The genetic relationship between *R. microplus* and *R. decoloratus* ticks in South Africa and their population structure

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Highlights

- The mitochondrial COI gene is more informative than the nuclear ITS2 gene for Ixodes ticks.
- *R. microplus* from South Africa grouped with Asia and South America.
- *R. microplus* appears to be diverging into two populations along the coast of South Africa.
- *R. decoloratus* is more panmictic with no clear population structure.
- Population structure is essential for future vaccine and acaricidal control strategies.

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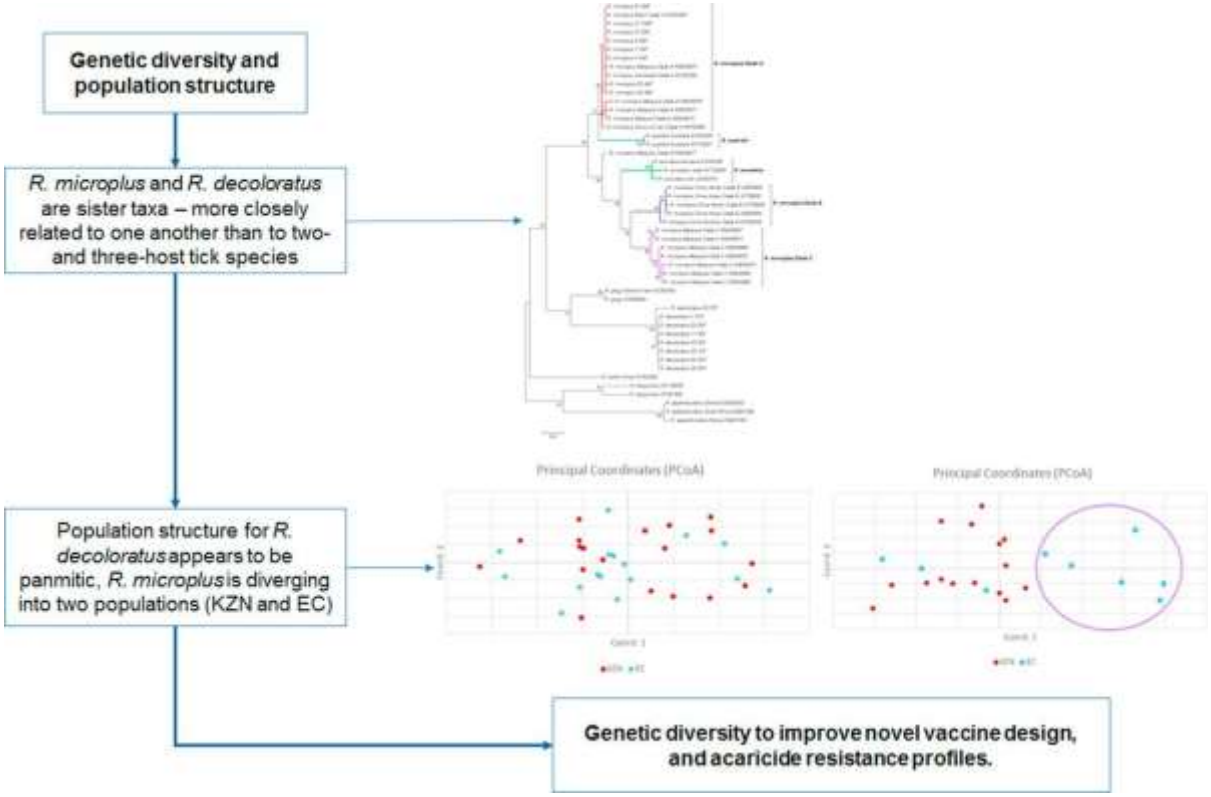
Abstract

Rhipicephalus microplus and *R. decoloratus* are one-host ticks that preferentially feed on cattle. They are capable of transmitting various tick-borne pathogens which may be detrimental to the agricultural and livestock industry in South Africa. Previous studies have shown that *R. microplus* forms five lineages in the *R. microplus* complex, segregating into different geographical areas based on mitochondrial markers. This study examined the phylogenetic relationship within and between *R. microplus* and *R. decoloratus* using the nuclear internal transcribed spacer 2 (ITS2) and mitochondrial cytochrome oxidase subunit I (COI) genes. The results showed that the nuclear ITS2 marker is informative for interspecific variation but lacks the resolution for intraspecific variation. Analysis of the mitochondrial COI gene revealed that *R. microplus* ticks from South Africa grouped into a clade comprised of ticks from Asia and South America. The population structure of these two tick species was also investigated using novel microsatellite markers. Population structure analyses revealed that both the *R. microplus* and *R. decoloratus* populations presented with two genetic clusters. *Rhipicephalus microplus* ticks from the Kwa-Zulu Natal (KZN) province belonged to cluster 1, and those from the Eastern Cape (EC) province predominantly grouped into cluster 2. No observable population structure was noted for *R. decoloratus*. The overlap of genetic clusters in both species could be attributed to inbreeding between the regions by unrestricted movement of cattle across provinces. Such movement promotes tick mobility, gene flow and the homogenisation of tick populations.

Keywords: *Rhipicephalus microplus*, *Rhipicephalus decoloratus*, recombination, phylogenetics, ITS2, COI, microsatellites, population structure.

Graphical abstract



1. Introduction

Rhipicephalus microplus and *R. decoloratus* ticks are of economic importance in South Africa. These ticks severely burden cattle farmers, and may directly affect cattle condition and result in the spread of tick-borne diseases [1]. Of the two tick species, *R. microplus* has the ability to transmit *Babesia bovis* (lethal Asiatic babesiosis), making it of great concern in the agricultural industry [2]. *Babesia bigemina*, which causes milder African babesiosis, is transmitted by *R. decoloratus* [3]. Both tick species are adept in transmitting *Anaplasma*, a gram-negative bacterium resulting in anaplasmosis.

Compared to *R. microplus*, *R. decoloratus* maintains a larger geographical distribution within the country, and appears to be adaptable to more arid regions [4, 5]. By comparison, *R. microplus* seems to prefer the coastal regions of the country, displaying a discontinuous distribution in the more temperate regions [5-7]. Recent studies documented the adaptive ability of *R. microplus* ticks as they move into previously unsuitable environments to displace the native *R. decoloratus* tick species

[8-10]. The spread of *R. microplus* to previously unoccupied areas is of great economic concern, since South African cattle are immunologically naïve to *B. bovis* [1].

The increase in the geographical spread, discontinuous distribution and adaptation to varying climatic zones can contribute to genetically diverse strains within the same species. Genetic diversity can lead to genetically distinct populations that could explain the inconsistent efficacy of the Bm86 vaccine across different geographical areas [11]. Varying acaricide selection pressure may also drive the differentiation of populations further confounding the development of an effective control strategy [12]. For this reason, elucidation of phylogeny is imperative for effective tick control strategies in the future.

Several types of markers can be implemented for use in phylogenetic studies. These include both coding and non-coding loci. In most instances, species evolution is better represented when both coding and non-coding loci with different evolutionary rates are investigated [13]. Coding genes often used for tick phylogenetics include mitochondrial genes such as the 16S rRNA and 12S rRNA genes, with the 12S rRNA gene providing more resolution at genus and species level [14-16]. The mitochondrial cytochrome c oxidase subunit 1 (COI) gene has been successfully implemented to determine intraspecific variation in mites [17-20] and for phylogenetic inference between the morphologically similar tick species *Ixodes holocycus* and *Ixodes cornuatus* [21]. Non-coding loci that are often used in phylogenetic studies include the internal transcribed spacers (ITS) 1 and 2 of the nuclear rDNA gene. The ITS1 spacer has been used to determine both inter- and intraspecific variation in mites [22, 23], while the ITS2 spacer has been used for phylogenetic studies of several tick species [24] and was able to distinguish *Ixodes granulatus* from different geographical areas [25].

Recent studies showed that the mitochondrial COI gene provides better phylogenetic resolution than the nuclear ITS2 region in *R. microplus*. For example, COI sequences were used to show that *R. microplus* contains a cryptic species and displayed some population structure between distant geographical areas [26, 27]. Thus, *R. microplus* appears to form a species complex of five taxa, namely *R. australis*, *R. annulatus*, *R. microplus* clade A of Burger et al. (2014), *R. microplus* clade B of Burger et al. (2014),

and *R. microplus* clade C of Low et al. (2015). Burger et al. [26] also showed that *R. microplus* from clade B (Southern China and Northern India) is more closely related to *R. annulatus* than to *R. microplus* from clade A (Asia, South America and Africa). Additionally, the *R. microplus* complex is tentatively more closely related to *R. annulatus* than to *R. decoloratus* [26, 27]. Several studies showed the importance of investigating the role of recombination in the generation of genetic diversity [28-30]. For this reason, is it essential to explore the evolutionary history of recombination between phylogenetic markers as it could drastically influence the phylogenetic methodology and inferences made [30]. Ancestral recombination graphs (ARGs) are usually implemented for this purpose [31].

