Genotyping acaricide resistance profiles of *Rhipicephalus microplus* tick populations from communal land areas of Zimbabwe

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Abstract

Acaricide resistance is one of the greatest threats towards the successful control of vector ticks worldwide. Communal farmers of Zimbabwe use amitraz as the most common acaricide with occasional usage of pyrethroids and organophosphates. As a strategy towards developing an effective acaricide resistance management system in Zimbabwe, screening was done by genotyping *Rhipicephalus microplus* tick populations using molecular markers associated with resistance to these chemicals. The frequency of the mutant allele for the octopamine/tyramine receptor marker associated with amitraz resistance was high (0.55) and a large proportion 78.5% (288/367) of heterozygote genotypes were observed indicating balancing selection. Of the communal dipping tanks where *R. microplus* occurred 37.8% (39/103) showed complete resistance genotypes for amitraz. The carboxylesterase marker that has been associated with resistance to organophosphate and pyrethroids indicated no selection pressure in these chemical groups with a low frequency (0.052) of the mutant allele and 89.6% (329/367) sampled ticks showing homozygous susceptibility genotypes. Heterozygous genotypes were present at 27.2% (28/103) of the dipping tanks. The L641 mutation in the voltage–gated sodium channel gene associated with pyrethroid resistance was not detected in Zimbabwean samples. This would suggest a different mechanism of resistance to pyrethroids in these tick populations. Sequence analysis of the octopamine/tyramine receptor gene revealed the presence of other mutations in this region, it will be important to investigate their association with amitraz resistance. These results present the first molecular genotyping of resistance profiles of *R. microplus* tick populations from Zimbabwe.

Keywords: *Rhipicephalus microplus*, genotyping, acaricide resistance

1. Introduction

The deployment of acaricides is the most widely used strategy for the control of ixodid ticks affecting livestock in tropical and sub-tropical countries of the world (Abbas et al., 2014). The greatest challenge facing its implementation success is the accumulation of resistance in ticks to these chemicals (Rosario-Cruz et al., 2009). Subsequently, effective tick management is premised on the ability to detect and periodically carry out surveillance programmes on acaricide resistance (Ghosh et al., 2015).

Bioassays such as the Larval Packet Test (LPT) (Stone and Haydock, 1962), Larval Immersion Test (LIT) (Shaw, 1966) and the Adult Immersion Test (AIT) (Drummond et al., 1973) have traditionally been used to detect and monitor the acaricide resistance status of tick populations while the Larval Tarsal Test was recently introduced (Lovis et al., 2013). The increased length of time by which results from a bioassay are obtained, the need for live tick specimens and the inability to detect the genotype status of resistant individuals, negatively influences the effectiveness of these techniques. The development of molecular tools heralded by the design of an allele-specific PCR for the diagnosis of pyrethroid resistance in *R. microplus* ticks (Guerrero et al., 2001) has greatly improved acaricide resistance management. Molecular tools offer the distinct advantage that they are quick, do not require live tick specimens and enable the genotyping of the resistance status of tick populations.

Mutations have been identified in the voltage gated sodium channel gene in *R. microplus* which result in insensitivity to pyrethroids (Miller et al., 1999; He et al., 1999; Jonsson et al., 2010a; Morgan et al., 2009). An esterase in a pyrethroid resistant Mexican *R. microplus* strain was isolated and a mutation in the encoding gene was found by Hernandez et al. (2000), the same mutation was found in organophosphate resistant strains by Baffi et al. (2008). Mutations in genes encoding carboxylesterases will increase esterase hydrolytic activity on an acaricide (Jamroz et al, 2000). Chen et al. (2007) identified two mutations in a putative octopamine receptor gene that will result in target site insensitivity to amitraz. These mutations have enabled the development of molecular markers (Guerrero et al, 2001; Morgan et al., 2009; Hernandez et al, 2002; Baron et al., 2015) diagnostic for pyrethroid, organophosphate and amitraz resistance.

Ticks are an important constraint to livestock production in (sub)-tropical countries with annual losses estimated at USD$18.7 billion (De Clercq et al.,2012) and resource-poor communal farmers who own approximately 80% of the cattle are most affected (Rushton et al., 2002). These costs are related to their direct effects as blood sucking parasites which result in productivity losses and damage to hides affecting their quality and value on the market (Rajput et al., 2006). Indirect effects are foreseen in their being: vectors of pathogens which affects humans and animals, costs incurred for treating tick-borne diseases and controlling the vectors (de Castro, 1997). In Zimbabwe the most important tick-borne diseases are heartwater, babesiosis, anaplasmosis and theileriosis and these account for 60 percent of livestock mortalities annually (Sungirai et al., 2016).

