Resolution of the taxonomic status of *Rhipicephalus (Boophilus) microplus*

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Abstract

Rhipicepahlus (Boophilus) microplus is an obligate feeding, hard tick of great economical importance in the cattle industry. Every year billions of dollars of loss is attributed to *R.(B)* microplus, mainly through loss of cattle due to pathogens transmitted such as *Babesia* and *Anaplasma*, but also through damage to hides from blood-feeding. There is conflicting evidence regarding the taxonomic status of *R.(B) microplus*, however the most recent published research has been in support of the reinstatement of *R.(B) australis* as a species distinct from *R.(B) microplus*. The way in which some members of the scientific community have responded to the designation of separate species has implications for vaccine and acaricide research. In this study, we aimed to resolve the taxonomic status of *Rhipicephalus (Boophilus) microplus*, using morphological and phylogenetic approaches. 1,650 *Rhipicephalus (Boophilus) microplus* ticks from Australia, Thailand, South Africa, North and Central America and South America were used in this study. 340 specimens consisting of 170 *R.(B) annulatus* (USA) and 170 *R.(B) decoloratus* (South Africa) were also used. To maximize the information obtained from morphological observations, three methods were used; a binary scoring system based on previously described features, a standard morphometric method, and the more novel approach of geometric morphometrics. For the phylogenetic analysis three genes were used; the mitochondrial gene *COX1* and two functional nuclear genes; *Bm86* and *βAOR*.

Morphological scoring is the process of assigning a binary value to any feature as being present or absent, or satisfying a logical comparator. For this study the scoring matrix was based on previously described sets of morphological criteria used for discriminating among species. Each of the populations for which samples were obtained was tested using four two-way analyses, each of which was designed to test whether a sample should be classified as one of two possible species: *R.(B) australis* versus *R.(B) microplus*; *R.(B) microplus* versus *R.(B) annulatus*; *R.(B) microplus* versus *R.(B) decoloratus*; and *R.(B) annulatus* versus *R.(B) decoloratus*. The scoring system was highly repeatable for the differentiation of males and females of *R. (B) annulatus* and *R.(B) decoloratus* from both of *R.(B) microplus* and *R.(B) decoloratus*. However, in the case of *R.(B) australis* and *R.(B) microplus*, clear differentiation was not achieved for either male or female ticks. Among females, the Australian population were classified almost evenly as *R.(B) australis* and *R.(B) microplus*, with 8 individuals showing a mixture of features and therefore not able to be classified. Ticks from the rest of the regions were mainly classified as *R.(B) microplus*, which is to be expected as *R.(B) australis* is reported in Australia. However, only the Mozo isolates were classified as solely *R.(B) microplus*. The remaining regions included several ticks with mixed features. Six ticks from South Africa, and four of the Juarez isolate were classified as *R.(B) australis*. Among the males

an entirely different pattern emerged. Most male ticks from all geographical locations were classified as either *R.(B) australis* or showed a mixture of both features, with only a small number scoring as *R.(B) microplus*.

Morphometrics is the linear measurement from one anatomical landmark to another and is a widely-used technique for quantifying phenotypic variation. Twelve features based on previous morphometric work were used. The results obtained from this study varied according to stage and sex. For the larvae, the Fisher Pairwise comparison showed that the Australian ticks tended to have a shorter body length, idiosoma length and narrower scutum width. Among the remaining morphological features, there were no consistent patterns in the different populations and species. A principal components analysis (PCA) was undertaken and in PC1 the strongest feature was scutum length and hypostome length. In PC2 the strongest feature was idiosoma length. The PCA of larval stage ticks didn't provide conclusive evidence that *R.(B) australis* is a distinct species from *R.(B) microplus*, and there was no obvious grouping based on region at all, even when including *R.(B) decoloratus* and *R.(B) annulatus.* In relation to the male ticks studied, the Fisher Pairwise comparison and the PCA showed that Australian males (presumed *R.(B) australis*) were significantly different from the other isolates. As with the larvae, no patterns were seen in the other populations, based on species or region. In PC1 palpal length measures were the strongest features for differentiation and in PC2 the length of the ventral basis capituli had the strongest effect. The adult female samples yielded a mixed result. There was no real trend in the size of Australian ticks observed from the Fisher Pairwise comparison. However, *R.(B) decoloratus* tended to be smaller for most of the morphological features tested and *R.(B) annulatus* tended to be larger. This observation was inconsistent with the results from the PCA, in which there was grouping of Australian ticks. Measures of palpal length, width of the basis capituli and the length of the dorsal basis capituli were the strongest for differentiation in PC1. In PC2 the length of the ventral basis capituli was the strongest feature for differentiating populations.

Geometric morphometrics is the quantitative representation of shape using coordinates in the form of landmarks, instead of measurements and is intended to give the shape of the feature independent of size. Hence it is useful for eliminating the effect of size distortion occurring with physiological changes. Geometric morphometric analysis did not clearly and consistently enable the differentiation of any of the populations of ticks in this study. Each feature differed among samples in different sets of pairwise relationships.

Mitochondrial cytochrome oxidase subunit I gene (*COX1*) has been presented as a suitable mitochondrial gene to clarify complex groupings that were not resolved when using other mitochondrial genes. *COX1* has also been proposed to be the main gene for

differentiating between *R.(B) microplus* and *R.(B) australis*. The aims of this study were to confirm whether *COX1* can be used to resolve complex relationships within the *R.(B) microplus* clade and to determine whether there is justification for the view that *R.(B) australis* is a distinct species from *R.(B) microplus*. Maximum likelihood trees were constructed with a Bootstrap analysis. A relaxed clock Bayesian analysis was then undertaken to estimate topology and divergence timings, using three ticks found in amber covering three genera: *Amblyomma*, *Hyalomma* and *Ixodes* to calibrate the clock. These analyses suggest that *R.(B) microplus* is a clade, containg five subspecies including *R.(B) annulatus*, *R.(B) australis*, and three, regionally based clades of *R.(B) microplus*: 1. All the South and Central American isolates together with isolates from Cambodia, Thailand, and some of those from Malaysia; 2. Indian and the remaining Malaysian isolates; 3. Most of the Chinese isolates. *R.(B) decoloratus* shares a common ancestor with *R.(B) microplus* and *R.(B) annulatus* however it is clearly divergent, appearing to be more related to *R. bursa*. All proposed groups of *R.(B) microplus* also appear to have evolved within the same time scale (within the last 20 million years).

Bm86 is the name given to a midgut glycoprotein that is the target antigen of the only commercially available vaccine against ticks. All the prior work on this gene has been conducted using cDNA and suggests a high degree of sequence variation and the presence of different isoforms. The aim was to use genomic DNA to examine the regional variation in the *Bm86* sequences and to determine whether *Bm86* variation segregated according to the recently proposed taxonomic re-classification of *R.(B) microplus* and *R.(B) australis*. After extensive optimization, it was found that all primer sets, including those previously published and those designed in this project, failed on the extracted genomic DNA from all isolates. High variability in the published cDNA sequences indicated an extremely high mutation rate, which could potentially be linked to variation in the function of the protein and its utility as a vaccine immunogen. Analysis of sequence alignments from publicly available databases did not allow grouping of samples by either geographical location or proposed taxon. These findings are in apparent contradiction to claims by other researchers that regional variation in the efficacy of the vaccine is associated with regional variation in sequence.

Beta-adrenergic octopamine receptor (*βAOR*) is a G-protein coupled receptor (GPCR) located on the neuronal cell surface and believed to be the main target of the acaricide amitraz. Polymorphism in *βAOR* has been associated with amitraz resistance. 121 samples from our isolates were sequenced for a length of 183 base pairs in position 95-277, among which eight SNPs were identified, five of which had not been previously described. It was found that geographical populations did not group based on the *βAOR* gene. Six of the SNPs were non-synonymous. When the 2-D structure of the putative

βAOR protein was predicted, it was found that all non-synonymous single nucleotide polymorphisms (SNPs) caused a change to a residue in the *βAOR* cytoplasmic pore. Chi-square analysis showed that populations that are known to be resistant to amitraz were significantly more likely to have one of the non-synonymous SNPs, regardless of geographical location.

In summary, when using the morphological analyses alone, it is not possible to consistently differentiate *R.(B) microplus* from the proposed *R.(B) australis*. Analysis of the *COX1* gene supports the differentiation of *R.(B) australis* from *R.(B) microplus*, however when the gene is analyzed across all isolates, *COX1* also showed support for *R.(B) microplus* being a species complex made up of three regional groups, *R.(B) australis* and *R.(B) annulatus*. The *Bm86* gene was not amenable to analysis of gDNA and the analysis of published cDNA sequence was not informative and showed no clear regional or taxonomic variation. The shared SNPs between the previously documented Australian amitraz-resistant population and our South American amitraz-resistant isolates provide support for a role of *βAOR* in amitraz resistance. The presence of the same SNP arising independently in resistant isolates on two continents suggests strong selection at this locus. The three novel SNPs that were found in amitraz-resistant populations, having amino acid residues located on the intracellular loop 1, provide further support for the link between genotype and functional resistance.