The use of microsatellite markers is a popular strategy to address population structure within a species [32, 33]. Sympatric speciation was shown for *R. australis* populations using microsatellite makers developed by Koffi, Risterrucci (34) in New Caledonia [35]. Microsatellite results showed little to no population structure for other tick species including *Ixodes ricinus* [36], *R. microplus* [37] and *R. appendiculatus* [38]. Microsatellite markers developed specifically for *R. microplus* [39] displayed variation in their flanking regions [40], the presence of null alleles [34] and difficulty in amplification [41]. This indicates the urgent need for the identification of novel, well characterized and robust microsatellite markers.

In this study, we aimed to determine the phylogenetic relationship between *R. microplus* and *R. decoloratus* ticks in South Africa. Previous phylogenetic analyses did not include samples from South Africa for both tick species. The markers used for the study included the non-coding nuclear ITS2 and the coding mitochondrial COI genes. This study also aimed to infer population structure for both tick species using novel microsatellite markers. Phylogeographic inferences made by Burger et al. [26] using the COI gene were investigated to determine which clade *R. microplus* from South Africa belongs to. Identifying the level of genetic diversity and population structure of *R. microplus* and *R. decoloratus* could improve future tick vaccine design. This can be achieved by targeting specific populations based on their genetic compositions [42]. Therefore, investigating the population structure of these two tick species in South Africa is important for their future control, as well as the control of their associated tick-borne diseases.

2. Materials and Methods

2.1. Area of study and sample collection

The sampling area was focused around the coastal regions of Kwa-Zulu Natal (KZN) and the Eastern Cape (EC) provinces in South Africa (Fig 1), which showed the most variation and very little population structure for *R. microplus* in a previous study [40]. *Rhipicephalus microplus* and *R. decoloratus* ticks were collected by Zoetis Pty Ltd representatives with consent from each farmer. Upon collection, each farmer completed a questionnaire and placed the collected ticks in 70% ethanol. These samples were shipped to the University of Pretoria for further analysis.

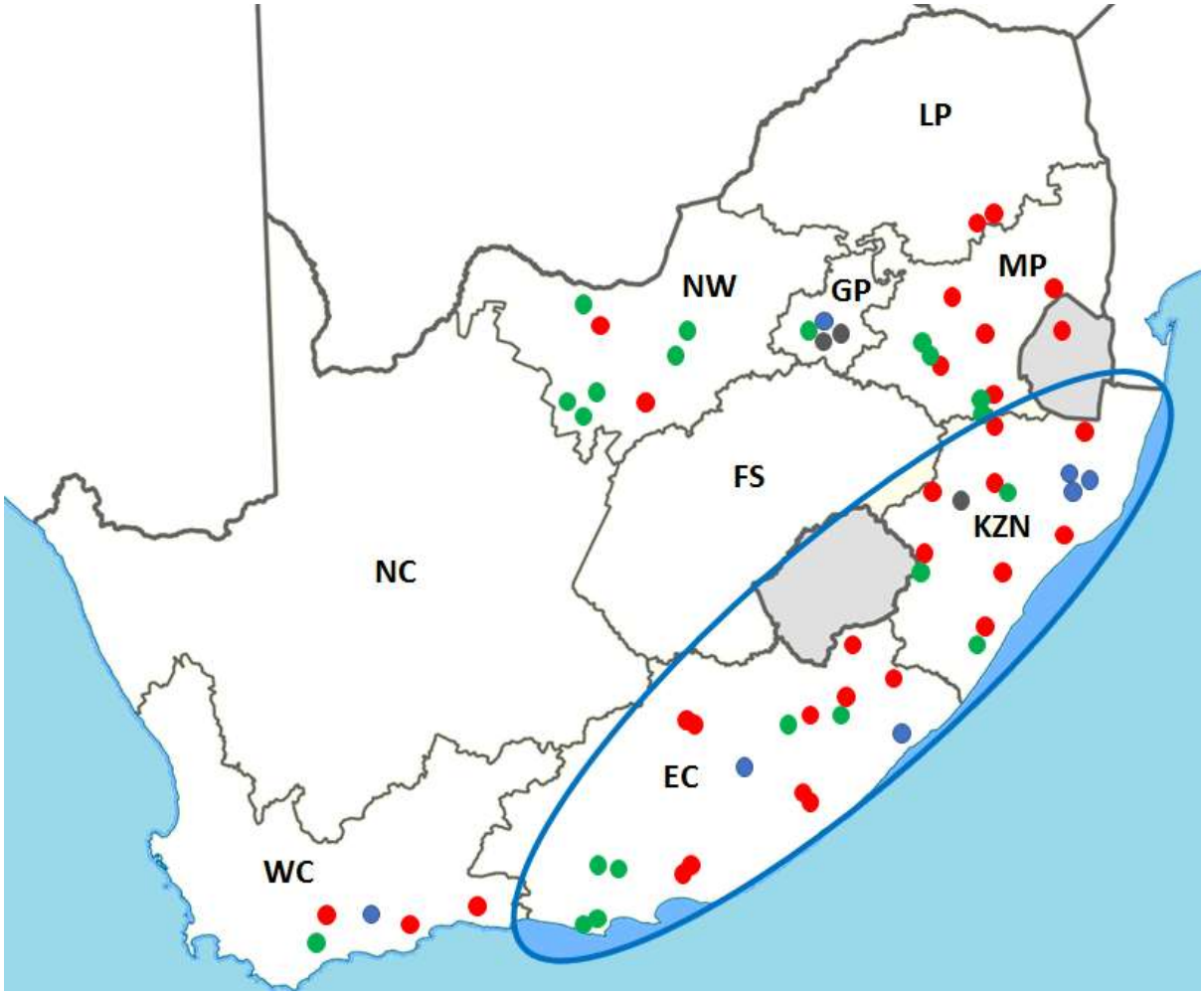


Figure 1: Distribution of tick collections along the coastal regions of South Africa. Only the areas in the blue circle were considered, since this area showed the most genetic variation in a previous study. The blue dots represent areas where only *R. microplus* ticks were collected, the green dots are representative for *R. decoloratus*, red dots are areas where both tick species were found, and grey dots are where other tick species occurred.

2.2. Identification and genomic DNA isolation

Rhipicephalus microplus and *R. decoloratus* ticks were initially identified using microscopy, during which the hypostome dentition of females and the adanal spurs of males were distinguished [1, 43]. This was followed by molecular confirmation using ITS2-PCR-RFLP [44], during which each tick species displays a characteristic restriction profile. Genomic DNA (gDNA) was extracted from all confirmed *R. microplus* and *R. decoloratus* tick samples using a previously published protocol [45]. The quality of DNA was assessed using agarose gel electrophoresis and a spectrophotometer (Nanodrop-1000, Thermo Fisher Scientific, USA).

2.3. PCR and sequencing

Amplification of the nuclear ITS2 marker and the mitochondrial COI marker was carried out using published primers and annealing temperatures (Supplementary Table S1). All PCR amplification reactions were performed using the Econo*Taq*® PLUS GREEN 2X Master Mix (Lucigen, USA). Each reaction contained 1.25 U Econo*Taq* DNA polymerase (0.1 units/µl), 200 µM dNTPs, 1.5 mM $MgCl_2$, a proprietary PCR enhancer/stabilizer, and agarose gel loading buffer. Each primer was added to a final concentration of 10 pmol and 200 ng of template DNA was added to each reaction. All PCR reactions were performed using a GeneAmp 2700 thermocycler (PE Applied Biosystems, USA) and visualized using 1% (w/v) agarose gel electrophoresis.

All amplified PCR products were purified using the GeneJET™ PCR Purification kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Amplicons were sequenced according to the standard dye terminator sequencing strategy by Macrogen Inc. (Netherlands) in a 96-well plate. Sequences were analysed using BioEdit sequence alignment editor 7.2.0 [46], and multiple sequence alignments were constructed using the online MAFFT program (http://mafft.cbrc.jp/alignment/software/) [47].

2.4. Identification and optimization of microsatellites

Microsatellites were identified for *R. microplus* by screening bacterial artificial chromosome (BAC) clones (obtained from Professor Felix Guerrero, United States Department of Agriculture) with msatcommander-0.8.2-WINXP [48]. Microsatellites

from *R. decoloratus* were selected from a *de novo* assembled transcriptome (transcriptome data available at the University of Pretoria) using the microsatellite identification (MISA) tool [49] to predict potential polymorphic microsatellites. Fifteen microsatellites for each species were selected for further analysis and primers were designed for each locus using Oligo® 7 Primer Analysis Software.

Due to difficulty in amplification or lack of polymorphism, several microsatellite markers were discarded. The remaining microsatellite markers used for further analysis are shown in Supplementary Table S2. All PCR amplifications were performed using the Econo*Taq*® PLUS GREEN 2X Master Mix (Lucigen, USA) and visualized using 3% (w/v) agarose gel electrophoresis. Amplified markers were purified using the GeneJET™ PCR Purification kit (Thermo Fisher Scientific, USA) and sequenced at Inqaba Biotec (Pretoria, South Africa). All sequences were analysed using BioEdit and MAFFT.

