Genetic diversity, acaricide resistance status and evolutionary potential of a *Rhipicephalus microplus* population from a disease controlled cattle farming area in South Africa

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Abstract

The Southern cattle tick, *Rhipicephalus microplus* is a hematophagous ectoparasite of great veterinary and economic importance. Along with its adaptability, reproductive success and vectoring capacity, *R. microplus* has been reported to develop resistance to the major chemical classes of acaricides currently in use. In South Africa, the Mnisi community in the Mpumalanga region offers a unique opportunity to study the adaptive potential of *R. microplus*. The aims of this study therefore included characterising acaricide resistance and determining the level and pattern of genetic diversity for *R. microplus* in this region from one primary population consisting of 12 communal dip-stations. The level of acaricide resistance was evaluated using single nucleotide polymorphisms (SNPs) in genes that contribute to acaricide insensitivity. Additionally, the ribosomal internal transcribed spacer 2 (ITS2) gene fragments of collected individuals were sequenced and a haplotype network was constructed. A high prevalence of alleles attributed to resistance against formamidines (amitraz) in the octopamine/tyramine (OCT/Tyr) receptor (frequency of 0.55) and pyrethroids in the carboxylesterase (frequency of 0.81) genes were observed. Overall, the sampled tick population was homozygous resistant to pyrethroid-based acaricides in the voltage-gated sodium channel (VGS) gene. A total of 11 haplotypes were identified in the Mnisi *R. microplus* population from ITS2 analysis with no clear population structure. From these allele frequencies it appears that formamidine resistance in the Mnisi community is on the rise, as the *R. microplus* populations is acquiring or generating these resistance alleles. Apart from rearing multi-resistant ticks to commonly used acaricides in this community these ticks may pose future problems to its surrounding areas.

Keywords: *Rhipicephalus microplus;* acaricide resistance; population genetics; internal transcribed spacer 2, ITS2; evolutionary potential

Introduction

In the wake of an ever increasing human population, the demand for livestock derived products as a source for nutrition and other commodities is growing steadily, especially for developing countries (Abbasi and Abbasi, 2015; Sibhatu et al., 2015). The threatened sustainability of nutrition and other commodities is hindered by ticks and tick borne diseases affecting various economic and agricultural sectors (Jongejan and Uilenberg, 2004; Sibhatu et al., 2015; Wright et al., 2014). This highlights the need to understand the intricate vector-host-pathogen interface to enable the development of strategies to mitigate both the direct and indirect damages incurred by ticks and their associated diseases (de la Fuente et al., 2007; Guerrero et al., 2006).

The Southern cattle tick, *Rhipicephalus microplus*, in particular, is recognised as the most economically devastating tick species worldwide (Guerrero et al., 2006). This hematophagous ectoparasite is of primary concern to the cattle industry, although it has been shown to parasitize other domestic animals as well (de la Fuente et al., 2007; Walker et al., 2003). Its success and impact as an invasive species can be attributed to several factors, including: high adaptability as it can displace endemic species (Adakal et al., 2013; George et al., 2004; Nyangiwe et al., 2013; TØnnensen et al., 2004); vector competence for several pathogenic species (incl. *Anaplasma*, *Borrelia* and *Babesia spp*.) (de la Fuente et al., 2007; Jongejan and Uilenberg, 2004; Lynen et al., 2007; Madder et al., 2011); as well as rapid development of resistance to major chemical classes of acaricides (De La Fuente et al., 2000; Li et al., 2007; Rajput et al., 2006; Rodriguez-Vivas et al., 2011).

In 1979, Baker and co-workers reported the first incidence of resistance to acaricide treatment for *R. microplus* in South Africa (Baker et al., 1979). Since then, acquisition of resistance has become increasingly problematic (Lovis et al., 2013). Resistance in field populations can be gained within two years for *R. microplus* (Rodriguez-Vivas et al., 2011). This is due to this species' high reproductive potential, a short life cycle that enables 4 to 6 generations to be produced per year along with the intensive misuse of acaricides that sustains a high level of selection pressure on these populations (Chevillon et al., 2013, 2007; Rodriguez-Vivas et al., 2011). Therefore, adaptive resistance enables *R. microplus* to potentially evade the effects of acaricides. The principal mechanism of developing adaptive resistance in arthropods is via mutations in the acaricide target(s), rendering the target site(s) insensitive to the active compound (Chevillon et al., 2013, 2007). Metabolic resistance can also arise via genetic changes within a tick population, resulting in increased metabolism or sequestration of the acaricide (Guerrero et al., 2012). In this regard, enzyme families

involved in detoxification (or secondary metabolism) such as cytochrome P450's, esterases and glutathione S-transferases are generally up-regulated in metabolic resistance (Guerrero et al., 2012).