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I would like to thank my supervisory team; Nicholas Jonsson, Roderick Page and Roman Biek. In particular Nick, who guided me through the vast majority of this work, putting up with my ‘stupid questions’ in good humour. I would also like to thank Caline Koh-Tan, for all her guidance and help in the lab, as well as the work she put into optimizing the *Bm86* protocol while I was on maternity leave, and Elizabeth Kilbride for all her help with optimizing the DNA extraction protocol.

I would like to warmly thank all the projects collaborators for providing all the ticks for this work; Constantin Constantinoiu for the Australian ticks, Robert J Miller for the North American ticks, Nkululeko Nyangiwe for the South African ticks, Guilherme Klafke for the South American ticks and finally Sathaporn Jittapalapong for the Thai ticks. Without them, this project wouldn’t have been possible.

Finally I dedicate this work to my husband, who has had to put up with a stressy wife, a few sleepless nights and my endless chat about ticks, where he had no idea what I was saying half the time. Whose support has seen me through this project to its completion.

“I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.”

Printed Name: Christina M. Berry

Signature: _____

CHAPTER 1

Introduction

Ticks are obligate, blood feeding ectoparasites that feed on a wide range of vertebrate hosts (Oliver, 1989). There are currently 896 recognized tick species worldwide (Guglielmone *et al*., 2010), made up of the families Argasidae (soft ticks), Ixodidae (hard ticks) (figure 1.1), and Nuttalliellidae which contains one species. The Ixodidae family contains approximately 702 species across 14 genera; however these numbers are in constant flux due to advances in molecular methods and their ability to resolve taxonomic disparities (Guglielmone *et al*., 2010).

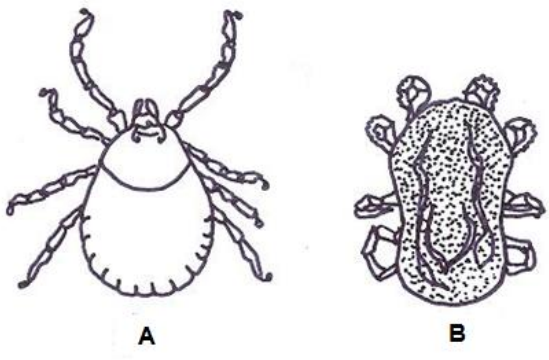


Figure 1.1: Dorsal view of the two main tick families: Ixodidae (A) and Argasidae (B) (Source: Authors own drawing).

Species of Ixodidae have been well documented to cause substantial economic loss to the livestock industry (Jongejan and Uilenberg, 2004). In particular, the Southern cattle tick *Rhipicephalus (Boophilus) microplus* is known to cause billions of dollars of loss to the cattle industry every year (Byford *et al*., 1992). *Rhipicephalus (Boophilus) microplus* is a single host tick and spends its entire parasitic phase on one host animal (normally cattle), often resulting in heavy infestations. This may cause substantial damage either through disease transmission or via direct mechanical damage due to blood-feeding activities (Jonsson, 2006).

The reinstatement of *R.(B) australis* as a species distinct from *R.(B) microplus* has been proposed in recent years, based upon mating studies (Labruna *et al*., 2009), molecular evidence (Labruna *et al*., 2009, Burger *et al*., 2014) and on observed morphological differences (Estrada-Peña *et al*., 2012). The taxonomic status of *R.(B) microplus* has assumed a more practical relevance than it deserves. This is because it has resulted in a tendency of researchers to use distinct species status of *R.(B) microplus* and *R.(B) australis* as an explanation for differences in the results among studies conducted in different regions on a tick that was previously considered to be a single species. An example of this is found in Baron *et al*. (2015). ""The mutation reported by Corley, Jonsson in the β-adrenergic-like octopamine receptor seems promising, but its restricted localization to only the central part of Queensland in Australia further substantiated our notion to investigate the OCT/Tyr receptor instead. Additionally, it has been reported that *R. microplus* in Australia may be a different species and has therefore been re-classified as *R. australis*." Whereas the first quoted sentence that relates to isolation and genetic drift is true and a reasonable concern, the belief that simply being classified as a distinct species should have any impact on the mechanism of drug resistance seems to be unreasonable. In other cases, referees acting for journals considering manuscripts on *R.(B) microplus* or *R.(B) australis* have requested verification that the species of ticks was one or the other of the two. This latter was the case in the publication of Kaewmongkol *et al*. (2015), among others. This presents an unreasonable challenge to researchers as so far, a non-molecular means of definitive differentiation of *R.(B) microplus* and *R.(B) australis* are not presently available.

Life cycles and host preference

Throughout the life cycle, ticks undergo three different stages of development; larva, nymph and adult, all of which require a blood meal in order to moult to the next stage (figure 1.2).

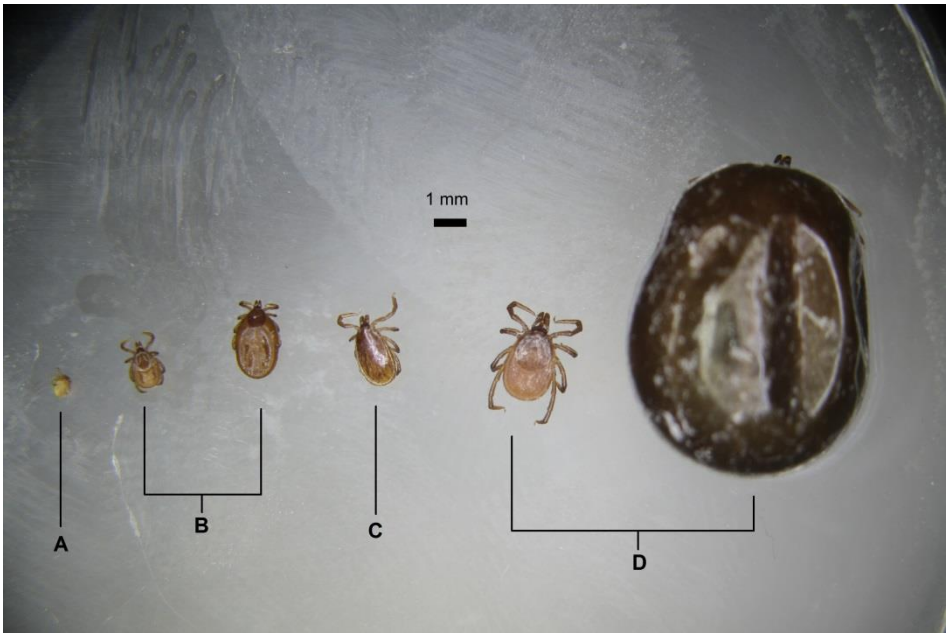


Figure 1.2: Life stages of *Ixodes ricinus* A: Larvae, B: Nymph (unfed and partially engorged), C Adult male and D: Adult female (unfed and engorged). (Source: Authors own photograph).

Larvae range in size depending on the species, but in general can be identified by their small size (approximately 0.05mm) and the presence of only six legs. Nymphs are larger in size to the larvae, but smaller than adults (adults can range in size 2-7mm) and have eight legs, at this stage they begin to resemble the adult females but can be distinguished by the absence of a genital aperture on the ventral surface (Walker *et al.,* 2003; Walker *et al*., 2005). Within the Ixodidae three different types of life cycle are observed, dependent on species adaptation to host availability and environmental conditions (Daniel *et al*., 1976). The most common of the life cycles is the three host cycle (figure 1.3), during which each stage takes a blood meal from a different host before dropping to the ground to moult to the next stage (McGarry, 2011). Adult males do not require taking a large blood-meal, unlike the females, and so spend a variable amount of time on the host taking small blood meals. The second type is the two host life cycle (figure 1.4), during which ticks moult on-host from larva to nymph, after which the nymph will then engorge and drop off the host before moulting to adult (Oliver, 1989). As

for the three-host life cycle, the time spent on host by the adult male will vary between species. Finally some Ixodidae species have a one host life cycle; after hatching on the ground a larva will attach to a host and proceed to complete its life cycle without dropping to moult. It is only after the adult females have mated, will the tick drop off to lay eggs and subsequently die (Sweatman, 1967). In the one host life cycle (figure 1.5), adult males will remain on-host longer than females, mating multiple times before they too drop off the host and die.

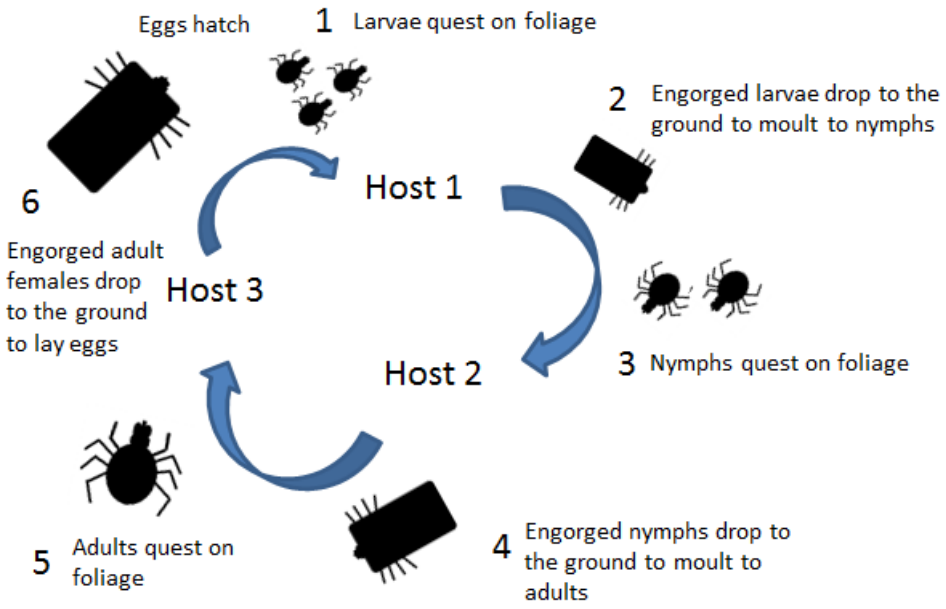


Figure 1.3: Schematic demonstrating a 3-host life cycles of the Ixodidae. (Source: Authors own drawing).