In Zimbabwe communal farming systems, tick control is based on the use of a plunge dip, where acaricide chemicals are diluted in large volumes of water and cattle will be submerged in the dip wash. Farmers have to bring their cattle for dipping weekly and fortnightly during the rainy and dry season respectively. The central government supplies acaricides to these communal farmers who pay a nominal fee to the Department of Veterinary Services to have their cattle participate in these tick control programmes (Sungirai et al., 2016). It then becomes important for the governments to be made aware of the status of acaricide resistance so as to take remedial action. It has been observed that communal farming systems are characterised by : absence of acaricide rotation practises, poor surveillance of acaricide resistance, lack of training on the judicious use of acaricides and indiscriminate selling of these chemicals without the recommended active ingredients (Mendes et al., 2013). This may increase the selection pressure for acaricide resistance which may be difficult to reverse once it has been established.

Studies on acaricide resistance have been neglected in Africa although interest in the field is growing (Adakal et al., 2013; Adehan et al., 2016; Baron et al., 2015; Robbertse et al., 2016; Wyk et al., 2016). Much work on acaricide resistance has been reported from Central America, South East Asia, the Caribbean and Australia with the focus on *R. microplus* (*R. australis* in Australia) largely due to its undisputed global importance (Abbas et al., 2014). This tick species developed resistance to the major classes of acaricides in use in these areas, although the level of resistance differs between areas (Lovis et al., 2012).

The purpose of this study was to genotype the acaricide resistance profiles of *R. microplus* ticks collected from cattle in communal areas of Zimbabwe. The hypothesis was that due to the overreliance on the same type of acaricides by farmers and government, *R. microplus* populations in different parts of the country are undergoing selection pressure for resistance to amidines, pyrethroids and organophosphates. The results of this study will provide vital information to policy makers to come up with alternative strategies for improved tick control in light of current acaricide resistance profiles. This is the first report on the genotyping of acaricide resistance status in this tick species in Zimbabwe.

1.1 Materials and Methods

1.1.1 Biological materials and DNA extraction

Following a nationwide tick survey conducted in Zimbabwe (Sungirai et al., 2017), 383 *Rhipicephalus microplus* ticks collected at 103/322 communal dipping tanks were used for this study. The identity of *R. microplus* ticks which could not be resolved through morphology was confirmed using the ITS2 PCR-RFLP test (Lempereur et al., 2010). Whole genomic DNA was extracted from *R. microplus* ticks using the QIAamp genomic DNA kit (Qiagen, Hilden , Germany).

1.1.2 PCR conditions for the octopamine/tyramine receptor gene, carboxylesterase genes and voltage-gated sodium channel genes

The PCR assay conditions for the amplification of the octopamine/tyramine receptor gene, carboxylesterase and voltage-gated sodium channel genes genes were carried out as described by Baron et al. (2015), Hernandez et al. (2002) and Morgan et al. (2009) respectively. An additional PCR assay as described by Stone et al. (2014) was done to amplify a large fragment of the voltage-gated sodium channel gene for subsequent sequencing.

Each of the molecular markers were amplified for the 383 tick samples in a programmable thermocycler (Biometra©, Göttingen, Germany). For the carboxylesterase and octopamine / tyramine receptor genes, Restriction Fragment Length Polymorphism (RFLP) was done after amplification as described by Faza et

al. (2013) and Baron et al. (2015) respectively. The PCR and restriction digest products were loaded with dye on a 3% (w/v) agarose gel together with a 100 bp DNA super ladder (Thermo Fisher Scientific©, Waltham, Massachusetts, USA) for sizing the fragments. The gel was stained in 0.001% ethidium bromide solution for 30 minutes and the fragments were visualised using the Gel Doc™ XR+ gel documentation system (BioRAd©, Hercules, California, USA). Each reaction was run with a non-template control (nuclease free water) and positive controls of *R. microplus* which were resistant and susceptible to pyrethroids as well as amitraz. These were obtained from the Department of Genetics, University of Pretoria, South Africa.

1.1.3 PCR cloning and sequencing

Samples showing homozygous (susceptible and resistance) as well as heterozygous genotype profiles were selected for cloning and subsequent sequencing of the cloned products. Three samples for each of the profiles for the carboxylesterase gene, 4 for the voltage-gated sodium channel (all susceptible) and 10 for the octopamine/tyramine receptor (Figure 2) were used. The QIAquick PCR purification kit (QIAGEN) was used to purify the PCR product according to the manufacturer's instructions. The TOPO® TA Cloning Kit (Thermo Fisher Scientific) was then used to clone the purified PCR products by chemical transformation. The positive clones were then sent to the VIB Genetic Service Facility at the University of Antwerp for forward and reverse sequencing.

1.1.4 Data Analysis

Frequency counts were done for all the genotypes present for each marker and these were expressed as a proportion of the total number of individual ticks which had positively amplified. This enabled calculation of the overall proportion of the genotypes as well as the proportion at the dipping tank level. To test for evidence of selection pressure against the acaricides in use, Hardy-Weinberg Equilibrium (HWE) was examined for the markers where all the genotypes were recorded. This was done using the chi-square test by the software R and a package called "HardyWeinberg" (R Development Core Team, 2013). The forward and reverse sequences were edited using the BioEdit software (Hall, 1999). The final edited sequence was aligned with reference samples of the respective genes to check for the presence of mutations using the software ClustalW (Larkin et al., 2007). These were the para-sodium channel gene, GenBank Accession Number: AF134216.2; for the carboxylesterase, the Gonzalez strain Accession Number : AF182283; and for the octopamine/tyramine receptor gene, the Santa Luisa strain Accession Number: EF490688.1; as well as the Gonzalez strain Accession Number: EF490687.1. Maps showing the geographic distribution of genotypes at the communal dipping tanks where *R. microplus* occurred were generated using QGIS (QGIS Development Team, 2013).