All markers were initially amplified and sequenced for more than one sample to ensure that the correct amplicon was generated. Multiplex Manager version 1.0 [50] was used to predict the best multiplex arrangement for each panel with the final chosen panel shown in Supplementary Table S3. Multiplex reactions were performed in 12 µl reaction volumes using the Platinum® Multiplex PCR Master Mix (Thermo Fisher Scientific, USA) and fluorescently labelled primers (Thermo Fisher Scientific, USA). All multiplex reactions were analyzed using 3% (w/v) agarose gel electrophoresis before GeneScan analysis.

GeneScan 96-well plates were assembled by pipetting 1 µl of the multiplex reaction, and the addition of 10 µl HiDi and GeneScan™ 500 Liz® size standard (Thermo Fisher Scientific, USA) in a 70:1 ratio. All GeneScan runs were performed on an AB13500XL series apparatus (Applied Biosystems, USA) at the University of Pretoria. Fluorescent peaks and allele sizes were analyzed using GeneMarker 2.6.3 [51].

2.5. Phylogenetic and population genetic analysis

To determine the evolutionary histories within genes, ancestral recombination graphs were constructed using SNAP Workbench [52]. Sequence alignments were converted into haplotypes by excluding indels and violations of the infinite site model. The branch

and bound Beagle algorithm in SNAP Workbench was implemented to infer the minimal number of recombination events within the gene that could explain the data [31]. Phylogenetic trees were constructed in MEGA5 using the Maximum Likelihood method [53, 54] with the inclusion of several other GenBank Accession entries reported in previous studies [26, 27]. A representative sample from each haplotype determined from the ancestral recombination graphs was included in the phylogenetic analysis.

Population genetic analysis was performed using GenAlex version 6.502 [55]. Genetic diversity was investigated by analysing expected heterozygosity (H_e) and observed heterozygosity (H_o) from allelic frequencies by locus and by population. Genetic differentiation was investigated within and between populations using analysis of molecular variance (AMOVA) with 999 permutations, as well as Principal Coordinate Analysis (PCoA).

Population structure was investigated using a Bayesian clustering algorithm in STRUCTURE 2.3.4 [56, 57], during which the admixture and correlated allele frequency model was implemented. A burn-in of 100 000 generations and 100 000 iterations was used for the analysis with the upper level of *K* set to ten runs. The optimal number of genetic clusters was inferred from its second order rate of change, ΔK [58], which was calculated using STRUCTURE HARVESTER [59]. The initial population was divided into two cohorts, consisting of individuals from Kwa-Zulu Natal and the Eastern Cape.

Bayesian clustering used in STRUCTURE requires that there is linkage equilibrium between loci. This was investigated and taken into consideration in two ways. Firstly, linkage disequilibrium was tested using the Multilocus 1.3b1 [60]. For these analyses, 100 000 data randomizations were performed to compare the observed data with randomized data that mimic linkage equilibrium. If the observed dataset displayed increased linkage disequilibrium compared to the randomized datasets, it was assumed that there is association between the loci. This was further supported by P-values. Secondly, discriminate analysis of principal components (DAPC) was used to enhance variations between groups and reduce variation within groups [61]. The optimal number of genetic clusters was determined using the Bayesian Information

Criterion (BIC). All DAPC analyses were performed using the ADEGENET v2.0.1 package [62] in R 3.3.2 (R Core Team 2016).

3. Results

3.1. Phylogenetic analysis of nuclear ITS2 marker

The nuclear ITS2 marker was amplified for 80 samples (40 *R. microplus* and 40 *R. decoloratus*) from Kwa-Zulu Natal (KZN) and the Eastern Cape (EC) provinces in South Africa. Sequence analyses revealed numerous polymorphisms, with 97 nucleotide substitutions differentiating *R. decoloratus* from *R. microplus* samples (Supplementary Fig S1). Ancestral recombination graphs were constructed for both *R. microplus* and *R. decoloratus* nuclear ITS2 spacers (Supplementary Fig S2). No recombination was detected within the nuclear ITS2 spacer in either of the two species. Ancestral recombination graphs showed five haplotypes for *R. microplus* and three for *R. decoloratus*. There was no correlation between the haplotype groupings and the geographic origins of the samples. Haplotype designations for each sample are shown in Supplementary Table S4.

The phylogenetic relationship based on the ITS2 marker, between South African *R. microplus* and *R. decoloratus* samples, along with additional GenBank Accession entries (Fig 2) showed a lack of resolution for the *R. microplus* complex as suggested in previous studies [26]. Thus, there was no clear separation of the species complex into its respective clades.

3.2. Phylogenetic analysis of the mitochondrial COI marker

The mitochondrial COI marker was amplified for 80 samples (40 *R. microplus* and 40 *R. decoloratus*). In the sequence alignment, there were 86 nucleotide substitutions differentiating *R. microplus* from *R. decoloratus* samples, with little variation detected within a species (Supplementary Fig S3). Amplification and sequencing of the mitochondrial COI marker was efficient for *R. microplus* samples. This was not the case for *R. decoloratus* samples, with sequences revealing a lack of specificity for the tick COI gene and resulting in amplification of *Anaplasma* spp. COI genes. For this reason, very few sequences were obtained for the COI marker for *R. decoloratus*. Nested primers were designed using the sequenced *R. decoloratus* COI gene as the

template to improve the specificity of COI amplification. Sequencing of these amplicons revealed that *Anaplasma* COI genes were still being amplified instead of *R. decoloratus*. Ancestral recombination graphs were generated for *R. microplus* COI sequences as well as the *R. decoloratus* sequences that were available (Supplementary Fig S4).

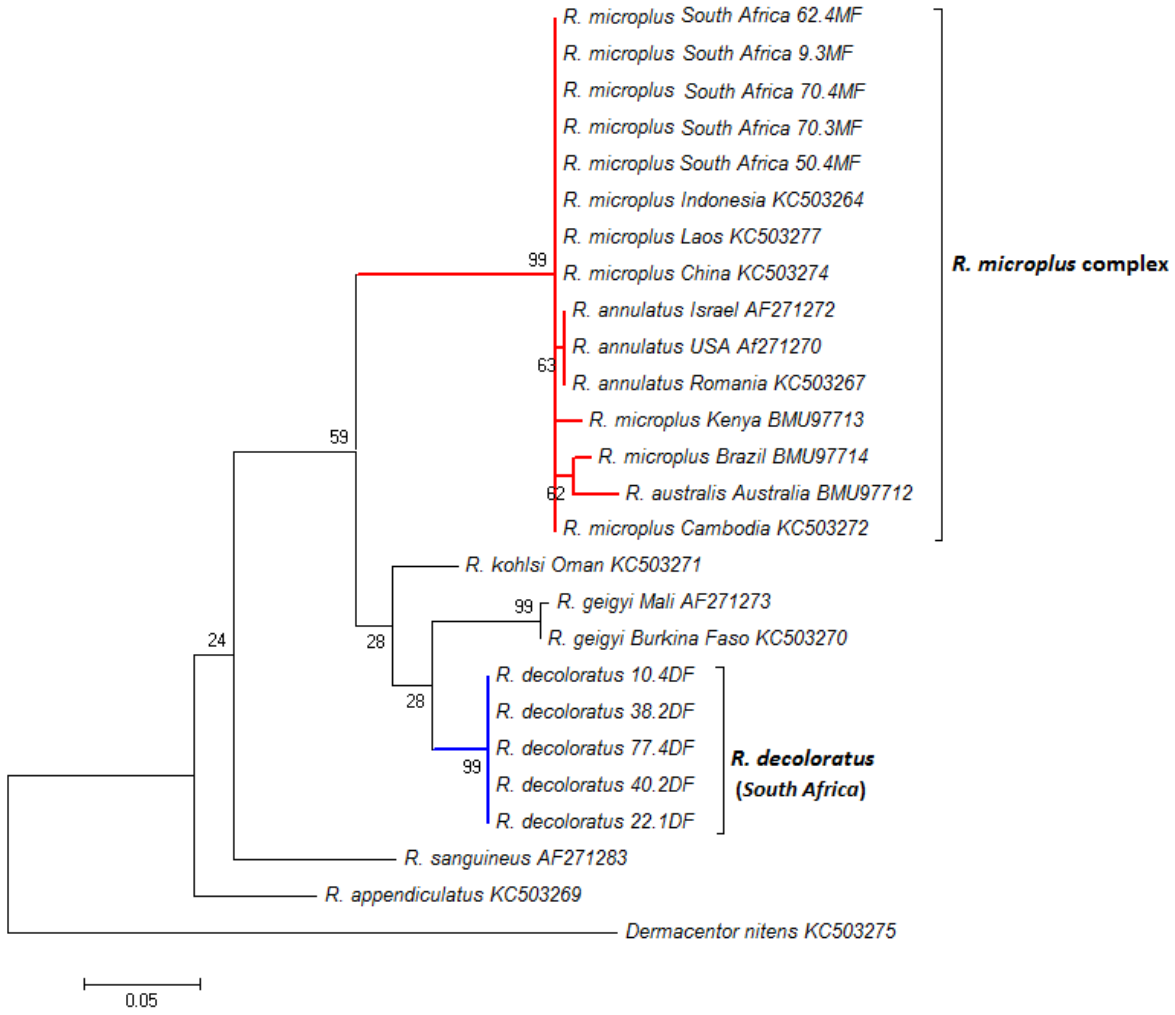


Figure 2: The maximum likelihood tree inferred from nuclear ITS2 sequences. Bootstrap values are indicated at each node. Samples sequenced in this study that represent South Africa are indicated with MF (*R. microplus*) and DF (*R. decoloratus*). Species names are followed by the location where they were collected from, and GenBank accession numbers. The *Rhipicephalus microplus* complex as published by Burger et al. [26] is indicated in red, and within it occurs *R. australis* and *R. annulatus*. The tree was rooted against *Dermacentor nitens*.