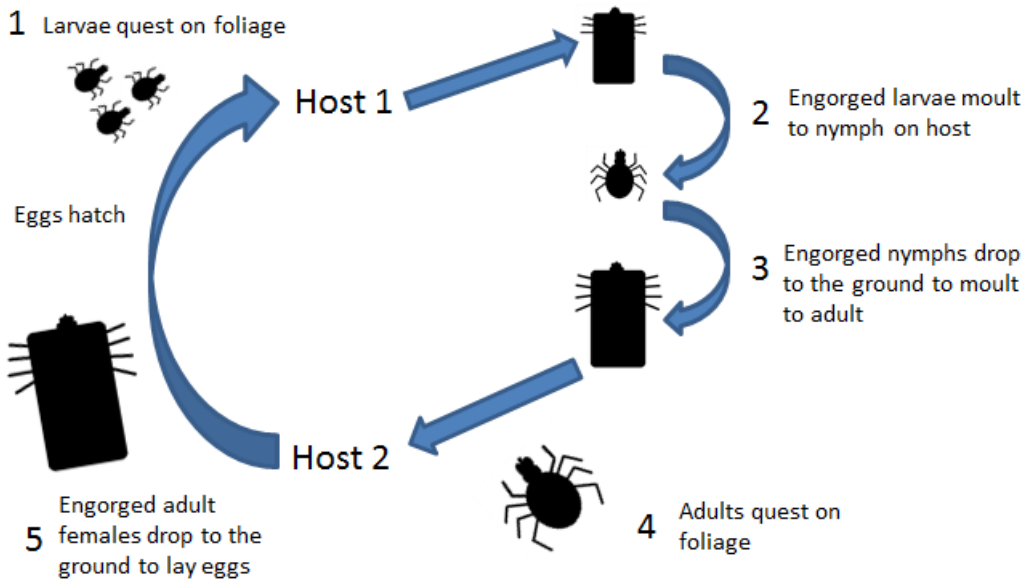


Figure 1.4: Schematic demonstrating a 2-host life cycles of the Ixodidae. (Source: Authors own drawing).

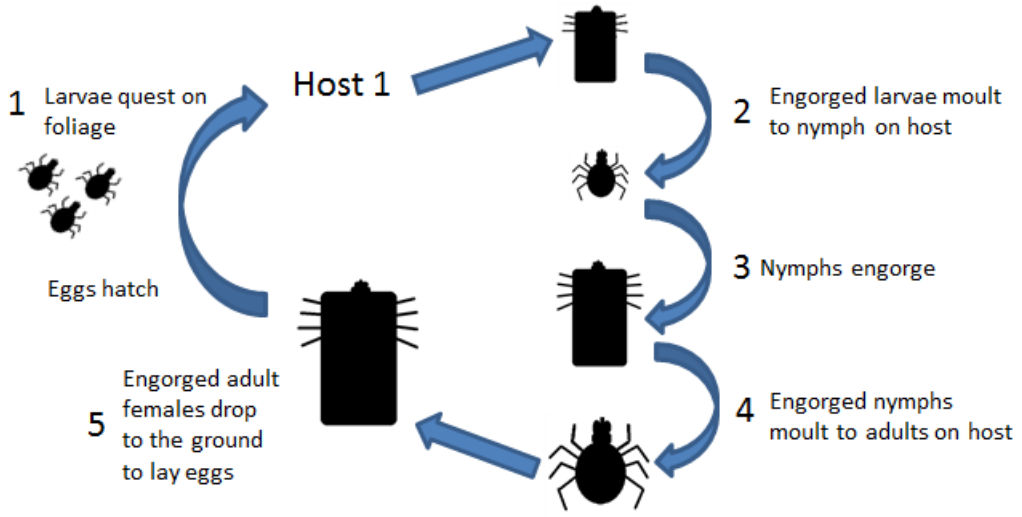


Figure 1.5: Schematic demonstrating a 1-host life cycles of the Ixodidae. (Source: Authors own drawing).

The Rhipicephaline ticks covered in this thesis; *R.(B) microplus*, *R.(B) australis*, *R.(B) annulatus* and *R.(B) decoloratus* belong to the genus *Rhipicephalus* and the sub-genus *Boophilus*. They are all one-host ticks which predominantly parasitize cattle and transmit a range of diseases (table 1.1-1.4).

Rhipicephalus (Boophilus) microplus

Table 1.1: Summary of life cycle and hosts of *R.(B) microplus* (Walker *et al.*, 2003)

Common names	**Hosts**	**Life cycle**	**Pathogens transmitted**
Southern Cattle tick, Asiatic cattle tick, Cattle tick	Primarily Cattle Also found on: horses, sheep, deer and water buffalo Rarely found on: marsupials, goats, dogs, cats and pigs	One host: Parasitic phase 18 – 35 days, typically males are on host for longer than 35 days. Life cycle can be completed in 2 months	*Babesia bovis* *Babesia bigemina* *Anaplasma marginale* *Borrelia theileri*

Rhipicephalus (Boophilus) annulatus

Table 1.2: Summary of life cycle and hosts of *R.(B) annulatus* (Walker *et al.*, 2003)

Common names	**Hosts**	**Life cycle**	**Pathogens transmitted**
Blue cattle tick, Texas cattle fever tick, Cattle fever tick, American cattle tick	Primarily Cattle Also found on: horses and deer Rarely found on: sheep, goats, humans and dogs	One host: Parasitic phase approximately 3 weeks. Life cycle can be completed in 2 months.	*Babesia bigemina* *Babesia bovis* *Anaplasma marginale*

Rhipicephalus (Boophilus) decoloratus

Table 1.3: Summary of life cycle and hosts of *R.(B) decoloratus* (Walker *et al.*, 2003)

Common names	**Hosts**	**Life cycle**	**Pathogens transmitted**
Tropical cattle tick, Blue tick	Primarily Cattle Also found on: horses and antelope Rarely found on: sheep, goats and humans	One host: Parasitic phase approximately 3 weeks. Life cycle can be completed in 2 months.	*Babesia bigemina* *Anaplasma marginale* *Borrelia theileri*

Rhipicephalus (Boophilus) australis

Table 1.4: Summary of life cycle and hosts of *R.(B) australis* (Barker and Walker, 2014)

Common names	**Hosts**	**Life cycle**	**Pathogens transmitted**
Cattle tick	Primarily Cattle Also found on: horses, sheep, deer and water buffalo Rarely found on: marsupials, goats, dogs, cats and pigs	One host: Parasitic phase 18 – 35 days, typically males are on host for longer than 35 days. Life cycle can be completed in 2 months.	*Babesia bovis* *Babesia bigemina* *Anaplasma marginale* *Borrelia theileri*

Identification of individual species

There are a number of morphological features that can be used in the identification of tick species, in order to use these features, it is important to understand where/what the features are and the variations that can occur. For a full guide to the morphological terms please see the appendix.

Rhipicephalus (Boophilus) microplus

Adult female *R.(B) microplus* has broad oval porose areas. The internal margin of palp article i is short and distinctly concave with no protuberance. The ventral surface of the hypostome has a typical 4+4 tooth arrangement. Coxa I have distinct, short internal and external spurs, with indistinct spurs on the rest of the coxae. The genital aperture forms a broad 'U' shape and is positioned between coxae II. Dorsal setae are said to be short and slender, forming clusters of medial alloscutal setae in 2-3 rows (figure 1.6) (Barker and Walker, 2014, Walker *et al.*, 2003).