1.2 Results

1.2.1 Single Nucleotide Polymorphisms (SNPs) in the Octopamine / Tyramine receptor gene

A total of 20 SNPs were found in the open reading frame of the G-protein coupled octopamine / tyramine receptor gene (Figure 1). Five of these mutations were transversion mutations in nucleotide positions; 22 (A->C), 141(C->G), 171(G->C), 207 (A->C) and 213 (C->A) while the rest were transition mutations (A<->G and C<->T). Eight nucleotide substitutions were similar to those found in the Santa Luisa strain (resistant) and these included the mutations at nucleotide position 22 (A to C) and 65 (T to C) which are associated with resistance to amitraz (Baron et al., 2015; Chen et al., 2007; He et al., 1999). These mutations are at nucleotide positions 157 and 200 in Baron et al. (2015). Also of importance is the SNP at position 39 in Figure 1 which has been found to be in linkage disequilibrium with the SNP at position 65 and found in resistant samples (Baron et al., 2015).

The RFLP profiles for the sequenced samples are shown in Figure 2. The expected RFLP profiles (Figure 2) were 409 bp for homozygous resistant genotype, 186 bp and 223 bp for the homozygous susceptible genotype, and 409 bp, 223 bp and 186 bp for the heterozygous genotype. Six different RFLP profiles consistently occurred (Figure 1 and Table 1). As a result, 32 samples were sequenced in order to verify for the presence of SNPs at the expected positions.

A check was done to ascertain whether other mutations observed were interfering with the restriction enzyme cutting site thus negatively affecting its utility as a molecular marker. This was done by carrying out a virtual RFLP of sequence data using the programme serial cloner (Perez, 2004). None of these mutations were found to be interfering with the restriction enzyme cutting sites.

To genotype the RFLP profiles, these were compared with corresponding sequences to check for the resistant published SNPs i.e. at position 22 and 65. Interestingly some more genotypes other than the ones described by Baron et al. (2015) were found at the SNPs. The RFLP profiles and the corresponding SNPs identified and the frequency of occurrence is shown in Table 1.

1.2.2 Single Nucleotide Polymorphism in the Carboxylesterase gene

Mutations were detected in the homozygous resistant and heterozygous genotypes (Figure 3). The published SNP that is associated with resistance is at nucleotide position 1120 (G->A), which is present in the heterozygous ERG_61 and homozygous ERG_65 samples, but absent in the susceptible sample (ERG_001). There were other mutations in the susceptible sample and these occurred in 15 other samples sequenced but they did not interfere with the restriction site, hence they would not affect the utility of the RFLP marker for resistance detection. The corresponding RFLP profiles are shown in Figure 4.

1.2.3 Genotype Frequencies and Hardy Weinberg Equilibrium

A total of 383 tick gDNA samples were PCR amplified for the three molecular markers. Not all of the samples were positive for each marker (Table 2). There was a high frequency of amitraz resistance in individual tick samples although a higher proportion of these were heterozygotes (78.5%, n=288/367), with 14.8% being homozygous resistant (n=59/367) and 5.5% (20/367) being susceptible. The proportion of the amitraz resistance-associated alleles in the population was estimated at 55%.

For the carboxylesterase marker , 9.8% (n=36/367) were heterozygous whilst 0.54% (n=2/367) were homozygous resistant, the remaining 89.6%, (329/367) were homozygous susceptible. The proportion of the resistant allele for carboxylesterase in the population was estimated at 5.2%. No resistant samples either in the homozygous or heterozygous state were obtained for the L64I mutation. All of the samples were homozygous susceptible (n = 350). HWE revealed selection pressure ($p<0.05$) for amitraz resistance while there was no selection pressure for organophosphates and pyrethroids using the carboxylesterase gene marker ($p>0.05$). Since there were no other genotypes recorded for pyrethroid resistance HWE could not be computed for the L641 mutation.

Complete resistance (RR) for the octopamine/tyramine receptor marker (n=39/103, 37.8%, 95% Confidence Interval (C.I.) estimates 34-53%) and moderate resistance (SR) for the carboxylesterase marker (n= 28/103, 27%, 95% C.I., 31-50%) was recorded at more than a third and close to a third of the dipping tanks respectively, Table 3. The voltage gated sodium channel marker showed susceptible genotypes at all the dipping tanks. Figure 5 and 6 show the distribution of the genotypes with the "RR" genotype showing complete resistance for amitraz being found in all the provinces where *R. microplus* was collected while for the carboxylesterase marker the genotype was present in two provinces at one dipping tank each. The "SR" genotype at the carboxylesterase marker which shows moderate levels of resistance was found in all the provinces except Matabeleland North.