Acaricides are used extensively to control tick populations in South Africa. Resistant South African *R. microplus* populations have been reported as early as 1979 against arsenic-, organochlorine-, organophosphate- and amidine-based acaricides (Baker et al., 1979). More recently, amitraz resistance in South African *R. microplus* populations have been associated with mutations in the octopamine/tyramine (OCT/Tyr) receptor gene (Baron et al., 2015). Nucleotide substitutions within this receptor at positions 22 (A>C) and 65 (T>C) results in a corresponding amino acid change of threonine to proline and leucine to serine, respectively (Chen et al., 2007). These substitutions have been correlated to amitraz resistance in *R. microplus* using Larval Pocket Tests (LPT) but the functional implications of these substitutions remain to be elucidated (Baron et al., 2015). In regards to insensitivity to pyrethroid acaricides, single nucleotide polymorphisms (SNPs) in the carboxylesterase and the voltage-gated sodium channel (VGS) genes of *R. microplus* have been implicated in conferring resistance (Hernandez et al., 2000; Morgan et al., 2009; Rodriguez-Vivas et al., 2011). A SNP in the carboxylesterase gene at position 1120 (G>A) results in an amino acid change from aspartate to asparagine (Hernandez et al., 2000). Mutations in domain II S4–5 linker region of the VGS channel gene have been associated with pyrethroid resistance for *R. microplus* (Rodriguez-Vivas et al., 2011). An additional mutation at position 190 in the nucleotide sequence (C>A) results in an amino acid change from leucine to isoleucine that confers a moderate level of resistance to permethrin, cypermethrin and flumethrin (Morgan et al., 2009). The mutation in this domain has been shown to be present in the South African *R. microplus* population (Guerrero et al., 2012; van Wyk et al., 2015).

Currently, four similar bioassays are available to test for acaricide resistance each with their own advantages and disadvantages. The Adult Immersion Test (AIT), although providing results within 7 days, requires a large number of engorged females (Drummond et al., 1973). The Larval Pocket Test (LPT) (Stone and Haydock, 1962) and Larval Immersion Test (LIT) (Sabatini et al., 2001; Shaw, 1966) both involve the use of larvae and require 5-6 weeks to complete. Similarly, the Larval Tarsal Test (LTT) reports similar results as the two previous tests but circumvents direct contact with larvae (Lovis et al., 2011). In contrast, various molecular techniques such as target gene amplification, allele-specific PCR and probe-based quantitative PCR assays have been developed for detecting acaricide resistance in ticks (Chen et al., 2007; Guerrero et al., 2001; Hernandez et al., 2002; Rodriguez-Vivas et al., 2011; Wada and Iwamoto, 2009). These molecular based assays are sensitive and rapid alternatives to bioassays for determining the status of acaricide resistance within a

population to enable rational selection of appropriate treatments for parasite control (Jonsson et al., 2010). An advantage of PCR sequence based assays is the possibility of detecting known and novel mutations in the same gene. For these reasons, PCR-based assays have been used in this study.

To date, no study has reported on the concurrent evaluation of the genetic diversity and acaricide resistance status of a *R. microplus* population. This is essential as the potential for the success of an invasive species such as *R. microplus*, is also linked to the level of genetic diversity within a population (McNeely et al., 1990). This is based on the principal that a higher level of haplotype diversity is advantageous for populations to evolve in response to environmental changes. In contrast low levels of diversity are linked to the reduction of population fitness via inbreeding depression (Frankham, 1998, 1997). This leads to the expectation that levels of heterozygosity and fitness, at the population level, are correlated (Reed and Frankham, 2003). Typically, non-coding regions in the genome have been used as phylogenetic markers in tick populations (Barker, 1998; Murrell et al., 2001; Shaw et al., 2002). These include the second internal transcribed spacer region (ITS2) of the nuclear ribosomal gene cluster. Since the ITS2 region does not encode for proteins it is thought that it is under minimal selection pressure, which in turn allows for the accumulation of nucleotide substitutions (Barker, 1998; Coleman, 2003; Song et al., 2011). Thus, the genetic variation in this region may allow for the elucidation of evolutionary relationships within and between species.

The Mnisi community in the Mpumalanga region of South Africa offers a unique opportunity to study both the genetic diversity and level of acaricide resistance of *R. microplus.* The sub-tropical climate in combination with a high cattle host density provides suitable conditions for many tick species. Since this is a foot and mouth disease controlled area, with scheduled application of acaricides on livestock, it offers an opportunity to study the cattle tick, *R. microplus*, to help determine factors governing evolution of this tick species

In this study, the screening of acaricide resistance markers and the ITS2 region was employed to assess the level of acaricide resistance and genetic diversity of *R. microplus* respectively. From this foundation, further studies can elucidate the effect of the application of a selection pressure to the rate of development of resistance and evolutionary potential in this area. To our knowledge this is the first study to report on the concurrent evaluation of the genetic diversity and acaricide resistance status of a *R. microplus* population. Finally, from a social and economic view this study will also aid in the rational selection of effective chemical control and in conjunction with better management strategies, improve tick control in this area.

Methods

Area of study and sample collection

Adult female *R. microplus* ticks were collected from cattle at 12 communal dip stations (Fig. 1, panel A). Tick collection was performed according to the terms and provisions of the National Environmental Management: Biodiversity Act (Act of 2004, permit number: 005233). The Mnisi research area falls within the savannah ecosystem, overlapping with the adjacent Andover and Manyleti provincial game reserves. It is situated within a subtropical climate region and contains a high cattle host density, with 14 500 registered head of cattle in an area of approximately 30 000 ha. Tick control methods consist of weekly acaricide dipping regimes which consist of formamidine (amitraz) and pyrethroid based acaricides. Tick sample collection took place between March 2012 and May 2013 via random sampling from a number of cattle hosts at the respective dip stations.