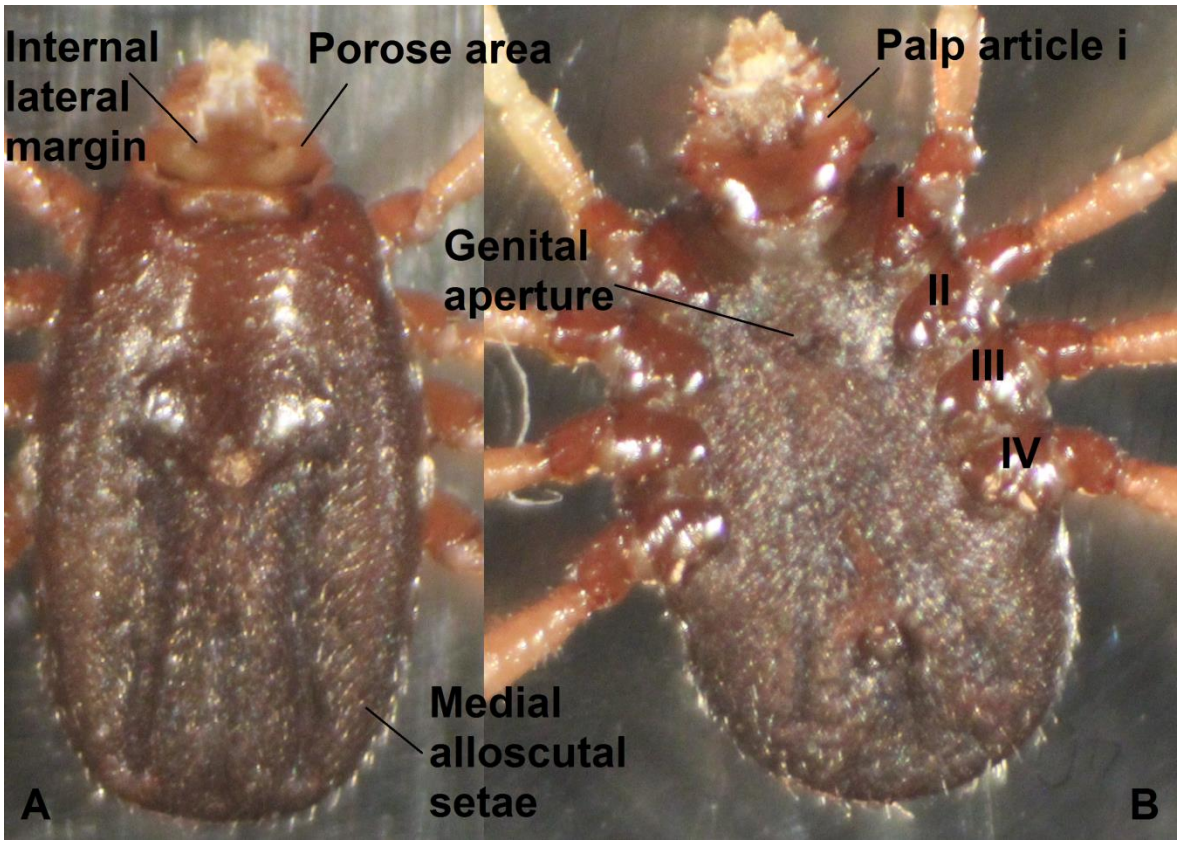


Figure 1.6: Morphological features of *R.(B) microplus* females; A: dorsal. B: ventral. (Source: Authors own photograph).

Adult males have distinct cornua on the posterior margin of the basis capitulum. Both adanal and accessory adanal shields are present, with indistinct spurs that are not visible from the dorsal aspect in unfed males. Adanal shields are squared in shape along the posterior border. A narrow caudal appendage is also present. Coxa I have a distinct anterior spur, as well as distinct internal and external spurs. Coxae II-IV has indistinct spurs. Several setae are present on the lateral margins of the ventral surface of the capitulum (figure 1.7) (Barker and Walker, 2014, Walker *et al.*, 2003).

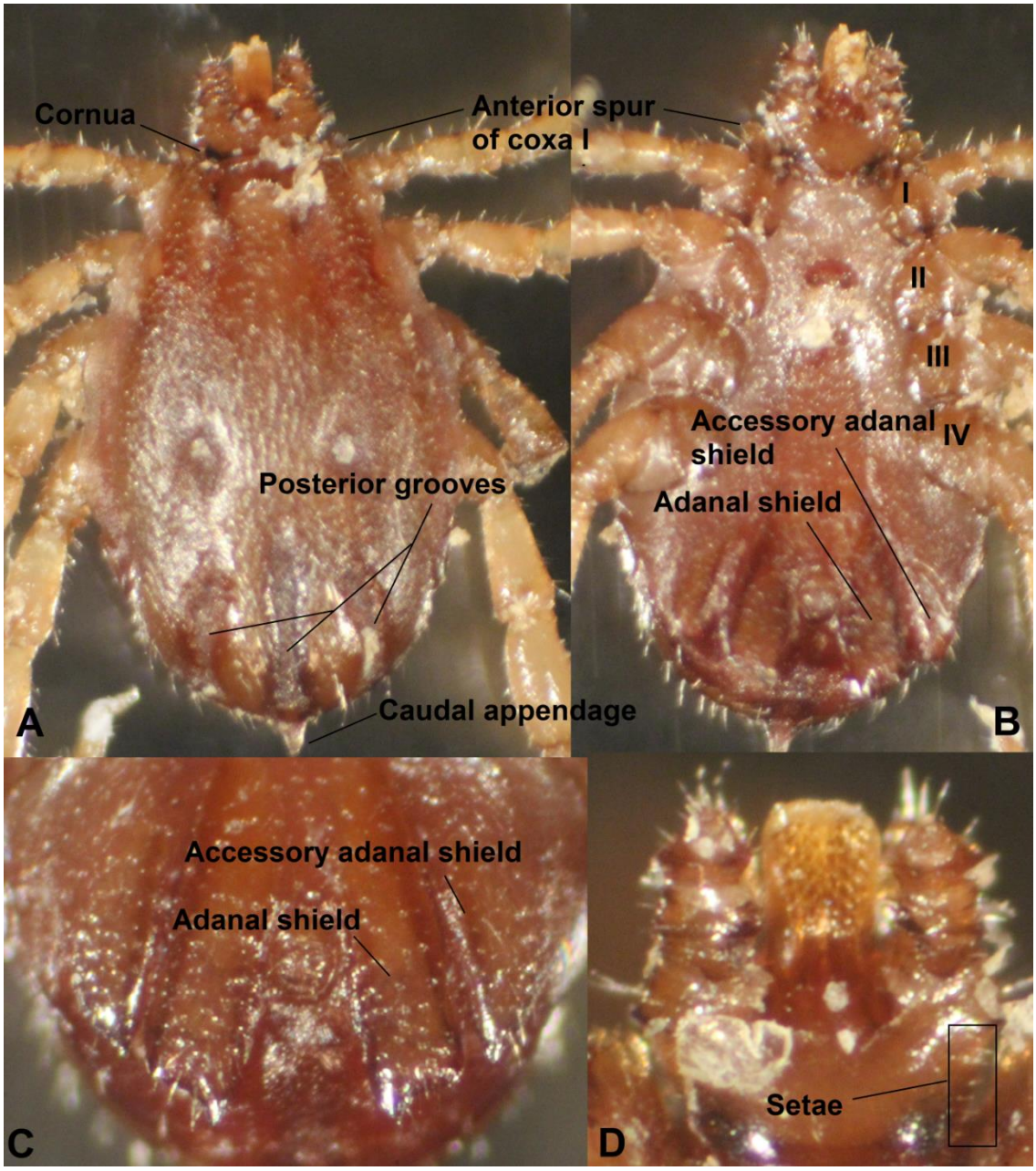


Figure 1.7: Morphological features of *R.(B) microplus* males; A: dorsal. B: ventral. C: close up of the ventral shields. D: setae on the lateral margin of the capitulum. (Source: Authors own photograph).

The larvae are broad oval in shape, with no cornua or palp article i. The basis capituli is broadly rounded on the ventral borders. The hypostome is short and broad with a 2+2 teeth configuration. The scutum is smooth, lacking setae and extends to take up approximately two thirds of the dorsal surface. Cervical grooves are present; however they are shallow. There is a short internal spur on coxa I with no spurs on the rest of the coxae (figure 1.8) (Barker and Walker, 2014, Walker *et al.*, 2003).

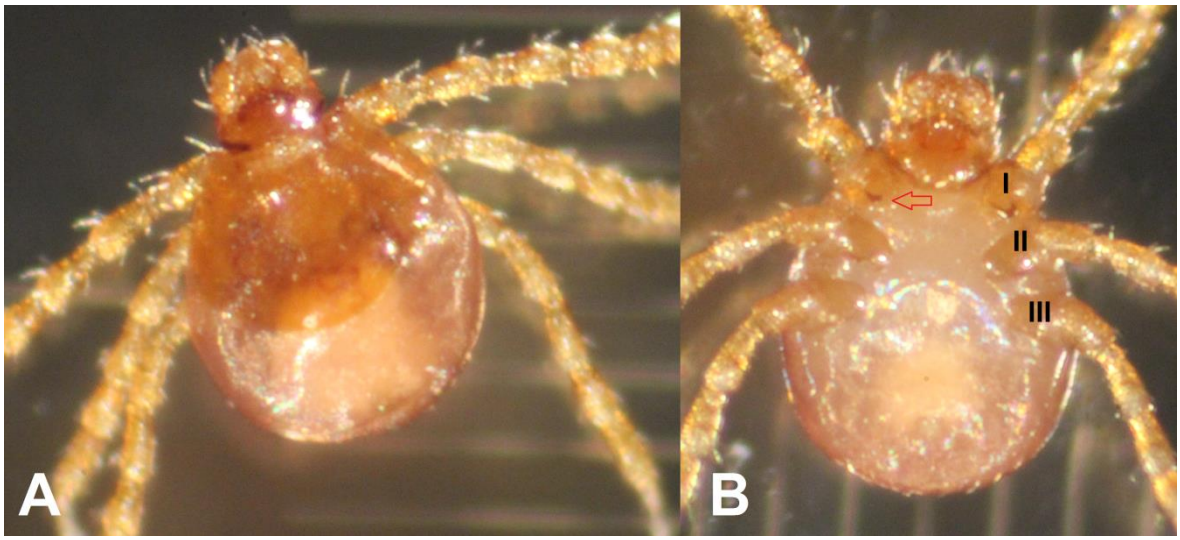


Figure 1.8: Morphological features of *R.(B) microplus* larvae; A: dorsal. B: ventral. Short internal spur of coxa I indicated by red arrow. (Source: Authors own photograph).