1.2.4 Multiple Resistance

Multiple resistance was defined when an individual tick displayed homozygous resistant (RR) genotypes for the octopamine/tyramine receptor marker and “RR” and “SR” for carboxylesterase marker. Heterozygous individuals for amitraz were not considered resistant as these have shown to be susceptible. Five individual ticks showed “RR” for the octopamine / tyramine receptor and “SR” for the carboxylesterase and this was observed at 4 communal dipping tanks. Two of these tanks were located in Manicaland and two in Mashonaland Central. Interestingly 28/36 (77.8%) individuals with “SR” genotype for carboxylesterase marker had the same genotype for the octopamine/tyramine receptor marker.

1.3 Discussion

The findings of this study showed that there is high frequency of the resistant allele associated with amitraz resistance in the *R. microplus* population while the mutant allele associated with pyrethroid and organophosphate resistance has a low frequency. This suggests that the *R. microplus* population in Zimbabwe is undergoing selection pressure towards amitraz resistance and remains susceptible to the pyrethroids and organophosphates.

There was a large proportion of heterozygous genotypes associated with amitraz resistance observed in this study. These results are comparable to a study carried out by Baron et al. (2015) in South Africa where the frequency of the mutant allele was high together with the proportion of heterozygous genotypes. This could be

attributed to balancing selection and the inherent fitness costs associated with resistance to amitraz (Corley et al., 2013). Amitraz-resistant strains have been found to lack fitness (Jonsson et al., 2010b) and are recessively inherited (Fragoso-Sanchez et al., 2011), this means that the heterozygous individual will be susceptible to amitraz and the homozygous resistance genotypes will have a selective disadvantage. This leads to a slow rate of fixation of amitraz resistance alleles in the population (Li et al., 2005). Subsequently, balancing selection will act on the population hence a large proportion of heterozygous genotypes. The lack of fitness of amitraz-resistant tick populations could be exploited using an acaricide rotation strategy (Jonsson et al., 2010b). Nonetheless, close to 40% of the dipping tanks showed complete resistance to amitraz, this could be attributed to the high frequency of use of amitraz by communal farmers as reported by Sungirai et al. (2016). The complete resistance genotypes were found in all the provinces in the country suggesting that the resistance allele is spreading in the country and this could be attributed to the movement of cattle together with *R. microplus* ticks (Sungirai et al., 2017).

The L641 mutation reported by Morgan et al. (2009) and associated with pyrethroid resistance was not recorded in this study while the carboxylesterase marker also associated with pyrethroid resistance had a low frequency of the mutant allele (five percent) with 10% of the individuals having heterozygous genotypes which were recorded at 27% of the dipping tanks. The carboxylesterase marker has also been associated with organophosphate resistance (Baffi et al., 2007 ; Faza et al., 2013). This low frequency of the resistance genotypes could be attributed to the reduced frequency of use of pyrethroids in communal farms due to their high costs which

makes them unaffordable to the resource poor communal farmers (Sungirai et al, 2016).

The continued use of organophosphates as an acaricide has been discouraged despite them being cheaper than both the pyrethroids and amitraz, due to a number of factors; their toxicity to both the humans and animals, chemical residues are present in meat and milk after slaughter and they can remain undegraded in the environment for close to 30 weeks (De Meneghi et al., 2016). Observations made during tick collection did indicate that the usage of this chemical group of acaricides was very low being applied topically on cattle in the so called hand dressing method which is practised when the tick burden is low and they are a few animals to treat. This explains why the resistant allele was absent in Matabeleland North province where it was observed that communal farmers virtually relied on amitraz for tick control. There is also a fitness cost associated with resistance to pyrethroids and organophosphate whereupon decreased usage of the chemicals might result in the loss of the mutant allele (Stone et al., 2014). Inheritance of resistance in organophosphates and pyrethroids is semi-dominant (Faza et al., 2013), with heterozygous individuals showing moderate levels of resistance. The presence of the heterozygous genotypes by the carboxylesterase marker at nearly 30% of the dipping tanks where *R. microplus* was found is a serious cause for concern.

The absence of a mutation in the sodium channel gene coupled with the presence of one in the carboxylesterase gene presents two contrasting scenarios towards resistance in pyrethroids. Apart from target site insensitivity, metabolic detoxification has been implicated in resistance to pyrethroids and organophosphates especially

the increased expression of esterase enzymes which would hydrolyse the acaricide (Rosario-Cruz et al., 2009) and this cannot be detected by molecular assays. It should be noted therefore that the absence of the L641 mutation may not necessarily mean the tick populations are not resistant but that the mechanisms of resistance may be different for the Zimbabwean tick population. In Mexico resistance mechanisms were found to vary depending on the tick population (Miller et al., 1999) with metabolic detoxification occuring when resistance levels were much lower (Hernandez et al., 2002). The same would apply for organophosphate resistance where apart from the mutation in the carboxylesterase gene studied here and metabolic detoxification reported in other studies (Li et al., 2005; Baffi et al., 2008), target site insensitivity has been reported in the acetylcholine esterase gene (Temeyer et al., 2009; Jyoti et al., 2016). The results of our study presents an opportunity to further understand resistance mechanisms in *R. microplus* isolates from Zimbabwe.