Biological materials used in the study

Adult female *R. microplus* ticks collected from each dip station in the Mnisi area were confirmed based on morphological characteristics up to genus level. Species identification was further conducted microscopically based on taxonomic descriptors (Walker et al., 2003) and morphological characteristics specific to *R. microplus* and *R. decoloratus* (Madder and Horak, 2010). Species identification was confirmed using the ITS2 PCR-RFLP test (Lempereur et al., 2010). Laboratory strains of *R. microplus* and *R. decoloratus* from Clinvet International (Pty) Ltd. (Bloemfontein, South Africa) were used as positive and negative controls. Genomic DNA (gDNA) isolation from 64 female *R. microplus* ticks was performed using a modified NaCl-based protocol (Baron et al., 2015).

Amplification and sequencing of target gene fragments

To evaluate the level of acaricide resistance in the population, SNP detection was done for genes known to contribute to acaricide resistance or insensitivity. Published primers (Chen et al., 2007), were used for the amplification of the OCT/Tyr gene fragment at an annealing temperature of 56°C. For the amplification of the carboxylesterase gene fragment, published primers (Hernandez et al., 2002, 2000) were used with an annealing temperature of 68°C. Allele specific PCR (AS-PCR) was carried out in two separate reactions using the published

primers as described by Guerrero et al. (2001) to detect both the resistant and susceptible alleles in domain II S4-5 linker region of the VGS channel. The annealing temperature of both reactions was optimised at 60°C.

Target gene amplification was performed using an Applied Biosystems® 2720 Thermal Cycler and verified using the ChemiDoc™ XRS+ System with 3% agarose gels stained with ethidium bromide (0.375 µg/ ml). The column-based PureLink™ Pro 96 PCR Purification Kit (Invitrogen) was used according to manufacturer's instructions. Sequencing was performed on an Applied Biosystems (ABI) 3730XL DNA Analyzer (Macrogen Inc., Netherlands) according to the standard dye terminator cycle sequencing strategy.

Sequence alignment and data analysis for acaricide resistance genes

Nucleotide sequence quality was assessed and processed using the BioEdit sequence alignment editor version 7.2.0 (Hall, 2013). Each sequence was manually curated to determine the reliability of base calling. Areas with low confidence were removed before assembling the multiple sequence alignments using the online alignment tool MAFFT version 6 (http://mafft.cbrc.jp/alignment/software/) (Katoh and Standley, 2013). The confirmed OCT/Tyr gene fragments were compared via sequence alignment to NCBI sequence entries for susceptible (Accession: EF490688.1) and resistant (Accession: EF490687.1) *R. microplus* ticks. Confirmed carboxylesterase gene fragments were aligned with NCBI sequences entries for susceptible (Accession: AF182282.1) and resistant (Accession: AF182283.1) *R. microplus* ticks.

Sequence alignment and data analysis for the ITS2 gene fragment

Consensus sequences were assembled using the forward and reverse sequences from the ITS2 sequence data. Multiple sequence alignments were performed as described previously using the following parameters in MAFFT: G-INS-i (global pairwise algorithm), 1PAM/k=2 scoring matrix (for closely related sequences) with gap opening penalties set at 1.5 and the offset value at 0.5. The sequence alignment was imported into DnaSP version 5.1 where haplotypes were computed using default parameters (Librado and Rozas, 2009). Nuclear data was phased using the Bayesian PHASE algorithm (Stephens and Donnelly, 2003) in DnaSP version 5.1 based on 1000 burn-in iterations and a 95% confidence interval (Librado and Rozas, 2009). Haplotype (Nei, 1987) and nucleotide diversity (Tajima, 1993) was calculated for the data set in DnaSP version 5.1. Values of Tajima's D were calculated from

the total number of segregating sites and used to assess population expansion (Tajima, 1989). Estimation and testing of Tajima's D statistic was done using MEGA version 5 (Tamura et al., 2011). Departure from the expectation of a sudden expansion model was estimated by calculating Harpending's raggedness index (Harpending, 1994) in DnaSP version 5.1. The relationships between haplotypes were investigated by generating a rooted haplotype network using the statistical parsimony software TCS (Clement et al., 2000) and the Median Joining algorithm in Network 4.6.1.1 (Fluxus Technology). To visually represent the geographical distribution of the haplotypes of the Mnisi *R. microplus* population, a map was plotted with the relative haplotype data at each geographical point.

Results

Amplification and analysis of acaricide resistance associated genes

A total of 64 individual ticks from 12 dip stations were subjected to genomic DNA extraction and target gene amplification (summarised in Table 1). Sequence information for the OCT/Tyr receptor gene fragment was obtained for 31 samples (62 alleles). The presence of SNPs conferring resistance to amitraz was evaluated and the frequencies of susceptible (AA/TT), heterozygous (AC/TC) and resistant (CC/CC) genotypes were calculated as 0.4516, 0.5161 and 0.0322, respectively. The relative distribution of these genotypes showed that the resistance alleles are starting to spread across several dip stations (Fig. 1, panel B).