Rhipicephalus (Boophilus) australis

Based on the re-description outlined by Estrada-Peña *et al*. (2012); adult female *R.(B) australis* has broad oval porose areas. The internal margin of palp article i is short and distinctly concave with no protuberance. The ventral surface of the hypostome has a typical 4+4 tooth arrangement. Coxa I have distinct, short internal and external spurs, with indistinct spurs on the rest of the coxae. The genital aperture forms a broad 'U' shape and is positioned between coxa II. Dorsal setae are said to be long and pale, forming clusters of medial alloscutal setae in 4-6 rows. Setae are also said to be present behind the eyes (figure 1.9) (Barker and Walker, 2014).

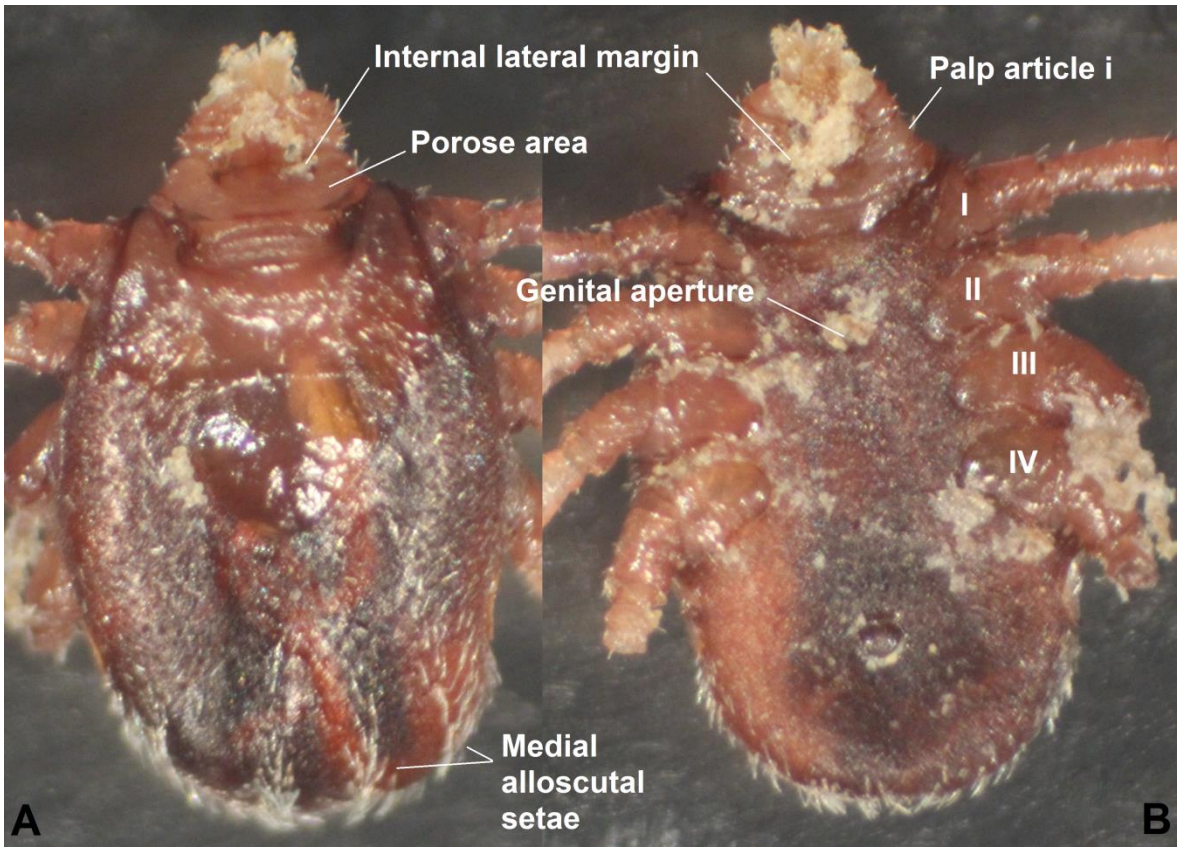


Figure 1.9: Morphological features of *R.(B) australis* females; A: dorsal. B: ventral. (Source: Authors own photograph).

Adult males have distinct cornua on the posterior margin of the basis capitulum. A spur is present on the ventral surface of palp article i. Both adanal and accessory adanal shields are present, with indistinct spurs that are not visible from the dorsal aspect in unfed males. A narrow caudal appendage is also present. Coxa I have distinct anterior spurs, visible from the dorsal aspect, as well as distinct internal and external spurs. Coxae II-IV has indistinct spurs. Setae may be present on the lateral margins of the ventral surface of the basis capitulum, when present they are very short (figure 1.10) (Barker and Walker, 2014).

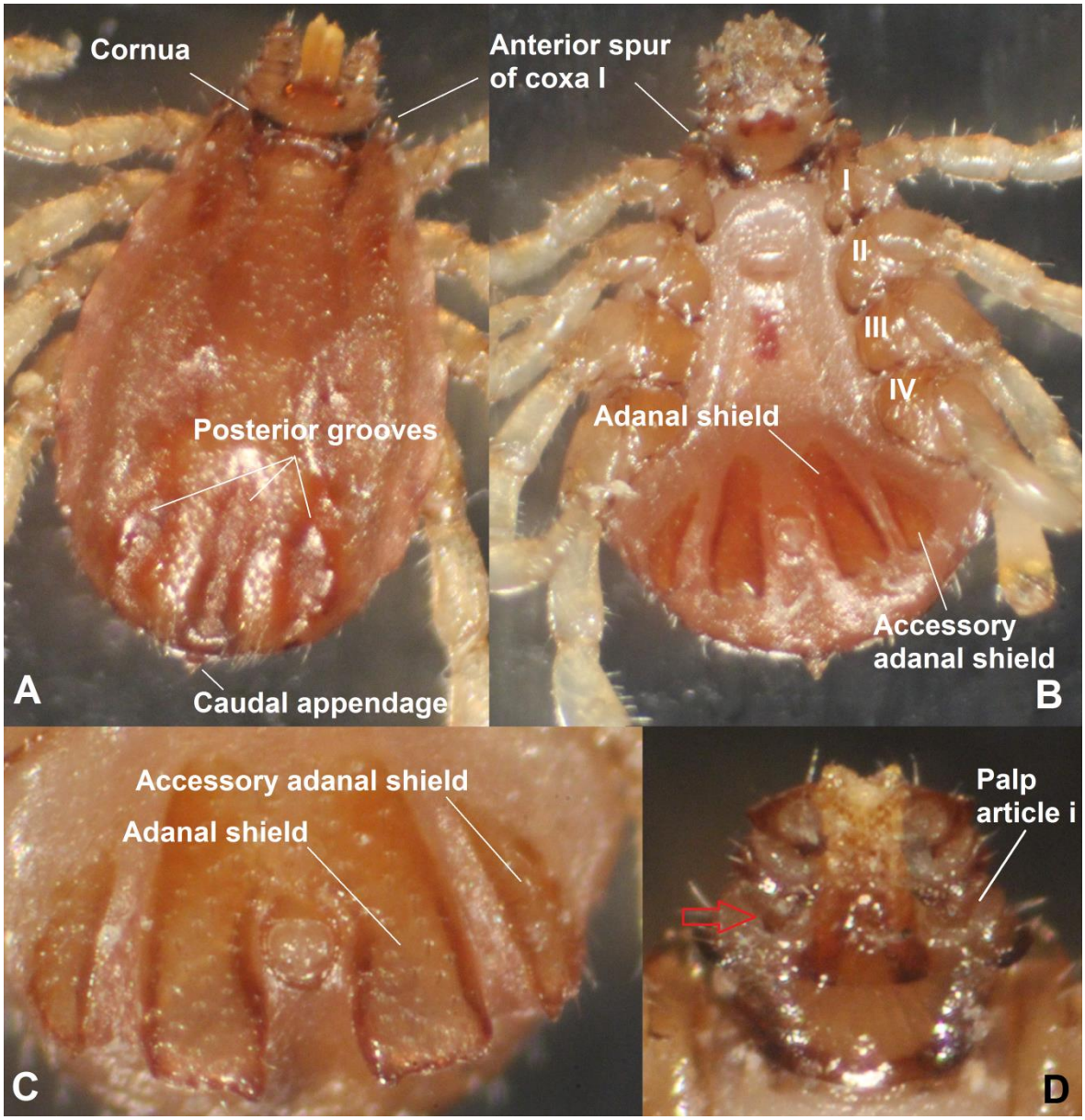


Figure 1.10: Morphological features of *R.(B) australis* males; A: dorsal. B: ventral C: close up of the ventral plates. D: ventral mouthparts (ventral spur of palp article i indicated by red arrow). (Source: Authors own photograph).