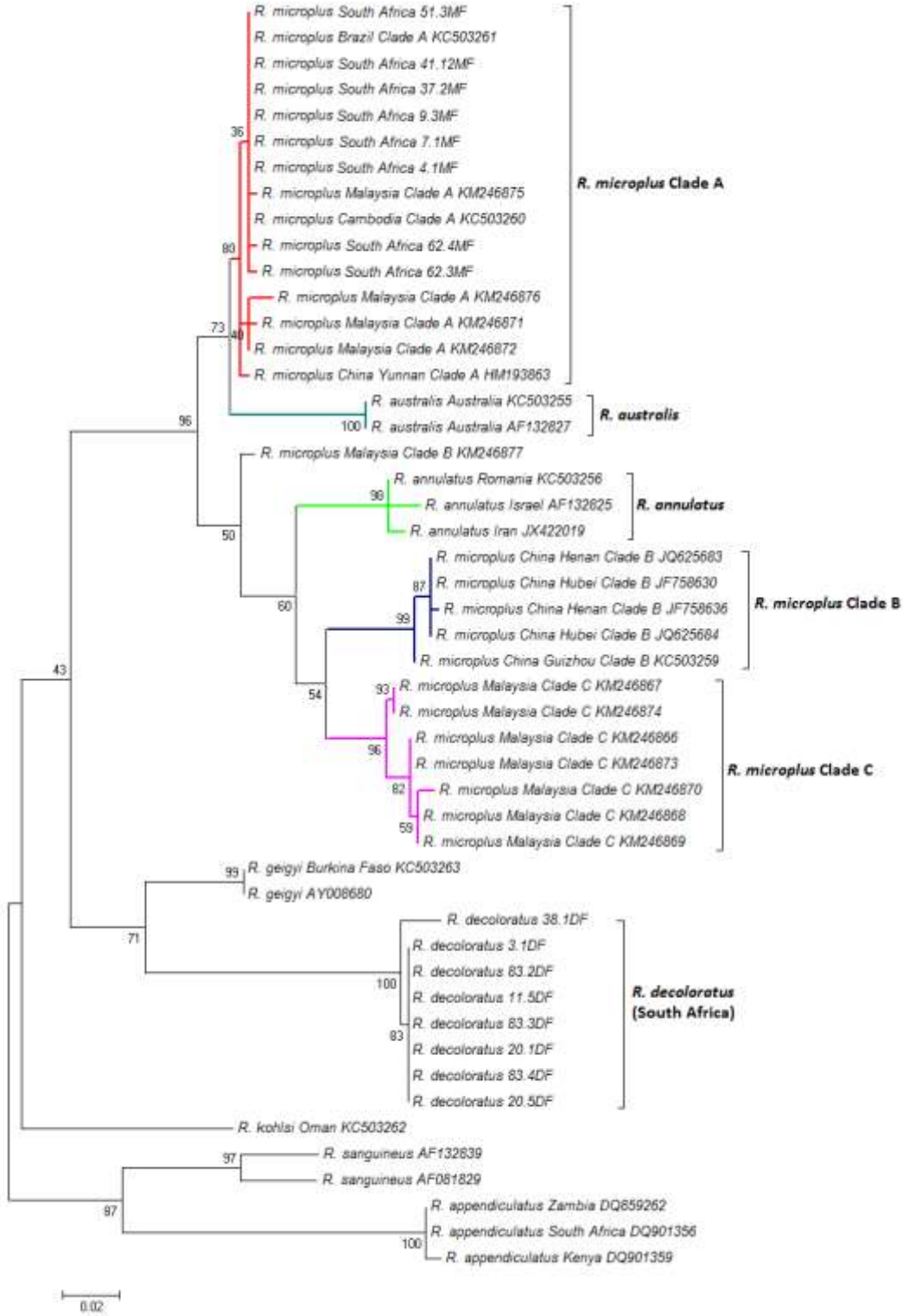


Figure 3: The maximum likelihood tree inferred from mitochondrial COI sequences. Bootstrap values are indicated at each node. Samples sequenced in this study that represent South Africa are indicated with MF (*R. microplus*) and DF (*R. decoloratus*) and their origin indicated by KZN (Kwa-Zulu Natal) and EC (Eastern Cape). Species names are followed by GenBank accession numbers and the location where they were collected from. The *Rhipicephalus microplus* complex as published by Burger et al. [26] is subdivided into clades A (red), B (blue), and C (purple). This cladistic complex included *R. australis* (turquoise) and *R. annulatus* (green).

Phylogenetic analysis was performed for the mitochondrial COI gene using *R. microplus* and *R. decoloratus* samples from South Africa, as well as the GenBank Accession entries reported in previous studies [26, 27] (Fig 3). This was done to decipher the clade allocation of South African ticks, as well as the relationship between *R. microplus* and *R. decoloratus*. Results revealed that *R. microplus* ticks from South Africa grouped into clade A of the *R. microplus* complex, along with ticks from Asia, South America and China. *Rhipicephalus microplus* ticks from clade A were more closely related to *R. australis*, while those from clade B were more closely related to *R. annulatus*. A low bootstrap value of 54% separated clade B from the Malaysian clade C. It is difficult to classify *R. decoloratus* into geographically defined clades due to the lack of sequence data available from other countries.

3.3. Assessment of genetic diversity using microsatellite markers

Heterozygosity, F-statistics and polymorphism was assessed by population for all the microsatellite markers for both *R. microplus* and *R. decoloratus* tick species (Table 1)

Table 1: Summary statistics of genetic variation for the two populations across all loci for *R. microplus* and *R. decoloratus* ticks in South Africa.

R. microplus									
Pop	**Locus**	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**uHe**	**F**
Total	All	24	5.250	3.580	1.328	0.557	0.660	0.691	0.228
KZN	C39A	15	5.000	4.018	1.487	0.933	0.751	0.777	0.000
	C50A	15	3.000	2.133	0.816	0.867	0.531	0.549	0.000
	P807F	15	6.000	4.369	1.609	0.400	0.771	0.798	0.481
	P804G	15	10.000	5.556	1.965	0.467	0.820	0.848	0.431
	C27A	15	5.000	1.779	0.903	0.533	0.438	0.453	0.000
	P801G	15	6.000	4.839	1.681	0.600	0.793	0.821	0.244
EC	C39A	9	4.000	2.656	1.117	0.778	0.623	0.660	0.000
	C50A	9	2.000	1.385	0.451	0.333	0.278	0.294	0.000
	P807F	9	6.000	4.500	1.611	0.778	0.778	0.824	0.000
	P804G	9	7.000	5.786	1.846	0.222	0.827	0.876	0.731
	C27A	9	3.000	2.571	1.011	0.222	0.611	0.647	0.636
	P801G	9	6.000	3.375	1.442	0.556	0.704	0.745	0.211
R. decoloratus									
Pop	**Locus**	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**uHe**	**F**
Total	All	35	6.000	3.134	1.302	0.528	0.630	0.649	0.214
KZN	69783	19	7.000	3.422	1.458	0.368	0.708	0.727	0.479
	50377	19	6.000	2.456	1.247	0.737	0.593	0.609	0.000
	53653	19	2.000	1.498	0.515	0.421	0.332	0.341	0.000

	47877	19	5.000	3.539	1.380	0.684	0.717	0.737	0.046
	52942	19	12.000	5.348	2.052	0.632	0.813	0.835	0.223
EC	69783	16	8.000	4.376	1.704	0.500	0.771	0.796	0.352
	50377	16	5.000	1.992	1.021	0.438	0.498	0.514	0.122
	53653	16	2.000	1.882	0.662	0.750	0.469	0.484	0.000
	47877	16	5.000	3.969	1.486	0.375	0.748	0.772	0.499
	52942	16	8.000	2.860	1.493	0.375	0.650	0.671	0.423

KZN: Kwa-Zulu Natal, EC: Eastern Cape, N: Number of samples, Na: Number of different alleles, Ne: No. of Effective Alleles = 1 / (Sum pi^2), I: Shannon's Information Index = -1* Sum (pi * Ln (pi)), Ho: Observed heterozygosity, He: Expected heterozygosity, uHe: Unbiased Expected Heterozygosity = (2N / (2N-1)) * He, F: Fixation Index = (He - Ho) / He = 1 - (Ho / He).