Of the 30 samples from which the carboxylesterase gene fragment was successfully amplified, sequencing was successful for 15 (30 alleles). A large amount of sequence diversity was observed throughout the gene fragment, showing SNPs at a number of additional loci together with the SNP conferring pyrethroid resistance. The latter results in a non-synonymous nucleotide substitution at position 1120 (aspartate to asparagine). This SNP was identified in all sequence verified samples in either a heterozygous or homozygous state. The frequencies of the susceptible (GG), heterozygous (GA) and resistant (AA) genotypes were 0.0000, 0.3333 and 0.6667, respectively. The distributions of these genotypes indicate the rarity of the susceptible genotype across 5 dip stations (Fig. 1, panel C).

Finally, analysis of the allele specific PCR (AS-PCR) for the VGS channel indicated a homozygous resistant (AA) genotype for all 49 individuals (98 alleles) with a frequency of 1 across 10 of the dip stations (Fig. 1, panel D). This was confirmed through sequence

verification which revealed the presence of a nucleotide substitution at position 190 in the resistant tick samples. It appears as though some samples at dip stations display multiple resistance to more than one class of acaricide (Table 1).

Haplotype diversity and allele network analysis

A 380 bp sequence alignment was generated from the ITS2 profile of 41 ticks (82 alleles). Following sequence alignment of the individual isolates, a total of 8 variable sites were found between the alleles (summarised in Table 2). The sampled population differentiated into 11 haplotypes (*N*=11). Haplotype and nucleotide diversity was found to be high (0.7735) and low (0.0051) respectively, suggesting population expansion. Tajima's D was negative for the Mnisi population (-0.998) suggesting population expansion. Signatures of population expansion of the Mnisi *R. microplus* population is also suggested by the mismatch distribution and Harpending's ruggedness statistic (0.2466) (Fig. 2).

The ancestral population of Mnisi falls into the haplotype 1 category (Fig. 3). The number of unique haplotypes forming a star like distribution around the historical haplotype supports the theory of population expansion (Rogers and Harpending, 1992). Considering the relative distribution of haplotypes, it can be seen the greatest level of genetic diversity is localised to the central area (Fig. 4). The most Northern area of Mnisi is defined by the presence of only 3 haplotypes per dip station. The absence of a large number of haplotypes in this region suggests that there is no breeding of ticks from this region with the rest of the Mnisi *R. microplus* population following introduction to these areas.

Discussion

Acaricide resistance status of *R. microplus* in Mnisi

Previous work suggests that two nucleotide substitutions in OCT/Tyr receptors contributes to amitraz resistance based on phenotypic resistance observed in both Mexican and Brazilian *R. microplus* strains (Chen et al., 2007; Li et al., 2005; Rosado-Aguilar et al., 2008). A related study by Baron and colleagues in 2015 confirmed the presence of these two nucleotide substitutions in South African *R. microplus* field strains (Baron et al., 2015). These two associated resistance SNPs were found to both occur as heterozygotes in the Mnisi population.

With the abundance of heterozygosity in the population within this gene it can be hypothesised that balancing selection is acting on the population. The continued use of amitraz in the area ensures that a strong selection pressure is maintained for keeping these resistance conferring alleles in a heterozygous state, similar to results obtained in a Mexican field population (Rosado-Aguilar et al., 2008). The predominance of the heterozygosity at both SNP positions throughout the population can either point to slow allele fixation or to a high genetic load at these loci, i.e. a positive fitness cost. The assumption of a fitness cost being linked to a particular genotype may be associated with a significant disadvantage for homozygous resistant organisms when compared to a heterozygous or susceptible individual in a population (Kliot and Ghanim, 2012). The complex interaction between the continuous presence of amitraz selection pressure and the proposed fitness cost associated with a homozygous resistant genotype could potentially explain the heterozygous superiority observed. These results are similar to those published by Baron et al. 2015, where the South African *R. microplus* tick population was tested for the presence of these SNPs.

Considering resistance to pyrethroid-based acaricides in the VGS channel, several point mutations in this gene have been linked to pyrethroid insensitivity for numerous insect species (Dong, 2007). It is believed that pyrethroids keep the VGS channel in its open state leading to hyper-excitation in the tick's nervous system (Dong, 2007). The resistance mutation at base pair position 190 indicates a site that confers a mild form of resistance to pyrethroid-based acaricides (Jonsson et al., 2010). Synthetic pyrethroid resistance has also been associated with esterases (Baffi et al., 2007; Hernandez et al., 2002). Work from de Jersey and colleagues linked the involvement of esterases to pyrethroid-based detoxification in *R. microplus* (De Jersey et al., 1985). The enhanced activity of esterase(s) by insects has furthermore been linked to increased detoxification of insecticide-esters by a two-step process of sequestration and hydrolysis (Flores et al., 2005). In blow flies, a single amino acid substitution found shown to result the increased ability of a carboxylesterase enzyme to hydrolyse pyrethroids more efficiently (Newcomb et al., 1997). In ticks, a vast number of esterases have been shown to directly contribute to pyrethroid resistance in *R. microplus* (Chevillon et al., 2007) and *R. sanguineus* (Miller et al., 1999). Whether the resistance can be attributed to enhanced sequestration or hydrolysis remains to be elucidated.