In conclusion, the continued use of amitraz both by government and supplemental use by farmers has led to selection pressure for resistance. There are other mutations which have been discovered in the octopamine/tyramine receptor gene and it will be important to investigate whether they are associated with resistance. There are still opportunities in Zimbabwe for acaricide rotation using those chemicals which do not appear to be undergoing selection pressure among *R. microplus* at the moment.

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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Table 1: RFLP profiles, corresponding SNPs detected, their interpretation and frequency in the examined *R. microplus* samples.

RFLP profile	SNPs detected	Interpretation	% occurrence
409bp	CC/CC	Homozygous-resistant	59/367 (16.1%)
409bp, 223bp, 186bp	CC/TT, CC/TC	Heterozygous	35/367 (9.5%)
409bp, 223bp, 220bp, 190bp, 186bp	AC/TC, CC/TC, AC/TT	Heterozygous	70/367 (19.1%)
409bp, 219bp, 190bp	AC/TC, CC/TC	Heterozygous	183/367 (49.9%)
223bp, 186bp	AA/TT	Homozygous susceptible	1/367 (0.27%)
219bp, 190bp	AA/TT	Homozygous- susceptible	19/367(5.1%)

Table 2: Overall frequency of the genotypes detected in *R. microplus* from Zimbabwe

Molecular marker	Genotype			Hardy-Weinberg Equilibrium
	RR	SR	SS	
Octopamine/ Tyramine receptor gene (Amitraz)	59/367, 16.1% (12.3-19.8%)	288/367, 78.5% (74.3-82.7%)	20/367, 5.4% (3.1-7.8%)	χ^2=124.9 p<0.0001
Carboxylesterase (Organophosphate / Pyrethroids)	2/367, 0.54% (0-1.2%)	36/367, 9.8% (6.4-12.3%)	329/367, 89.6% (82.4-89.4%)	χ^2=0.2 p=0.65
Voltage gated sodium channel (Pyrethroids)	0	0	350/350 (100%)	cannot be computed

Table 3: Dipping tank level frequency of genotypes detected in *R. microplus* from Zimbabwe.

Molecular marker	Genotype		
	RR	SR	SS
Octopamine/ Tyramine receptor gene (Amitraz)	39/103, 37.8% (28.5-47.2%)	92/103, 89.3% (71-100%)	18/103, 17.5% (10.1-24.8%)
Carboxylesterase (Organophosphate/Pyrethroids)	2/103, 1 % (0-4.6%)	28/103, 27.2% (31.3-50%)	92/103, 89.3% (93.4-100%)
Voltage gated sodium channel (Pyrethroids)	0	0	103/103 (100%)