The variability indices (Table 1) indicated that both tick species had a slightly lower observed heterozygosity than expected. The fixation index (F) for *R. microplus* and *R. decoloratus* were effectively very similar. Results imply that there is genetic differentiation within the species which could potentially be due to population structure. Analysis of molecular variance (AMOVA) was done for each species to further investigate the variation among populations and within populations (Fig 4). This analysis indicated that most of the variation existed within individuals for *R. microplus* (79%) and *R. decoloratus* (81%). Very slight variation was observed among populations in *R. microplus* (4%), with no variation among populations detected in *R. decoloratus*. Variation among individuals was 17% and 19% for *R. microplus* and *R. decoloratus* respectively.

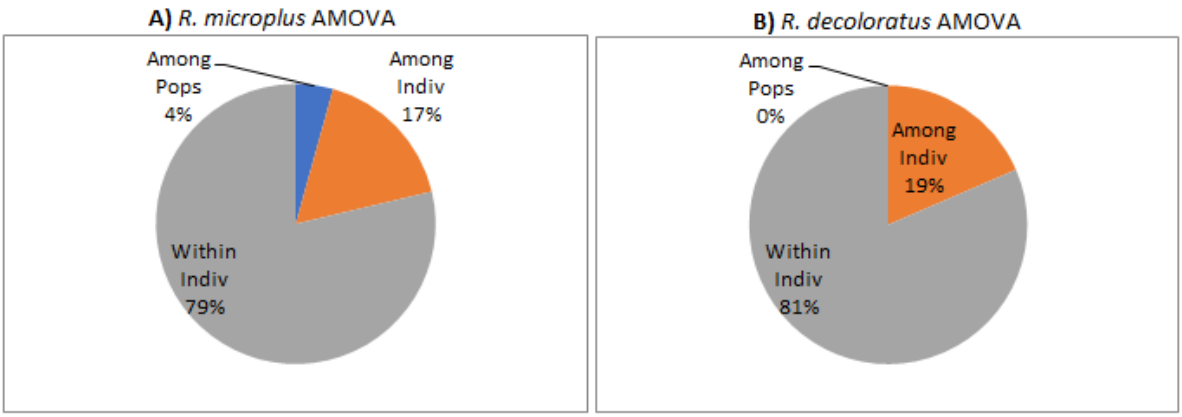


Figure 4: Analysis of molecular variance (AMOVA) determined for both A) *R. microplus* and B) *R. decoloratus* tick samples. A) For *R. microplus* and *R. decoloratus* most of the observed variation appears to be within individuals. There is very little variation detected among populations in both species.

3.4. Population structure of *R. microplus* and *R. decoloratus*

The results from STRUCTURE analyses suggested that three clusters (ΔK=3) exist within *R. microplus* (Fig 5A), although with little significance as delta K (ΔK) was only 0.9. The STRUCTURE plot (Fig 5B) further indicated that there was no clear differentiation between populations. Additional studies were conducted through DAPC analysis in attempt to further elucidate the population structure. These results suggested that two genetic clusters were present in *R. microplus* (Fig 5C) where the KZN population belongs to cluster 1 and the EC population belongs predominantly to cluster 2. PCoA analysis was performed on these clusters (Fig 5D) with results indicating that the EC population appeared to be separated from the KZN population. No correlation could be found between the outliers that occurred in EC population that shared a genetic background with cluster 1, although they could potentially represent migrants as shown in Fig 5D.

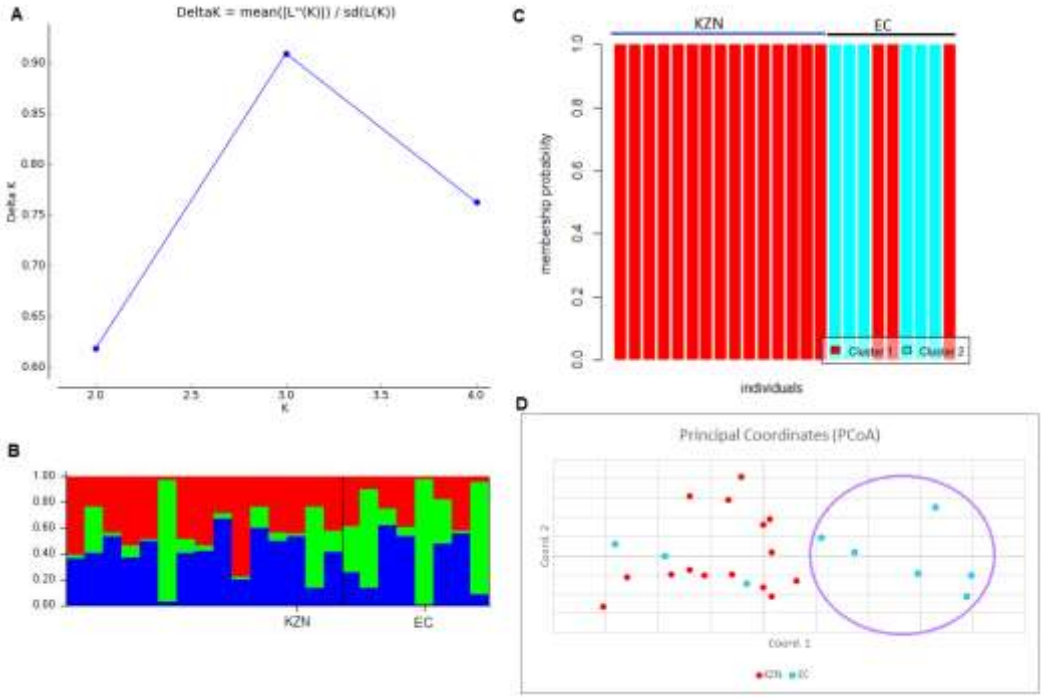


Figure 5: Predicted population structure of *R. microplus* ticks from the coastal regions in South Africa. A) The ΔK predicted using STRUCTURE suggested that there are three sub-populations. B) Clusters present in the Kwa-Zulu Natal population and Eastern Cape. Red – cluster 1, Green – cluster 2 and Blue – cluster 3. There is no observable difference between the two geographic populations and how their members cluster. C) The predicted clusters for each individual with relative membership probability to each cluster. Individuals to the left of the graph are from KZN while those on the right are from the EC. D) Principal coordinate analysis of the clusters shows that EC populations belonging to cluster 2 are separate from the KZN population. There are outliers in the EC population that group with the KZN population.

The population structure for *R. decoloratus* inferred using STRUCTURE suggested that the most probable number of clusters (ΔK) was two (Fig 6A). The STRUCTURE plot (Fig 6B) showed no genetic differentiation between the two geographic populations. DAPC analysis also suggested that two clusters were present in the population (Fig 6C). The membership probability of each individual to a specific cluster showed that the majority of the EC population belonged to cluster 1 and the KZN population to cluster 2, although there was overlap between the genetic clusters across the two populations. PCoA analysis further substantiated the lack of separation between the two populations, but rather an admixed genetic background (Fig 6D).