The Mnisi population was found to be homozygous resistant for the SNP in the VGS channel. A deficit in heterozygotes is indicative that these alleles are strongly driven by selection in the population. The paucity of the susceptible allele in the population can be linked to directional selection which favours the fixation of this resistance allele (Halliburton and Halliburton, 2004). It can therefore be postulated that the homozygous resistant genotype has a fitness benefit in the presence of pyrethroid selection pressure. Positive

selection occurs when a beneficial mutation arises within an organism, and the difference in fitness cost between the mutant allele and the wild-type results in a greater probability of passing that mutant allele to the offspring. If no fitness cost or benefit is associated with the allele, it may be stochastically fixed or lost in the population via genetic drift (Hughes et al., 1999). The lack of heterozygosity in the population can also be explained by the rapid fixation of the resistance allele, as with positive selection the heterozygous state is expected to be relatively short-lived in the population (Henning and Teuber, 1996).

Both heterozygous and homozygous genotypes were observed that the SNP associated with carboxylesterase mediated resistance in the Mnisi population. Since both the VGS channel and carboxylesterases are involved in pyrethroid resistance, it is noticed that homozygous resistance occurred in the entire population in the VGS channel prior to that of the carboxylesterase gene fragment. This may suggest directional selection favouring the pyrethroid resistant phenotype within the VGS channel. Based on the allele frequencies it can be hypothesised that it would be more energy efficient for target site insensitivity to occur before the up-regulation of pathways utilising carboxylesterases. Further work needs to be done to determine if the two resistance mechanisms are synergistic or additive.

Multi-acaricide resistance

A number of previous studies have reported tick strains with multi-acaricide resistance to diverse combinations of chemical classes. Fernández-Salas and colleagues (2011) found *R. microplus* individuals from Brazil to be resistant to four classes of acaricides namely, organophosphates, synthetic pyrethroids, amitraz and ivermectin (Fernández-Salas et al., 2011). Other examples of multi-acaricide resistance were reported by Benavides and colleagues (2000) to organophosphates, synthetic pyrethroids and amitraz, as well as by Aguirre and Santamaría (1996) to organochlorines and organophosphates. Kunz and Kemp (1994) also reported tick strains resistant to synthetic pyrethroids and amitraz. Additional epidemiological studies have found tick populations with diverse resistance to acaricides such as organophosphates, synthetic pyrethroids and/or amitraz (Mendesa et al., 2011; Rodriguez-Vivas et al., 2011). In the case of the Mnisi *R. microplus* population ticks from five dip stations displayed the resistance SNPs in the OCT/Tyr gene, the VGS channel and/or the carboxylesterase gene fragment (Table1). Noteworthy is the fact that three dip stations display pyrethoid and amitraz resistance with regards to SNPs in the VGS channel, carboxylesterase gene fragment and the OCT/Tyr gene (Table 1).

Haplotype diversity and allele network analysis

The high haplotype diversity contrasted to the low nucleotide diversity, the negative value of the Tajima's D statistic, the star like distribution of the haplotype network, the mismatch distribution and the Harpending's ruggedness statistic all support that the Mnisi *R. microplus* population underwent rapid recent expansion. A general pattern of relatively high haplotype diversity and low nucleotide diversity was observed, which could be attributed to population expansion following a bottleneck (Grant and Bowen, 1998). Low nucleotide variability could be attributed to recent coalescence, while the higher haplotype diversity could be explained by retention of mutations in a rapidly expanding population (Grant and Bowen, 1998). The Mnisi area had an excess of rare haplotypes as indicated by the negative Tajima's D statistic suggesting recent population expansion, purifying selection, or a selective sweep (Braverman et al., 1995; Simonsen et al., 1995). The estimate for Tajima's D for the *R. microplus* population is in range with other species in which population expansion is suspected for ticks (Mixson et al., 2006). Due to rare alleles contributing more to the number of segregating sites than to numbers of pairwise differences, negative values reflect an excess of low frequency polymorphisms. Signatures of population expansion are also supported by the mismatch distribution and Harpending's raggedness index. Population expansion increases the retention of new mutations (Watterson, 1985) and creates an excess of haplotypes that only differ by one or a few mutations (Rogers and Harpending, 1992; Slatkin and Hudson, 1991). Noteworthy is the observation that the majority of the rare, expanding haplotypes (6, 8, 9 and 10) can be linked to multiple acaricide resistance in the central dip stations of Mnisi. This correlation will be closely monitored in subsequent future studies of the Mnisi area.

Conclusions

The present study was aimed as a pilot study at evaluating the acaricide resistance status and the level of genetic diversity in a partially isolated *R. microplus* population within the Mnisi area. Even due to limited sampling the overall results indicated a high prevalence of SNPs associated with acaricide resistance in all three gene targets tested throughout the population. Approximately half of the ticks sampled proved to be genotypically resistant to amitraz. With regards to pyrethroids, all of the samples analysed proved to be homozygous resistant for the mutation in the voltage-gated sodium channel conferring reduced susceptibility (i.e. mild resistance). The threat of severe resistance to pyrethroids is evident in three of the dip stations where mutations in both the VGS channel and carboxylesterase

gene fragment is found. A number of dip stations with resistance to both amitraz and pyrethroids have also been detected, and this poses a problem as both of these acaricides are frequently used in the Mnisi area. Analysis of the ITS2 gene fragment indicated a high level of genetic diversity within the Mnisi *R. microplus* population with a number of expanding haplotypes. These results contribute to the understanding of acaricide resistance in the population and further more serves as a baseline for subsequent pilot study for monitoring the acquisition of acaricide resistant SNPs in a tick population. Future studies may include a combination of these different genetic markers for population and phylogenetic analysis to pinpoint the underlying evolutionary forces acting on the *R. microplus* population of Mnisi and to an extension, South Africa. Lastly, integration of this data coupled with continued surveillance of tick populations will allow for rational selection of acaricides and aid in better pest management within the area.