The morphological features of the larvae are the same as for *R.(B) microplus* (figure 1.11) (Barker and Walker, 2014).

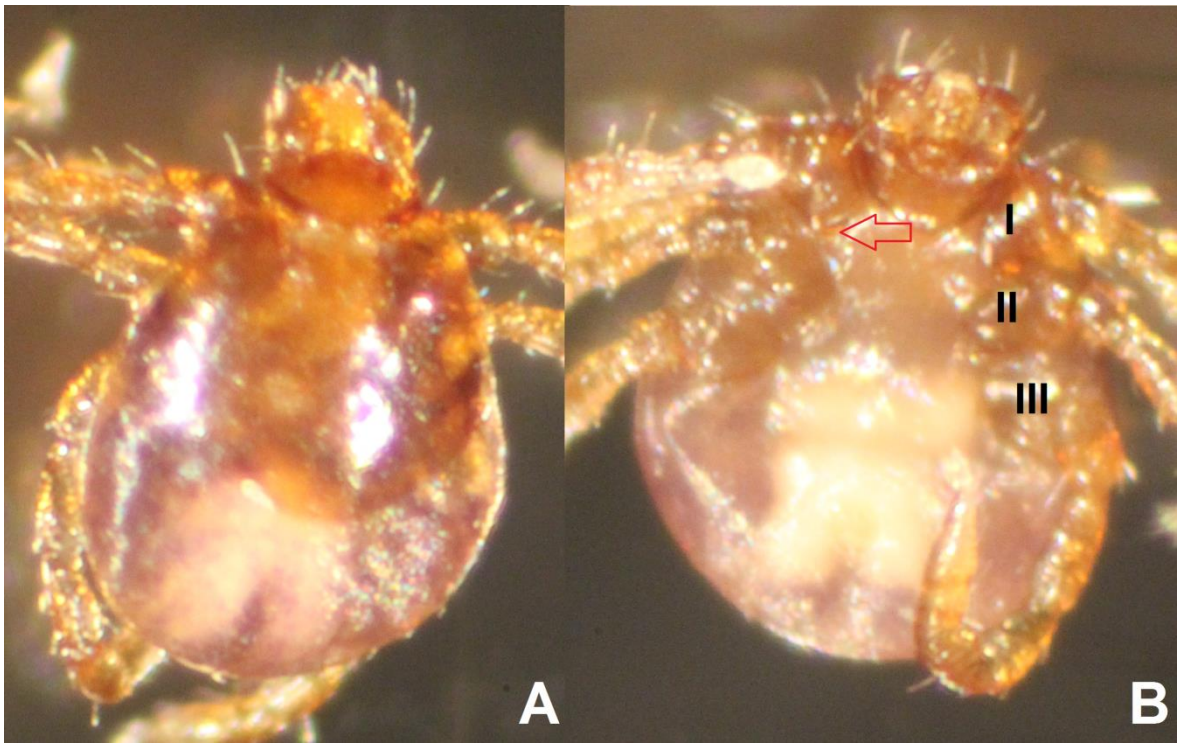


Figure 1.11: Morphological features of *R.(B) australis* larvae; A: dorsal. B: ventral. Short internal spur of coxa I indicated by red arrow. (Source: Authors own photograph).

Rhipicephalus (Boophilus) annulatus

Adult female *R.(B) annulatus* have broad oval porose areas. The tooth arrangement on the ventral hypostome is 4+4 and the internal margin of palp article i is long and slightly concave. Both internal and external spurs on coxa I are indistinct with spurs absent on coxae II-IV. The genital aperture forms a broad 'U' shape (figure 1.12) (Walker *et al.*, 2003).

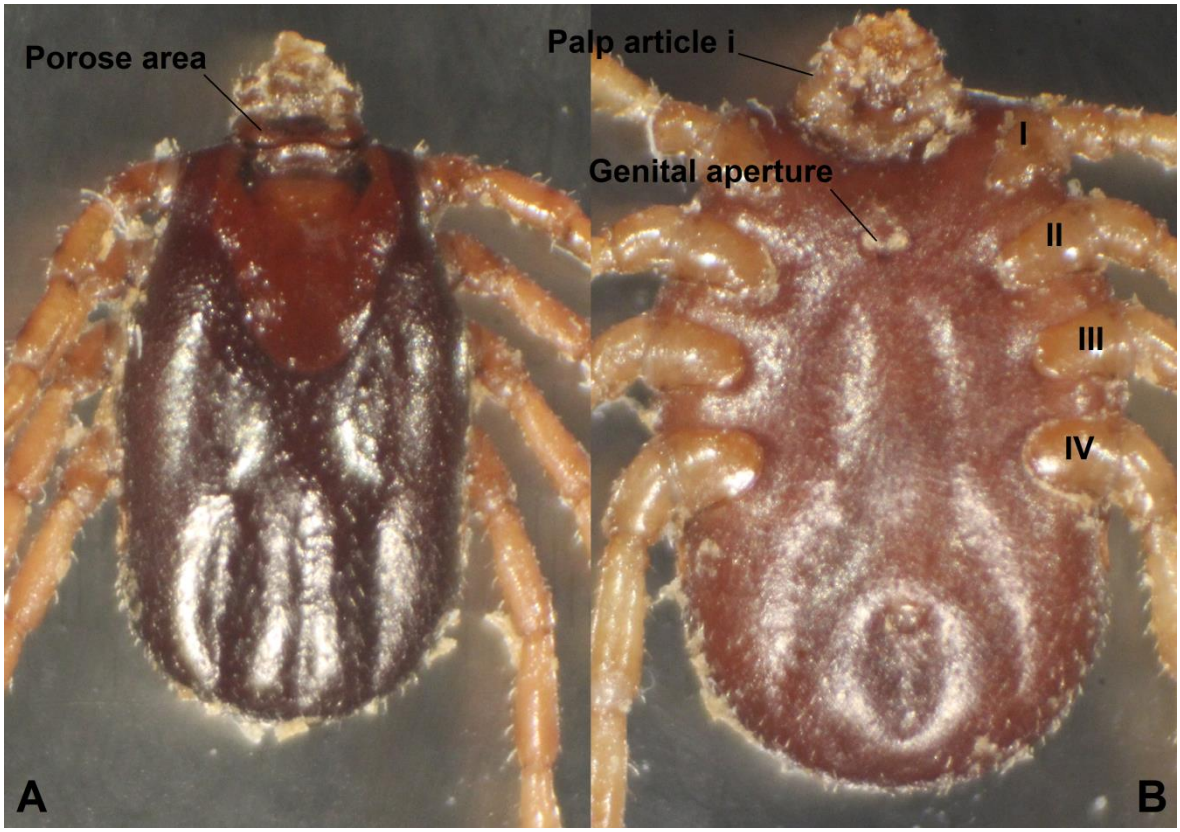


Figure 1.12: Morphological features of *R.(B) annulatus* females; A: dorsal. B: ventral. Lateral margin of palp article i obscured by debris. (Source: Authors own photograph).

Adult males have distinct cornua. On the ventral surface the posterior border of the adanal shield is rounded and spurs are indistinct. The accessory adanal shields are indistinct with indistinct spurs. The caudal appendage is also absent. Coxa I have short internal and external spurs, spurs are absent from the rest of the coxae (figure 1.13) (Walker *et al.*, 2003).