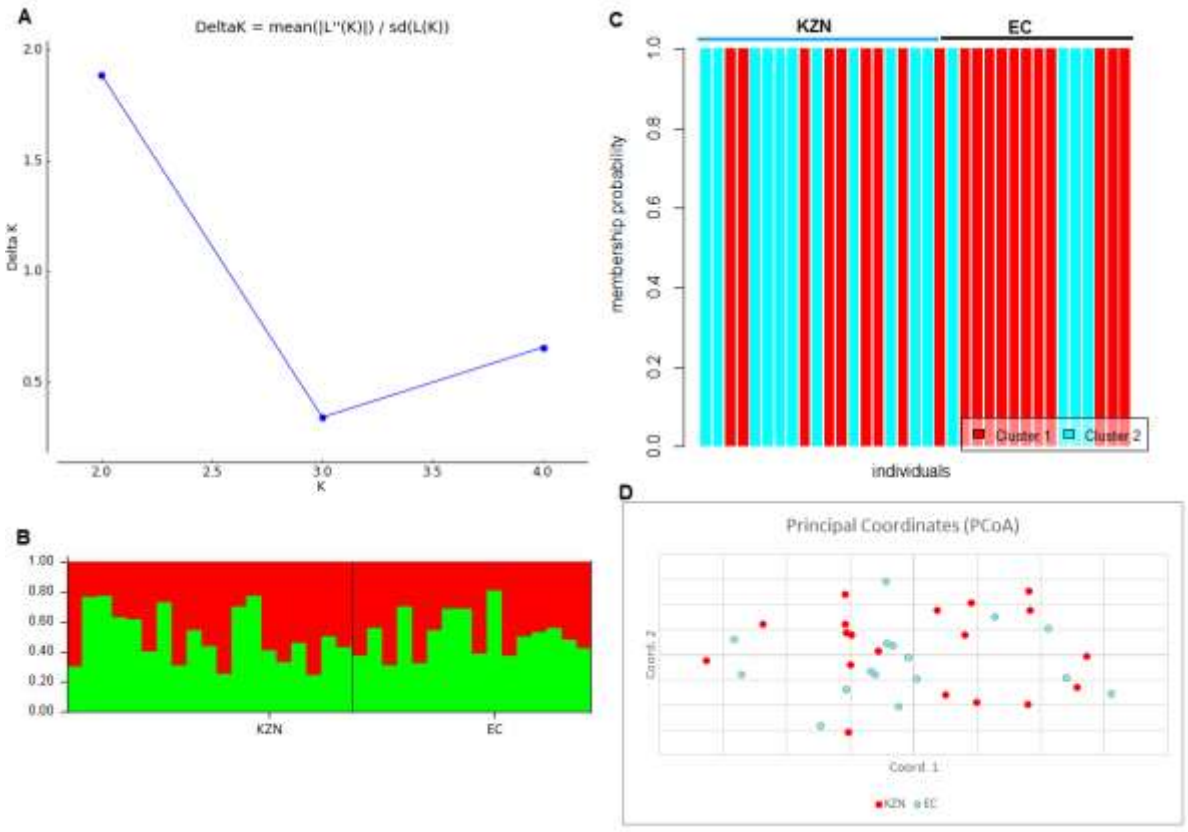


Figure 6: Predicted population structure of *R. decoloratus* ticks from the coastal regions of South Africa. A) The ΔK predicted using STRUCTURE suggested that there were two sub-populations. B) Clusters present in the Kwa-Zulu Natal population and Eastern Cape. Red – cluster 1, Green – cluster 2. There was no observable difference between the two populations and how they clustered. C) The predicted clusters for each individual with relative membership probability to each cluster. Individuals to the left of the graph are from KZN while those on the right are from the EC. D) Principal coordinate analysis of the clusters showed an admixed population.

4. Discussion

Due to the economic importance, adaptive nature and spread of *R. microplus* and *R. decoloratus* species into previously unoccupied areas, the phylogenetic relationship and population structure of these ticks were investigated. The population structure inferred for *R. microplus* along the coastal regions of South Africa, where cattle density is the highest, suggests genetic differentiation between the KZN and EC populations. Analysis using the mitochondrial COI marker indicated that *R. microplus* ticks from South Africa belong to clade A, along with samples from Asia and South America. In contrast, there was no observable population structure for *R. decoloratus.*

Molecular phylogeny allows for the resolution of genetic relationships between closely related species and has become a useful tool in several biological research fields [63]. Resolving the genetic relationship in arthropods has been notoriously difficult due to their deep divergence. Using several nuclear markers a recent study managed to provide some clarity for 75 arthropod species where every major arthropod lineage was represented [64]. Arachnida, specifically the Acari subclass, displayed low phylogenetic resolution with bootstrap values of less than 50% [64]. The lack of phylogenetic resolution within Acari makes the investigation of genetic diversity within and between populations problematic. Phylogenetic resolution of these families is further compounded by recent suggestions that gene duplications and/or whole genome duplications have occurred [65].

Much effort has been expended to elucidate the phylogeny of the Rhipicephalinae subfamily of Ixodidae. The ITS2 ribosomal RNA [66], cytochrome c oxidase subunit I (COI) and 12S rRNA [15] markers have been used in previous studies, and these markers could provide phylogenetic resolution at species level. Previous studies showed that the mitochondrial COI gene is phylogenetically more informative than nuclear ITS2 for *R. microplus* ticks [26].

In the current study, 80 alleles for both *R. microplus* and *R. decoloratus* were investigated for nuclear ITS2 and mitochondrial COI markers, resulting in a total of 160 alleles per phylogenetic marker. The nuclear ITS2 gene was highly variable across tick species, particularly within Rhipicephaline ticks, and this was in accordance

with previous research [24, 44, 67, 68]. The ITS2 marker could not resolve the *R. microplus* complex and illustrated that *R. microplus* displayed more variation compared to *R. decoloratus* within the marker.

The COI gene could distinguish the assemblage of *R. microplus* ticks from South Africa into clade A, along with samples from Asia and South America. *Rhipicephalus microplus* from clade A was more closely related to *R. australis*, while those in clade B were more closely related to *R. annulatus*. The separation of clades B and C was only supported by a moderate bootstrap value of 54%, while the separation of clades A and B was more significant with a bootstrap value of 96%. The grouping of *R. microplus* ticks from South Africa into clade A corroborates the hypothesis that *R. microplus* from Southeast Asia spread to Madagascar and later Southern Africa [69].

Mitochondrial markers have also been successfully implemented in elucidating population structure in *Ixodes ricinus* where two distinct populations could be detected from Britain and Latvia [70]. However, population structures inferred from mitochondrial genes spans large geographical areas and have limitations for providing structure within closely situated regions. For this reason, markers with improved phylogenetic and population genetic resolution are essential.

Amplification of the *R. decoloratus* COI gene was rather problematic, even with gene specific primers designed according to the generated PCR product. In most instances, the COI gene for *Anaplasma* was amplified instead, which suggests that future research should focus on designing more specific primers for amplification and sequencing. Alternatively, it could suggest higher copy numbers of the *Anaplasma* COI gene compared to the *R. decoloratus* COI gene. This problem was not encountered during amplification of the *R. microplus* COI gene. It is known that both *R. microplus* and *R. decoloratus* ticks can serve as vectors for *Anaplasma,* a gram-negative bacteria which infects red blood cells and causes anaplasmosis [1]. It could be hypothesized that perhaps the *R. decoloratus* ticks in South Africa display increased reservoir potential for bacterial *Anaplasma* compared to *R. microplus* and may be the main vectors for the transmission of anaplasmosis in cattle. This possibility should be further investigated.

Novel microsatellites were isolated and characterized for both *R. microplus* and *R. decoloratus*. The genetic diversity results indicated that both populations (KZN and EC) from each tick species displayed very similar levels of genetic differentiation based on their fixation indices. AMOVA analysis showed little to no variation among populations, but rather variation among and within individuals. The membership probability of individuals to each cluster predicted by DAPC showed that the *R. microplus* KZN population comprises cluster 1, while the EC population display genetic backgrounds of both clusters, although predominantly cluster 2. The overlap of clusters in the EC population suggests that there could be inbreeding between the two populations which is further illustrated in the pattern in the PCoA analysis. No population structure could be detected for *R. decoloratus*, although both STRUCTUTRE and DAPC suggested that two clusters were present. Both the KZN and the EC populations displayed an admixture of the clusters. There was no evidence of host, environment, acaricide usage, temperature or rainfall associated with population structure in either of the tick species (results not shown).

One of the main factors that could explain the lack of population structure is the free movement of cattle across geographic areas in South Africa, which promotes tick mobility and gene flow. Additionally, the lack of appropriate boundaries between the two populations and relatively short distances could also contribute to admixture. Perhaps these microsatellite markers would be able to distinguish population structure across a larger geographical range, as was evident from the mitochondrial COI gene tree. Lack of population structure and genetic differentiation between populations has previously been reported for ticks. In *R. appendiculatus* ticks across large geographical ranges, there was no population structure among field strains and this was attributable to host distribution and mobility [38]. A study of *R. australis* population structure revealed that the main variation that occurred was at farm level, and that there was no clear genetic differentiation between regions or amitraz resistance status [37].

In conclusion, it appears as though standard phylogenetic markers are unable to infer population structure, with the exception of the mitochondrial COI gene across large geographical areas. The COI gene can resolve *R. microplus* ticks into specific clades that appear to be geographically constrained. However, little population structure can

be inferred from this gene on a smaller geographical scale such as the coastal regions of South Africa. Microsatellites are effective to distinguish even subtle effects of population structure, whether it be distinct population structure or a lack thereof resulting in complete panmixis. The *R. microplus* species complex raises several taxonomic questions as to whether each clade can be classified as a separate species or a subpopulation of the same species. The current thinking is to view the clades as geographically isolated populations. This could be clarified in future with more in-depth techniques such as pan-genome phylogenetic studies between species and/or clades.