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Figures

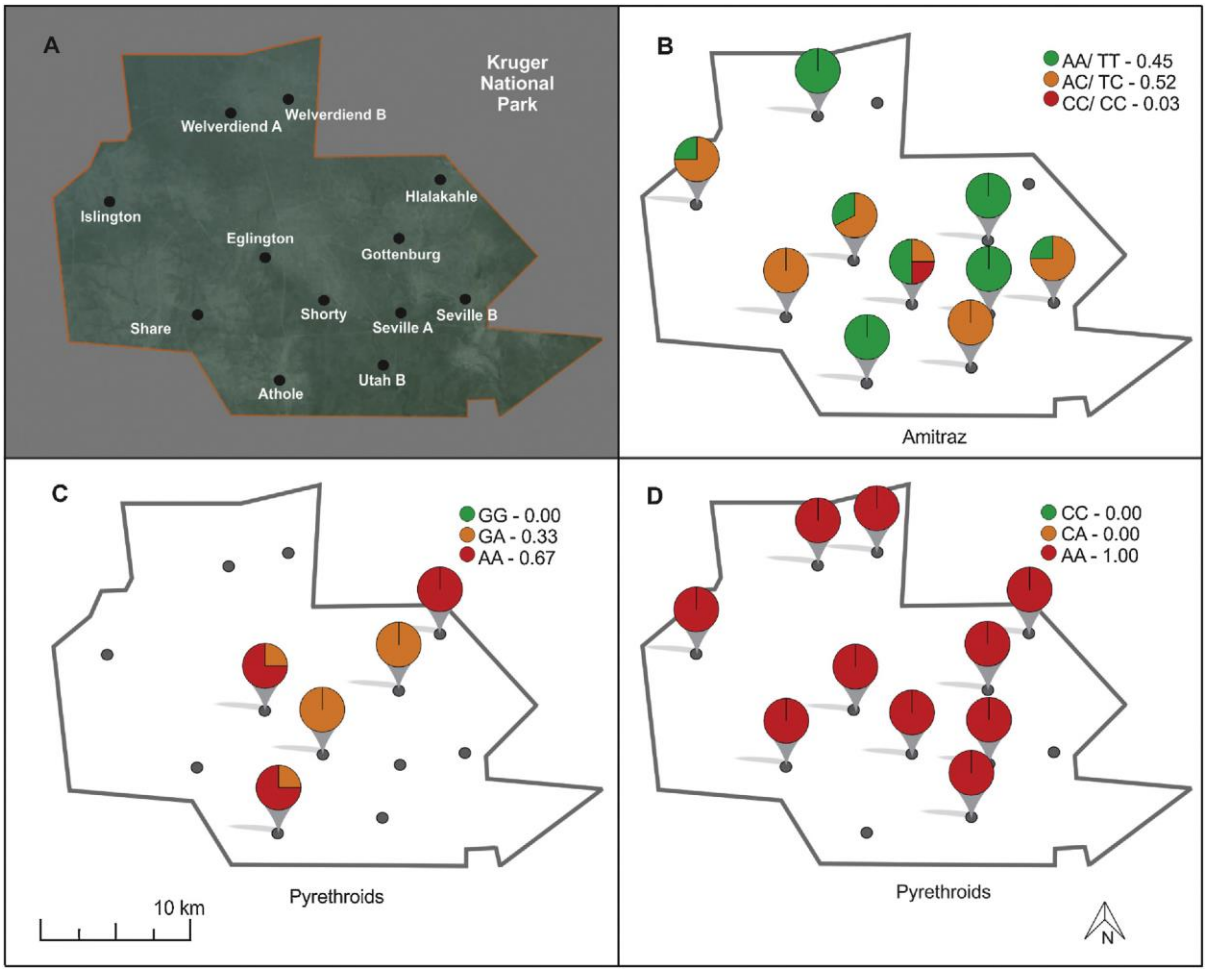

Fig. 1. The distribution of cattle dip stations in the Mnisi area and their related acaricide resistance alleles. (A) Topographical map of the Mnisi area with dip station distributions. (B) Genotypic frequencies and distribution of the OCT/ Tyr resistance alleles. (C) Genotypic frequencies and distribution of the carboxylesterase resistance alleles. (D) Genotypic frequencies and distribution of the VGS channel resistance alleles. Also indicated are homozygous susceptible (green), heterozygous (orange) and homozygous resistant (red) individuals.

