroid resistance in South Africa to accompany the usual phenotypic data obtained from LPTs.

3.3 Carboxylesterase and GABA-gated chloride channel

No significant evidence of resistance associated SNPs in either of the two genes encoding carboxylesterase and the GABA-gated chloride channel could be identified. Linkage studies were previously conducted between carboxylesterase and the sodium channel since published SNPs reported in both genes have been linked to pyrethroid resistance. However, this analysis illustrated that there was no association between the variable positions in either of these two genes. Linkage studies were not done for the GABA-gated chloride channel as no variable sites were present which is essential for this type of analysis.

3.4 Considerations and future perspectives

The prevalence of resistance appears to correlate with the density of *R. microplus* ticks found within a certain geographical area (*i.e.* Kwa-Zulu Natal displayed a higher tick density and hence an increased level of resistance). However, this was largely dependent on the collection efficiency of Zoetis (Pty) Ltd. representatives, since some collected more ticks than others. The prevalence of resistance within certain geographical areas did not seem to be related in any way to haplotypes generated from genotypic data. This limitation could be addressed in the future by performing more extensive sampling. This will clarify whether haplotypes move freely across geographical areas making their distribution uniform as seen in this study, or if discrimination between haplotypes can be made following screening of a larger sample size. Additionally, the SNPs reported in this study are being screened in *R. decoloratus* ticks across South Africa. It could therefore be suggested that perhaps interspecies comparisons of acaricide resistance between *R. microplus* and *R. decoloratus* could generate haplotype data that is significantly associated with certain geographical areas. This could be due to the observed phenomenon where *R. microplus* has previously displaced *R. decoloratus* (refer to Chapter 1, section 1.1.1).

The full effect of all SNPs reported in this study cannot be determined by sequence information alone and needs to be further analyzed through techniques such as *in silico* structural analysis, which is currently underway. The activities of the wild-type and mutant form of carboxylesterase from *R. microplus* exposed to pyrethroids are currently being tested

along with *in silico* docking studies. This will provide a more holistic overview of the SNPs reported in the carboxylesterase gene.

For improved comparisons of current results, adequate *R. microplus* reference strains need to be established or obtained from other institutions. Reference strains used in previous studies are shown in Table 3.1. Acquiring a few of these reference strains is currently underway.

Table 3-1 Summary of all *R. microplus* reference strains including their country of origin, which class of acaricide they are resistant to, as well as their mechanism of resistance. OP: organophosphate, SP: synthetic pyrethroid, A: Amitraz, ML: macrocyclic lactones, F: fipronil, Ti: Target site insensitive, Est: carboxylesterase resistance, GSH: glutathione-S-transferase resistance, cyt: cytochrome P450 resistance, AChE: acetylcholinesterase resistance, ABC: ABC transporter resistance, ↑: up-regulation of detoxification enzyme and NMR: no mechanism reported (Table adapted from R. van Wyk, MSc student, University of Pretoria).

Tick strain	Country	Resistant to	Mechanism	Reference
Gonzalez	USA	Susceptible	n/a	(Chen, He, Davey 2007)
Tuxpan	Mexico	OP	Ti [AChE(rBmAChE1)] + Est10↑	(Temeyer, Pruett, Olafson 2010)
Coatzacoalcos	Mexico	SP + OP	Est9↑ + cyt	(Guerrero, Davey, Miller 2001)
Corrales	Mexico	SP	Ti(sodium domain II leu>Ile)	(Guerrero, Lovis, Martins 2012)
San Felipe	Mexico	SP	Ti(sodium domain II leu>Ile)	(Li *et al.* 2008)
Pesqueria	Mexico	OP + A	GSH	(Li *et al.* 2008)
Tuxtla	Mexico	OP	Ti + Est↑ + cyt↑	(Guerrero, Lovis, Martins 2012)
Caporal	Mexico	OP	Ti + cyt↑(coumaphos)	(Li *et al.* 2008)
San Roman	Mexico	OP	Ti + cyt↑(coumaphos)	(Li *et al.* 2008)
San Alfonso	Mexico	OP + SP + A	GSH	(Fragoso-Sanchez *et al.* 2011)
Canestrini	Mexico	SP	Ti(sodium domain III Phe>Ile)	(Miller, Davey, George 1999)
Santa Luiza	Brazil	SP + A + ML	Ti(sodium domain II leu>Ile) + Ti(amitraz) (ML undetermined)	(Li *et al.* 2008)
Uberlandia	Brazil	OP + ML	AchE↑ (ML undetermined)	(Baffi, De Souza, Vieira 2007)
MuÑoz	Brazil	Susceptible	n/a	(Guerrero *et al.* 2007)
Jaguar	Brazil	SP + OP + ML + A	ABC	(Pohl *et al.* 2011)
G Goya	Argentina	OP	Ti(unidentified)	(Guerrero, Lovis, Martins 2012)
Mozzo	Uraguy	F	NMR	(Baffi, De Souza, Vieira 2007)