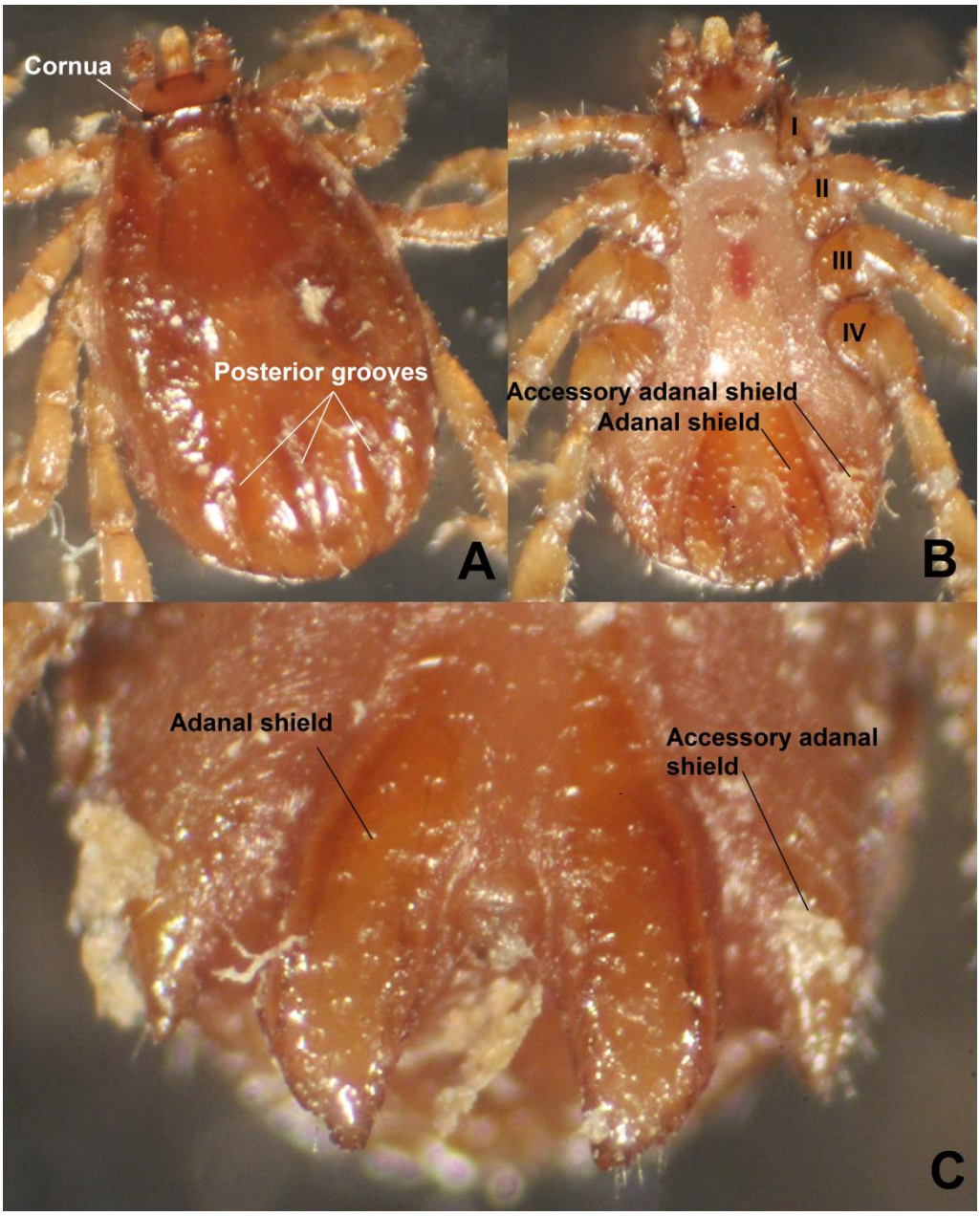


Figure 1.13: Morphological features of *R.(B) annulatus* males; A: dorsal. B: ventral C: close up of the ventral plates. (Source: Authors own photograph).

The body of the larva is broad oval with the basis capitulum slightly convex laterally and either straight or slightly convex along the posterior border. As with *R.(B) microplus* larvae, there are no cornua present and the hypostome is shorter than that of *R.(B) microplus*. Hypostomal teeth are arranged in a 2+2 formation. The scutum is smooth, lacking both punctations and setae. It takes up approximately half the dorsal surface and is broader then it is long. Cervical grooves are present and short (figure 1.14) (Walker *et al.*, 2003).

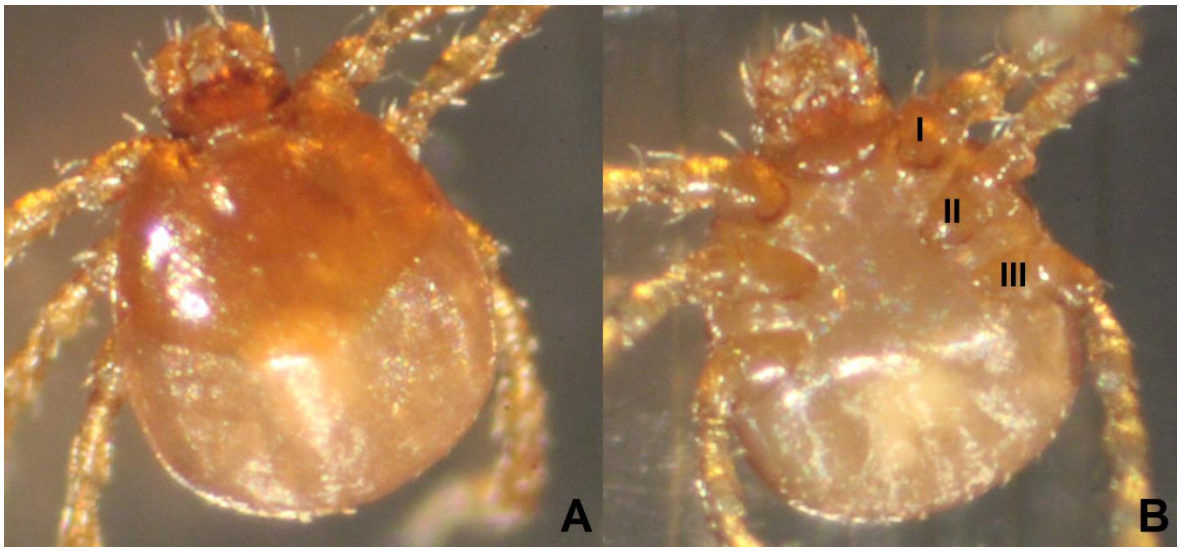


Figure 1.14: Morphological features of *R.(B) annulatus* larvae; A: dorsal. B: ventral. (Source: Authors own photograph).

Rhipicephalus (Boophilus) decoloratus

Adult female *R.(B) decoloratus* have narrow oval porose areas. The internal margin of palp article i has a protuberance with setae attached. The ventral hypostome has a 3+3 tooth arrangement. Coxa I have short distinct internal and external spurs, where coxae II-IV have indistinct external spurs. The genital aperture forms a narrow 'U' shape (figure 1.15) (Walker *et al.*, 2003).

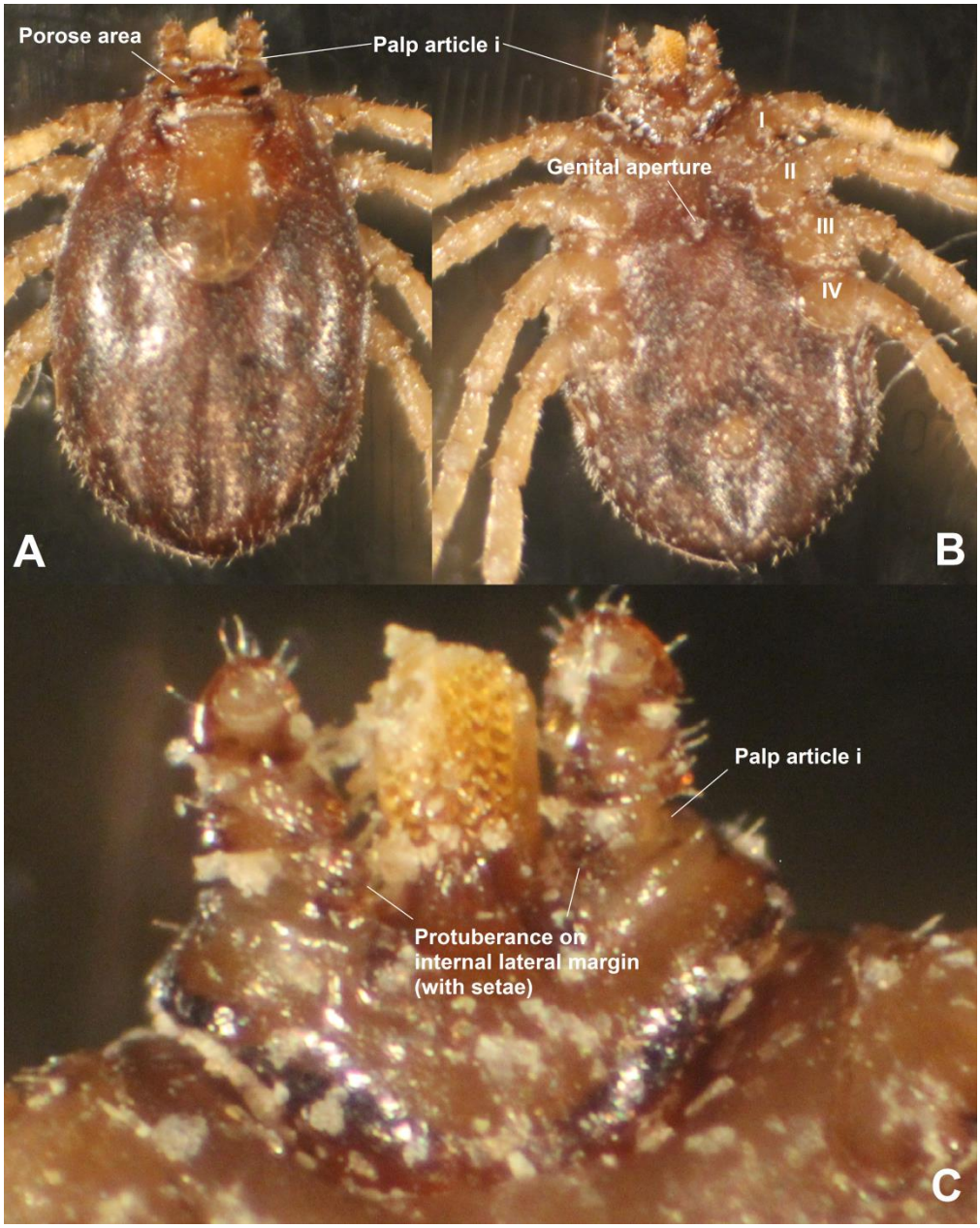


Figure 1.15: Morphological features of *R.(B) decoloratus* females; A: dorsal. B: ventral. C: close up of ventral mouthparts, protuberance on internal margin of article i indicated as red arrow. (Source: Authors own photograph).