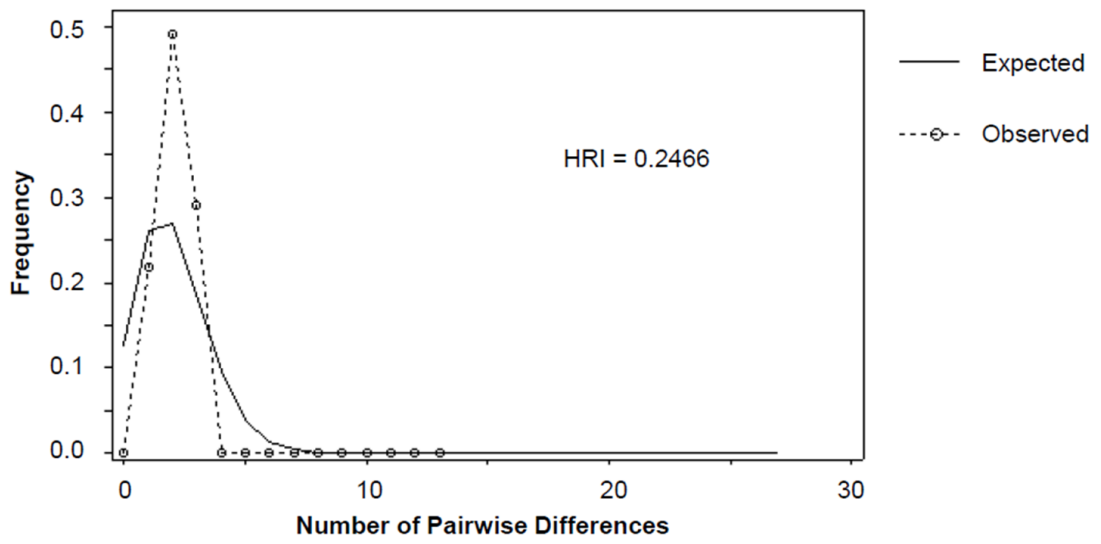

Fig. 2. The mismatch distribution of the pairwise number of differences among *R. microplus* haplotypes in the Mnisi area. The value of the Harpending's Raggedness Index (HRI) is given.

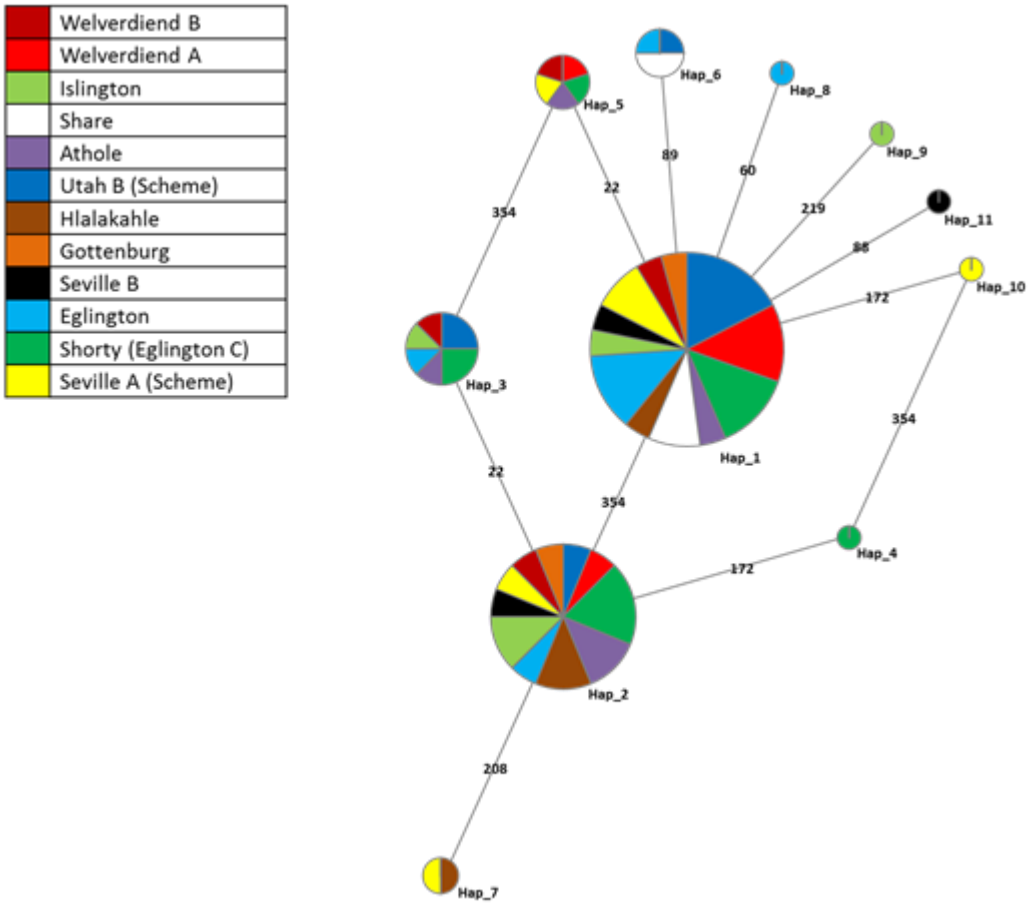

Fig. 3. Median joining and Statistical parsimony network of ITS2 haplotypes of *R. microplus* in the Mnisi area. The area of each circle is proportional to the frequency of a haplotype in the entire sample. Shading relates to the relative dip station at which the haplotypes occurred. In the cases where haplotypes were shared among dip stations, the shading is proportional to the frequency of the haplotypes in each region.