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Supporting information

Supplementary Table S1: Gene specific primers used for amplification of the nuclear (ITS2), mitochondrial (COI) and coding (OCT/Tyr) receptor gene regions as phylogenetic markers

Gene Name	Primer name	Primer sequence 5'-3'	Ta (°C)	Reference
ITS2	Boophits2 F	GCCGTCGACTCGTTTTGA	58°C	[34]
	Boophits2 R	TCCGAACAGTTGCGTGATAAA		
COI	LCOI490	GGTCAACAAATCATAAAGATATTGG	53°C	[42]
	HCO2198	TAAACTTCAGGGTGACCAAA AAATCA		

Supplementary Table S2: Optimal amplification conditions of microsatellite markers used for *R. microplus* and *R. decoloratus* ticks

***Rhipicephalus microplus* microsatellite markers**						
Locus name	**Repeat Unit**	**Forward primer 5'-3'**	**Reverse primer 5'-3'**	**Amplicon size (bp)**	**Ta (°C)**	**No. of cycles**
C27A	$(GCGT)_{11}$	TCTGACGATACCCCGAACTACAT	TACTACCGCGACAAGCACAATGA	344	55	40
C39A	$(CTGT)_{13}$	ATAGAAACACTTAAATCGCATAAC	GTCCCTTTGTTGCCGTTTAG	332	53	37
C50A	$(ATC)_5$	AAAATAAAAGCCAAGCGAGAAA	AGAATCAGTTATCCATCCGTATA	264	52	37
P801G	$(GAT)_{18}$	AACTGCCTTTCCTGTGAGTTCAA	CCCGATTCTTGGCCGATCTC	300	58	40
P804G	$(ATCT)_{17}$	CTCTATTTTCCCTTAGTGCTCAA	TCAGAAAGAAGCCTACTGATG	345	54	35
P807F	$(ATC)_6$	GCCACAAAGCTCGACCTAACTA	GACTGGGTTAACTGGCGGAACAA	322	58	35
***Rhipicephalus decoloratus* microsatellite markers**						
47877	$(AAT)_7$	AGCCAAACGACACCAACACAC	CCACTGCAGGCGCTTTTTCAA	355	62	37
52942	$(CAT)_9$	ACGCTTCTGTGTCATCTCATT	CTAGGGGGAGACAGAAGGTTA	296	58	37
50377	$(AGC)_{10}$	ACACATGTCAAAGTTCTGCCT	CTCACCAAAGCTATCGGCACT	230	58	35
53653	$(AGC)_7$	ACAATCAACGGCAGAGTTCCT	GACGAAGATGGCGACGAAGTA	178	57	35
69783	$(TGA)_{12}$	TGACTCGAAGGTGTAAGCAAC	AGAAGCATTGAATCGCACCAG	218	56	35

Supplementary Table S3: Multiplex reaction setup for each panel.

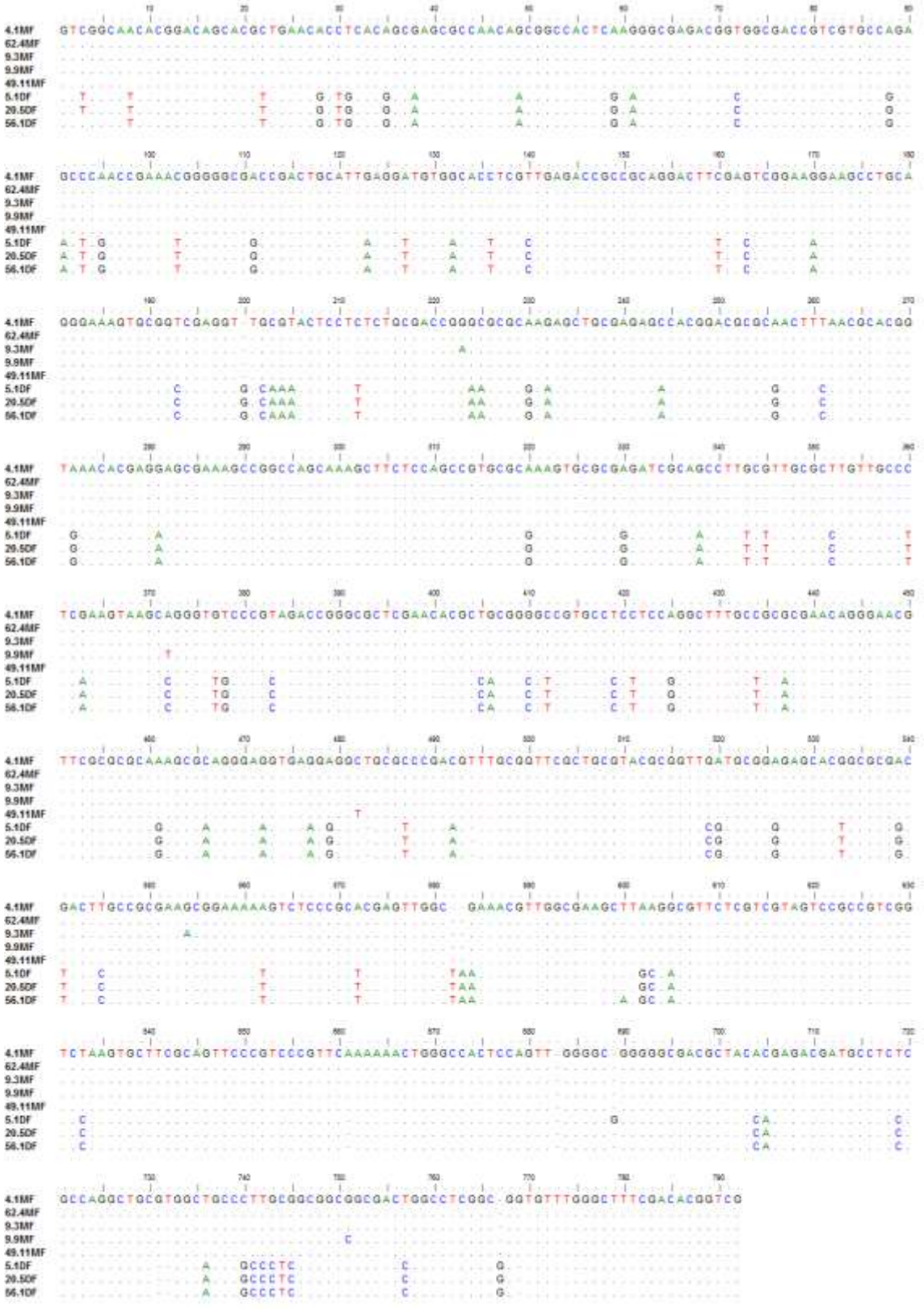
***Rhipicephalus microplus* multiplex panels**			
	Marker	**Fluorescent dye on forward primer**	**Ta (°C)**
Panel 1	C39A	6-FAM	53,3
	C50A	VIC	
Panel 2	P807F	6-FAM	55,5
	P804G	VIC	
Panel 3	C27A	VIC	57
	P801G	NED	
***Rhipicephalus decoloratus* multiplex panels**			
Panel 1	69783	VIC	58
	50377	NED	
	53653	PET	
Panel 2	47877	PET	59
	52942	NED	

Supplementary Table S4: Haplotype groupings of *R. microplus* and *R. decoloratus* based on the results from the ancestral recombination graphs for ITS2 and COI genes.

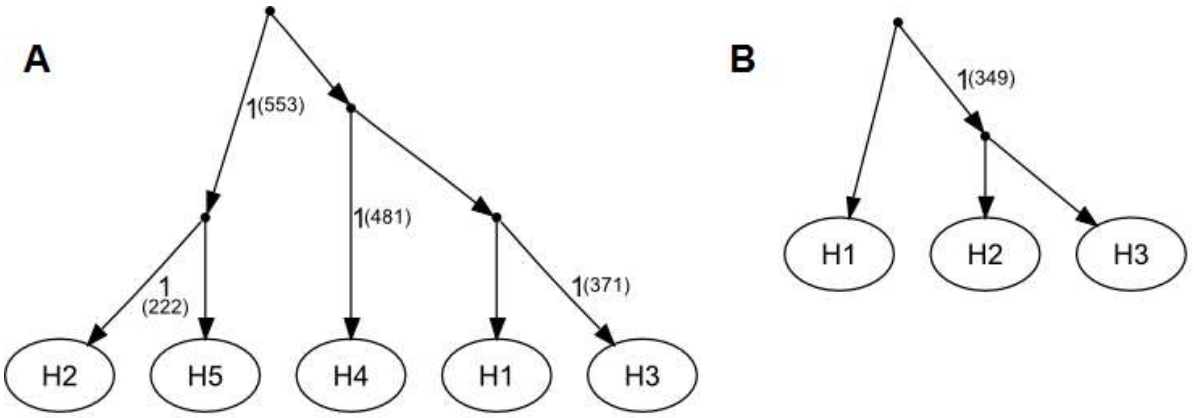
Tick species and gene	**Sample name**	**Haplotype ITS2**
***R. microplus* ITS2**	4.1MF	H1
	7.1MF	H1
	7.2MF	H1
	8.1MF	H1
	8.2MF	H1
	41.2MF	H1
	41.4MF	H1
	50.2MF	H1
	53.1MF	H1
	53.2MF	H1
	54.2MF	H1
	62.1MF	H1
	65.2MF	H1
	65.4MF	H1
	66.4MF	H1
	70.1MF	H1
	70.2MF	H1
	73.1MF	H1
	73.2MF	H1
	41.11MF	H1
	62.5MF	H1
	70.5MF	H1
	70.4MF	H1
	20.3MF	H1
	9.2MF	H1
	75.1MF	H1
	41.5MF	H2
	9.3MF	H2
	53.3MF	H2
	51.1MF	H2
	9.11MF	H2
	7.3MF	H3