Future work will include deep RNA sequencing (RNA-seq) of the entire transcriptome of amitraz treated *R. microplus* ticks to authenticate the true target site(s) of amitraz, as well as additional enzyme-based biological assays to further corroborate findings. This will allow for both novel SNP discovery, as well as expression level detection to determine whether target site insensitivity or metabolic detoxification are the main drive behind amitraz resistance.

Genome information for *R. microplus* is very limited due to its highly repetitive nature and consequent difficulties experienced with assembly of the genome (Moolhuijzen *et al.* 2011; Bellgard *et al.* 2012). Genomic DNA from gene-enriched regions has been assembled providing 1.8 Gbps of genome information. Genomic and transcriptomic data that is currently available represents only a 0.9X coverage of gene-coding regions from the tick genome (Bellgard *et al.* 2012). The genome size of *R. microplus* is estimated to be twice the size of the human genome (7.1 Gbps) with more than 70% being repetitive (Guerrero *et al.* 2010; Bellgard *et al.* 2012). The genome is essentially AT-rich with an estimated GC content of 44-48% (Bellgard *et al.* 2012). Therefore, once the genome of the *R. microplus* tick has been acquired, more efficient primers can be designed for screening of current reported SNPs, as well as novel SNPs that may arise from RNA-seq analysis. A summary of limitations and suggestions to overcome these limitations are shown in Figure 3.1.

In the current study, target-site insensitivity within the GABA-gated chloride channel resulting in dieldrin resistance was investigated. It is thought that MLs also target this channel (Guerrero, Lovis, Martins 2012), but no SNPs were found within this gene. It was recently reported that ivermectin targets Cys-loop receptors which include both the GABA and glutamate-gated chloride channels (Lynagh, Lynch 2012). Therefore, future work will include the development of a sequence-based screening method for SNPs in the glutamate-gated chloride channel.