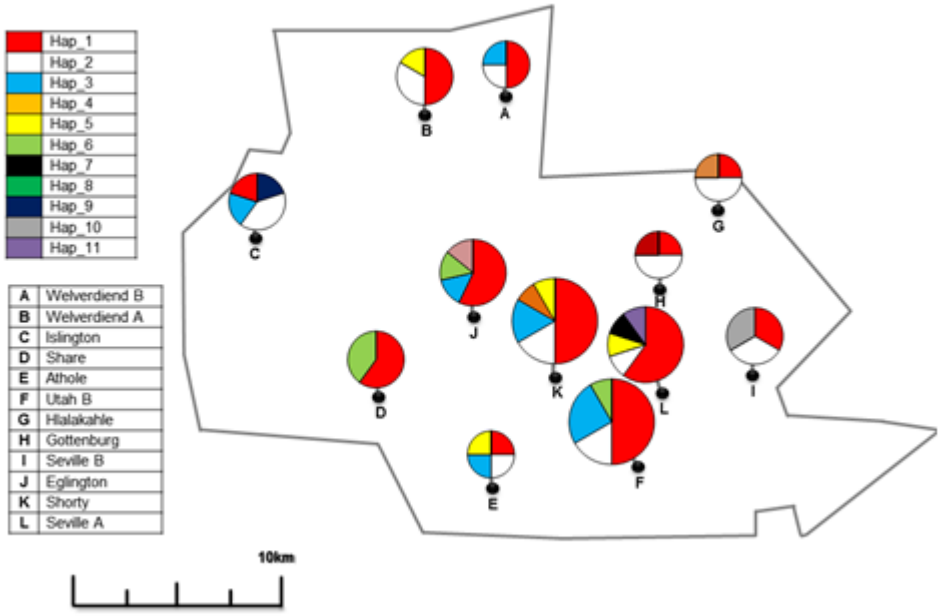

Fig. 4. The geographical distribution of haplotypes. Each circle represents a geographical point and its proportional haplotype contribution. Shading relates to the relative contribution of haplotypes at which the haplotypes occurred.

Tables

Table 1

Summary of samples processed and analysed per dip station. Numbers in brackets for the resistance assays indicate the number of: homozygous susceptible, heterozygous and homozygous resistant samples.

	Dip station	Number of samples processed	OCT/ Tyr resistance assay	Carboxylesterase resistance assay	VGS resistance assay	ITS2 assay
A	Welverdiend B	4	0 (0, 0, 0)	0 (0, 0, 0)	4 (0, 0, 4)	2
B	Welverdiend A	3	1 (1, 0, 0)	0 (0, 0, 0)	1 (0, 0, 1)	3
C	Islington*	6	4 (2, 2, 0)	0 (0, 0, 0)	6 (0, 0, 6)	3
D	Share*	6	3 (0, 3, 0)	0 (0, 0, 0)	6 (0, 0, 6)	3
E	Athole*	5	3 (1, 2, 0)	5 (0, 1, 4)	4 (0, 0, 4)	2
F	Utah B	6	2 (0, 2, 0)	0 (0, 0, 0)	0 (0, 0, 0)	6
G	Hlalakahle	7	0 (0, 0, 0)	2 (0, 0, 2)	7 (0, 0, 7)	2
H	Gottenburg	6	3 (3, 0, 0)	2 (0, 2, 0)	6 (0, 0, 6)	2
I	Seville B	4	4 (1, 3, 0)	0 (0, 0, 0)	0 (0, 0, 0)	3
J	Eglington*	6	5 (2, 3, 0)	5 (0, 1, 4)	6 (0, 0, 6)	4
K	Shorty*	6	4 (2, 1, 1)	1 (0, 1, 0)	6 (0, 0, 6)	6
L	Seville A	5	2 (2, 0, 0)	0 (0, 0, 0)	3 (0, 0, 3)	5
	Totals	64	31 (14, 16, 1)	15 (0, 5, 10)	49 (0, 0, 49)	41

*Dip stations where ticks displayed the resistance SNPs in the OCT/Tyr gene, the VGS channel and/or the carboxylesterase gene fragment.

Table 2

Haplotype frequencies and variable sites within the 380 bp alignment of the Mnisi *R. microplus* ITS2.

Haplotypes	Frequency	Nucleotide positions							
		22	60	88	89	171	207	218	353
Haplotype 1	0.37	G	T	G	C	A	G	T	G
Haplotype 2	0.25	.	.	.	.	.	.	.	A
Haplotype 3	0.13	A	.	.	.	.	.	.	A
Haplotype 4	0.02	.	.	.	.	T	.	.	A
Haplotype 5	0.08	A	.	.	.	.	.	.	.
Haplotype 6	0.06	.	.	.	T	.	.	.	.
Haplotype 7	0.03	.	.	.	.	.	A	.	A
Haplotype 8	0.02	.	C	.	.	.	.	.	.
Haplotype 9	0.02	.	.	.	.	.	.	A	.
Haplotype 10	0.02	.	.	.	.	T	.	.	.
Haplotype 11	0.02	.	.	C	.	.	.	.	.