	9.9MF	H3
	70.3MF	H3
	51.2MF	H3
	49.11MF	H4
	50.4MF	H4
	20.2MF	H4
	62.4MF	H5
	8.3MF	H5
	25.4MF	H5
	62.2MF	H5
	65.1MF	H5
	67.1MF	H5
	70.6MF	H5
	51.3MF	H5
	62.3MF	H5
***R. decoloratus* ITS2**	5.1DF	H1
	40.2DF	H1
	89.4DF	H1
	91.3DF	H1
	89.2DF	H1
	22.5DF	H1
	38.1DF	H1
	38.3DF	H1
	78.3DF	H1
	91.2DF	H1
	22.1DF	H1
	10.4DF	H2
	11.4DF	H2
	38.2DF	H2
	56.2DF	H2
	76.3DF	H2
	91.4DF	H2
	77.3DF	H2
	77.4DF	H2
	78.1DF	H2
	40.4DF	H2
	56.3DF	H2
	77.2DF	H2
	83.3DF	H2
	91.1DF	H2
	20.1DF	H2
	20.2DF	H2
	22.2DF	H2
	40.1DF	H2
	83.2DF	H2
	20.5DF	H3
	38.4DF	H3
	56.4DF	H3
	78.2DF	H3
	83.1DF	H3
	56.1DF	H3
	89.1DF	H3
	89.3DF	H3
	69.2DF	H3
	83.4DF	H3
***R. microplus* COI**	4.1MF	H1
	7.1MF	H1
	7.2MF	H1
	7.3MF	H1
	8.1MF	H1

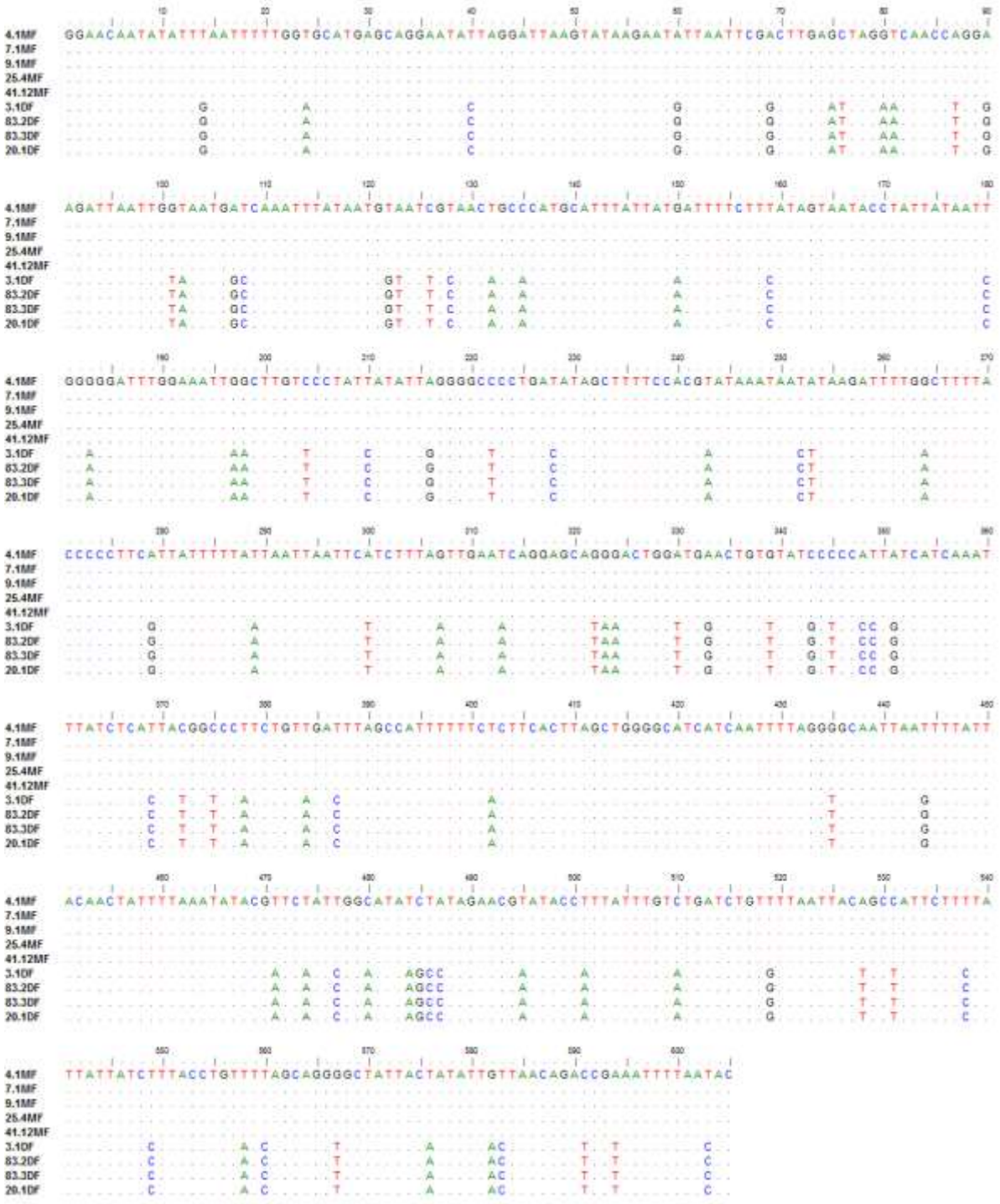
	8.2MF	H1
	8.3MF	H1
	9.1MF	H1
	9.11MF	H1
	9.3MF	H1
	20.2MF	H1
	25.3MF	H1
	25.4MF	H1
	37.1MF	H1
	37.2MF	H1
	37.3MF	H1
	41.2MF	H1
	41.12MF	H1
	51.1MF	H1
	51.2MF	H1
	51.3MF	H1
	54.1MF	H1
	54.4MF	H1
	58.1MF	H1
	58.2MF	H1
	58.3MF	H1
	62.2MF	H1
	70.4MF	H1
	70.5MF	H1
	70.6MF	H1
	93.2MF	H1
	8.1MF	H1
	8.2MF	H1
	75.1MF	H1
	75.2MF	H1
	66.10MF	H1
	51.8MF	H1
	62.3MF	H2
	62.4MF	H3
***R. decoloratus* COI**	3.1DF	H1
	83.2DF	H1
	11.5DF	H1
	83.3DF	H1
	20.1DF	H1
	83.4DF	H1
	38.1DF	H2
	20.5DF	H3



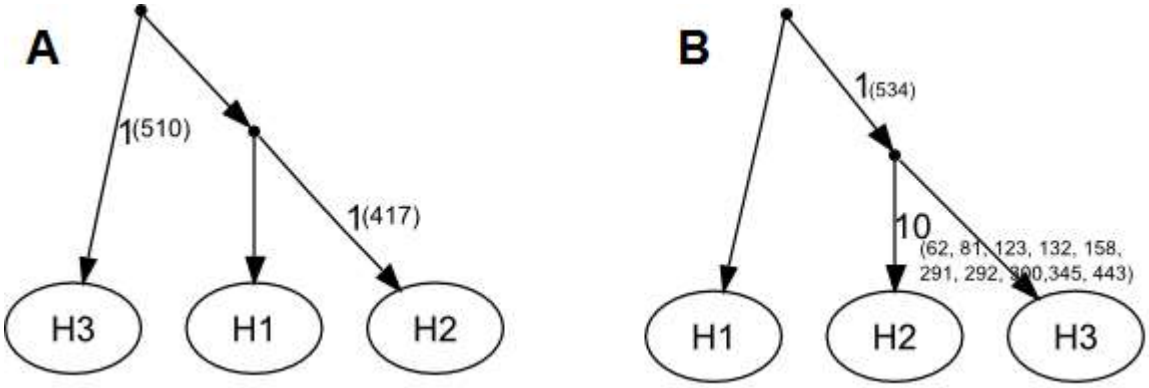
Supplementary Figure S1: Representative sequences for the nuclear ITS2 gene for *R. microplus* and *R. decoloratus*. MF indicates *R. microplus* samples and DF indicates *R. decoloratus* samples. The numbers represent the farms from which the samples originated. Only samples that displayed some variation are indicated in the figure.



Supplementary Figure S2: Ancestral recombination graphs for the ITS2 spacers of A) *R. microplus* and B) *R. decoloratus* in South Africa. No intra-spacer recombination events were detected. Haplotypes are indicated by the letter 'H'. For *R. microplus* samples, there were five haplotypes, while for *R. decoloratus* there were three haplotypes (Supplementary Table S4).



Supplementary Figure S3: Representative sequences for the mitochondrial COI gene for both *R. microplus* and *R. decoloratus*. MF indicates *R. microplus* samples and DF indicates *R. decoloratus* samples. The numbers represent the farms that the samples originated from.



Supplementary Figure S4: Ancestral recombination graphs for the mitochondrial COI gene of A) *R. microplus* B) *R. decoloratus.* No recombination events were. Haplotypes are indicated by the letter 'H'. Three haplotypes were generated for each species (Supplementary Table S4).