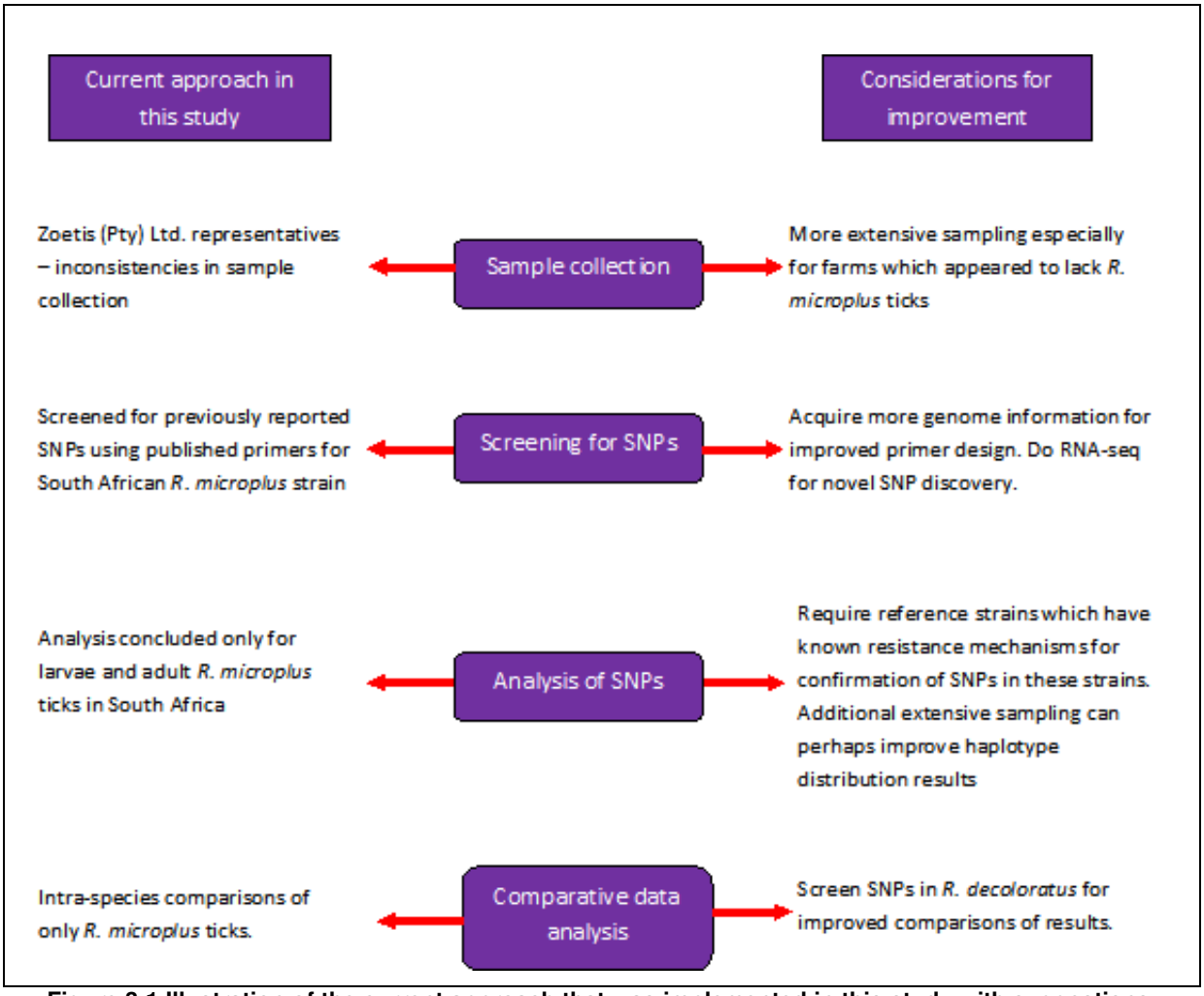


Figure 3:1 Illustration of the current approach that was implemented in this study with suggestions for improvement. Areas with major drawbacks are indicated in the above Figure, with the current approach that was taken indicated on the left, and the way forward for improvement indicted on the right.

In conclusion, farmers will receive feedback regarding the resistance genotypes that were found on their farms. Though no specific recommendations can be made, farmers can be advised to seek counsel from commercial suppliers to develop a tailored tick control program for their specific farm. This will aid in managing the acquisition of resistance in local tick populations. Additionally, this study provides the first comprehensive analysis of acaricide usage on farms across South Africa. Since acaricide usage coincides with selection for resistant alleles, commercial entities and farmers can use this information to identify products that are no longer viable options for certain areas or farms. Therefore, they could market specific products based on the local tick population, thus increasing their level of control and efficiency in product distribution.

Due to the inability of bioassays to detect resistance mechanisms, implementing sequence-based screening could be a viable alternative to define target-site insensitivity. Accompanied with synergistic assays to define the involvement of metabolic enzymes, a more holistic overview of acaricide resistance can be obtained. The potential of RFLPs as a diagnostic tool for amitraz resistance may have a positive economic impact on commercial industries allowing them to effectively supply farmers with the correct acaricidal treatments. Extending this assay to include other resistance markers will also allow farmers to continuously screen local tick populations, influencing their choice of treatment and ultimately improving resistance management and tick control programs within the country.

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