547

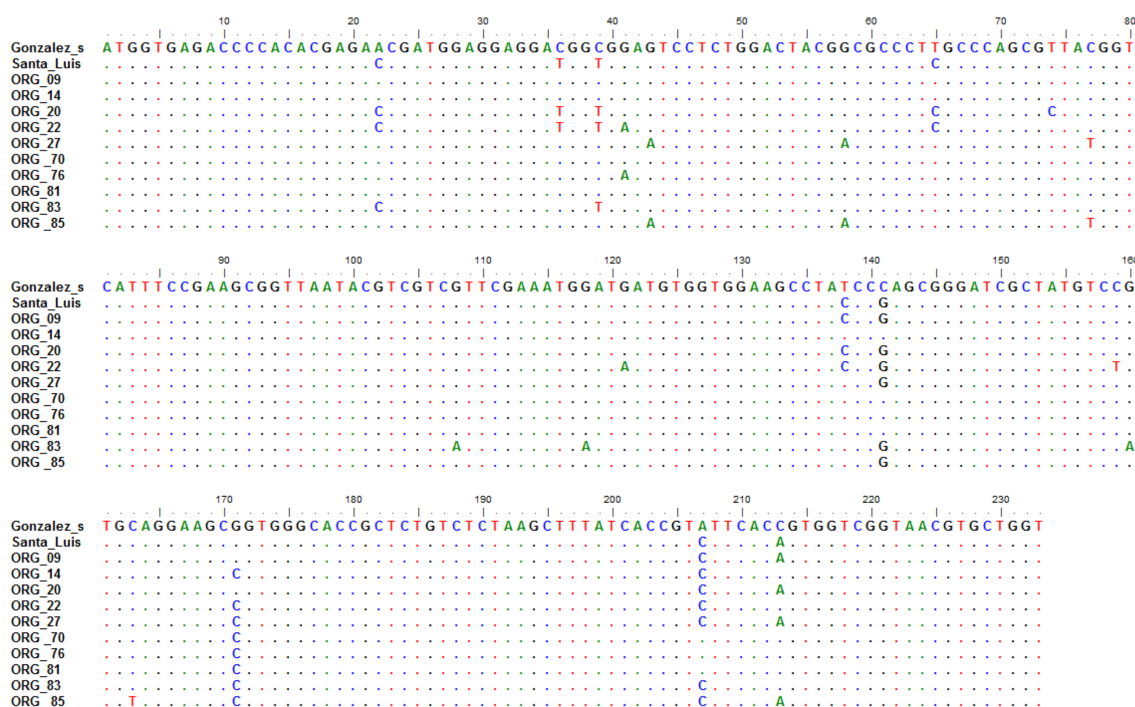

Figure 1: SNPs observed in the open reading frame of the *R. microplus* G-protein coupled Octopamine tyramine receptor gene (sequences from Zimbabwean samples ORG_09 to ORG_85) were compared with the reference Gonzales strain GenBank Accession No. EF490687.1 and the Santa Luisa strain GenBank Accession No. EF490688.1). SNPs associated with amitraz resistance are indicated at nucleotide positions 22 and 65.

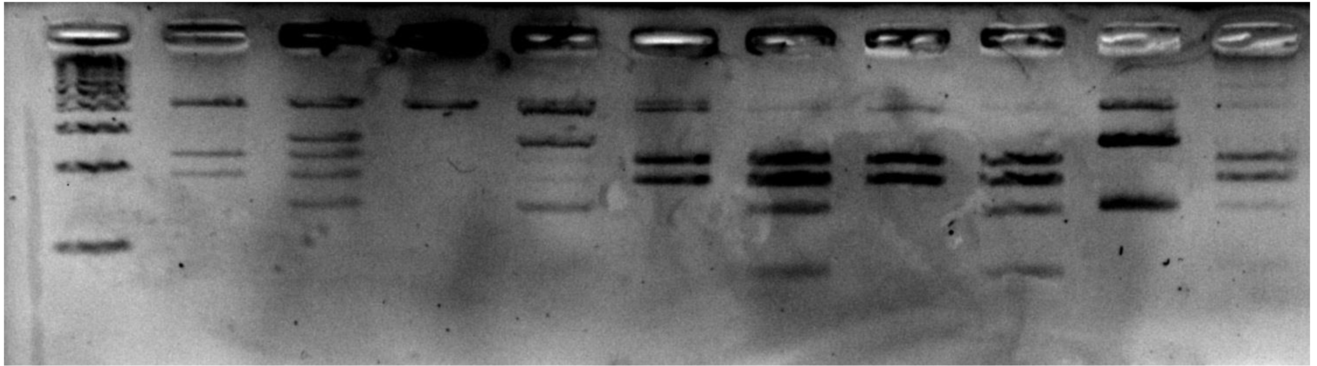

Figure 2: RFLP profiles of *R. microplus* samples sent for sequencing (from left to right 1st lane-100bp DNA super ladder, sample identities as in Figure 1; ORG_09, ORG_14, ORG_20, ORG_22, ORG_27, ORG_70, ORG_76, ORG_81, ORG_83, ORG_85).

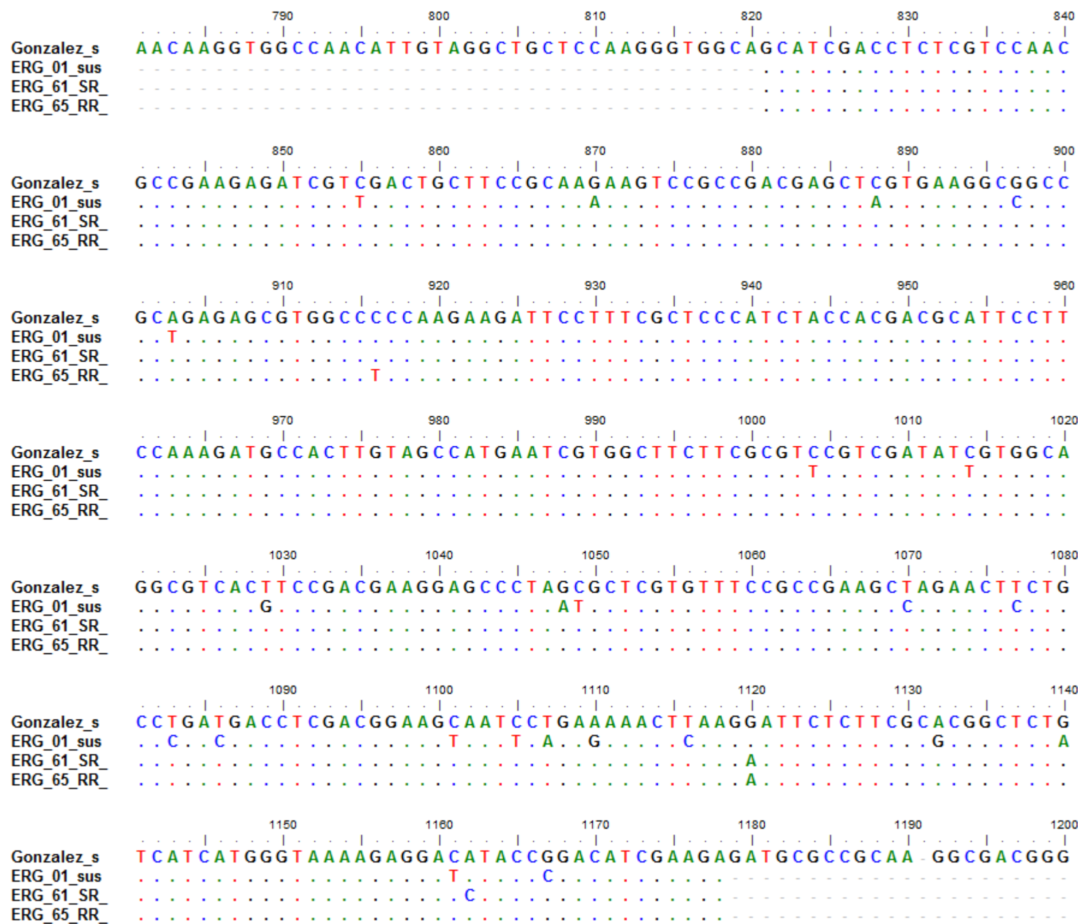

Figure 3: Open reading frame of *R. microplus* carboxylesterase gene for the 3 samples sequenced and compared to the susceptible Gonzalez Strain GenBank Accession Number: AF182283

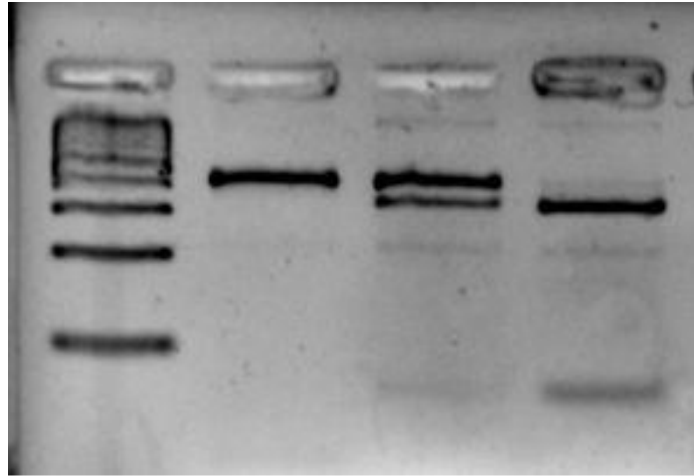

Figure 4**:** RFLP profile for three samples of *R. microplus* whose sequences are shown in Figure 3 from left right 100 bp DNA super ladder, ERG_001 (homozygous susceptible), ERG_061 (heterozygous) and ERG_065 (homozygous resistant)

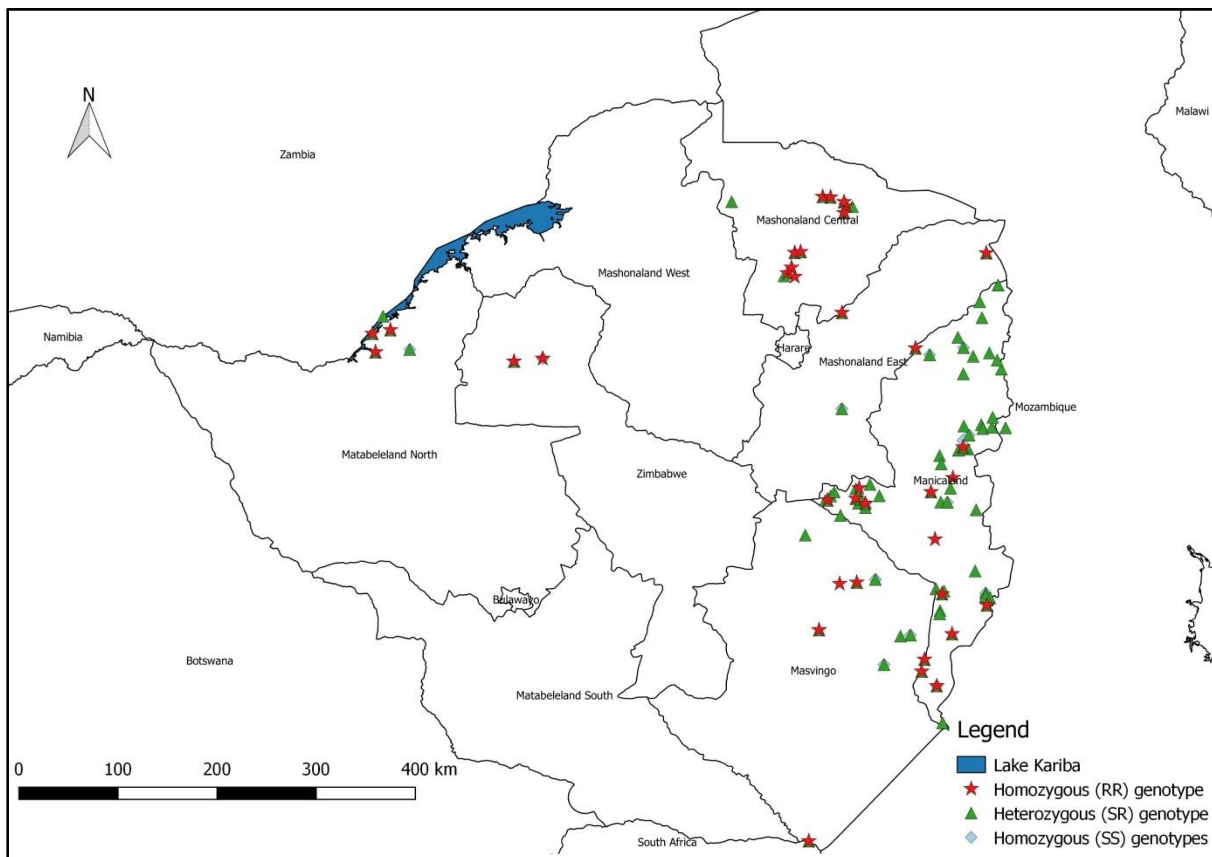

Figure 5: Distribution of genotypes at the octopamine/tyramine receptor marker associated with amitraz resistance for *R. microplus* at the communal dipping tanks where the tick species was collected in Zimbabwe

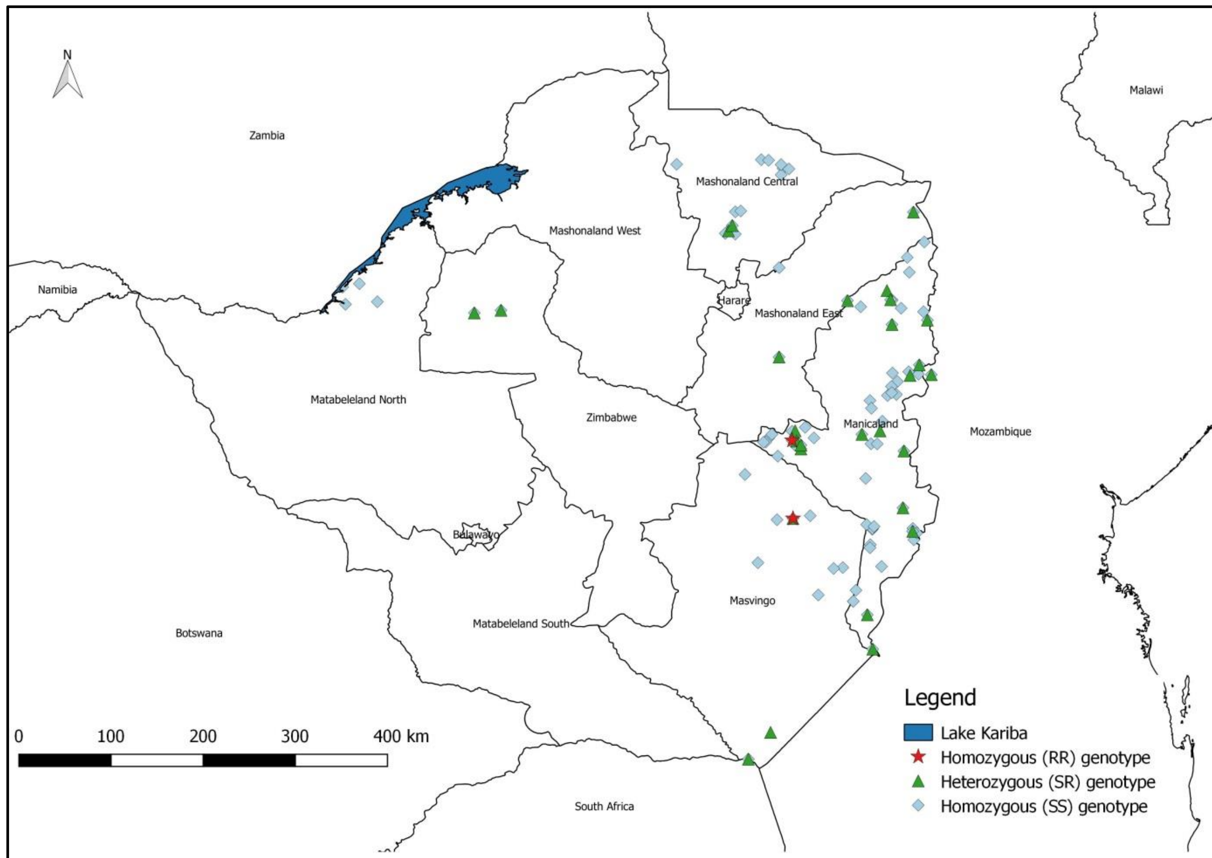

Figure 6: Distribution of genotypes at the carboxylesterase marker associated with pyrethroid and organophosphate resistance in *R. microplus* ticks at communal dipping tanks where the tick species was collected in Zimbabwe