Adult males have distinct cornua. The adanal and accessory adanal plates are distinct with distinct spurs that are visible from the dorsal aspect. The caudal appendage is present and narrow. Coxa I has distinct but short internal and external spurs (figure 1.16) (Walker *et al.*, 2003).

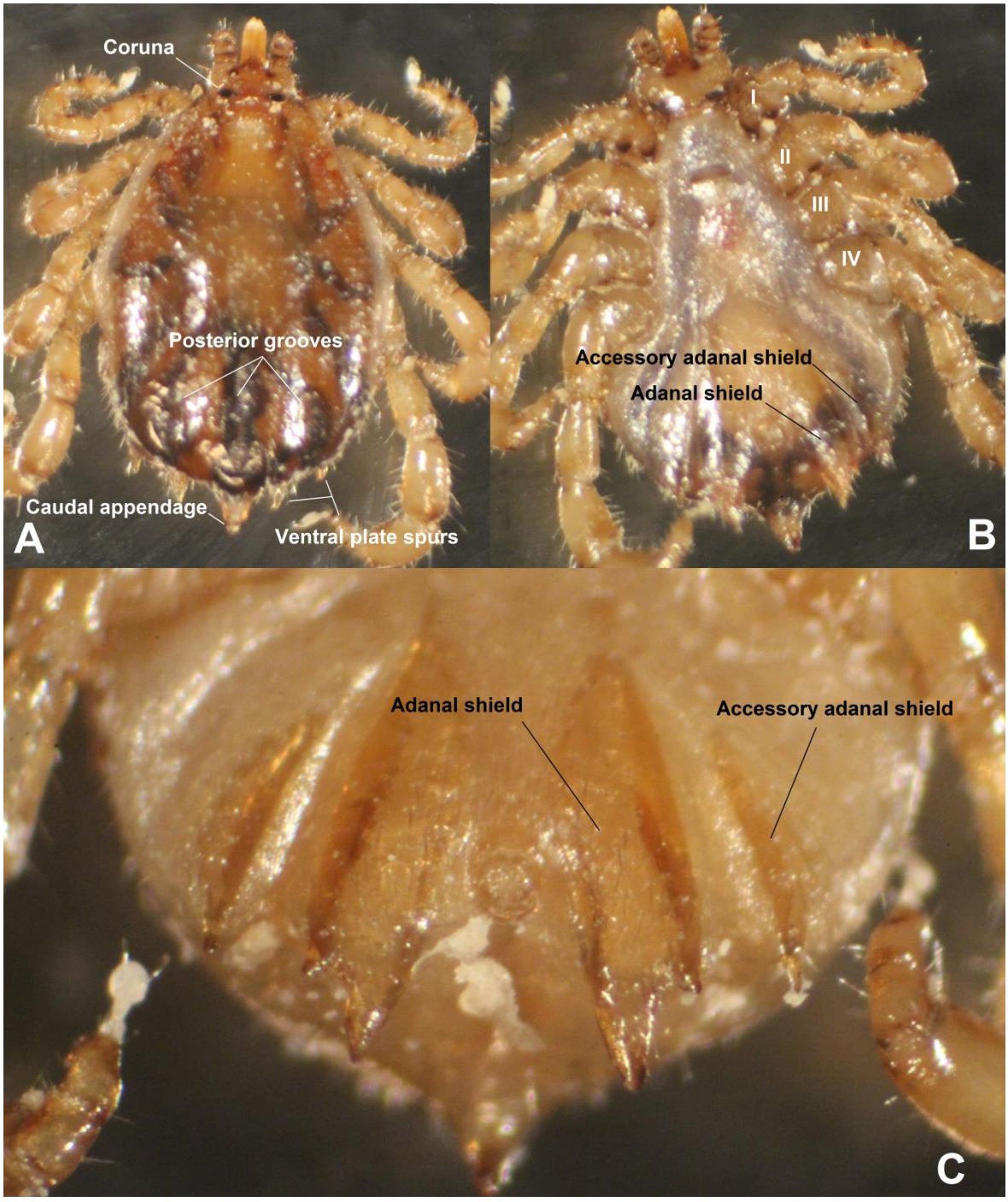


Figure 1.16: Morphological features of *R.(B) decoloratus* males; A: dorsal. B: ventral C: close up of the ventral plates. (Source: Authors own photograph).

Larvae are broad oval in shape, the scutum is broader then long, finely punctuated, containing fine setae. The scutum also has short, shallow cervical grooves and extends approximately half the dorsal surface. The capitulum is straight or slightly undulate along the anterior border and sharply convex and protuberant along the lateral borders. The ventral aspect of the basis capitulum is broadly rounded. Cornua are absent and the hypostome is short with a 2+2 tooth arrangement (figure 1.17) (Walker *et al.*, 2003).

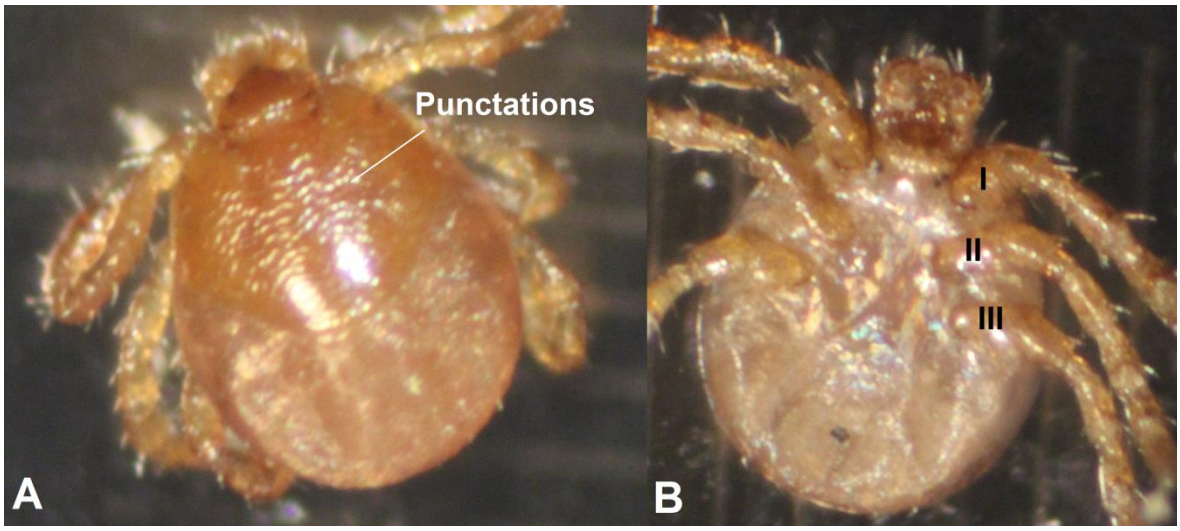
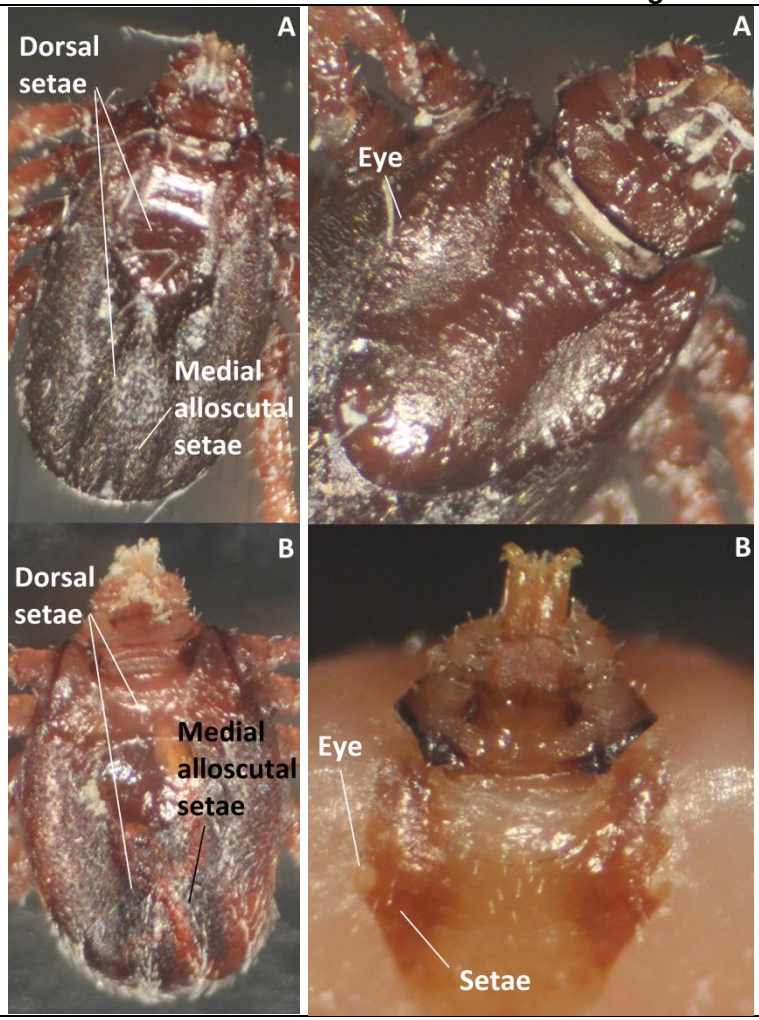


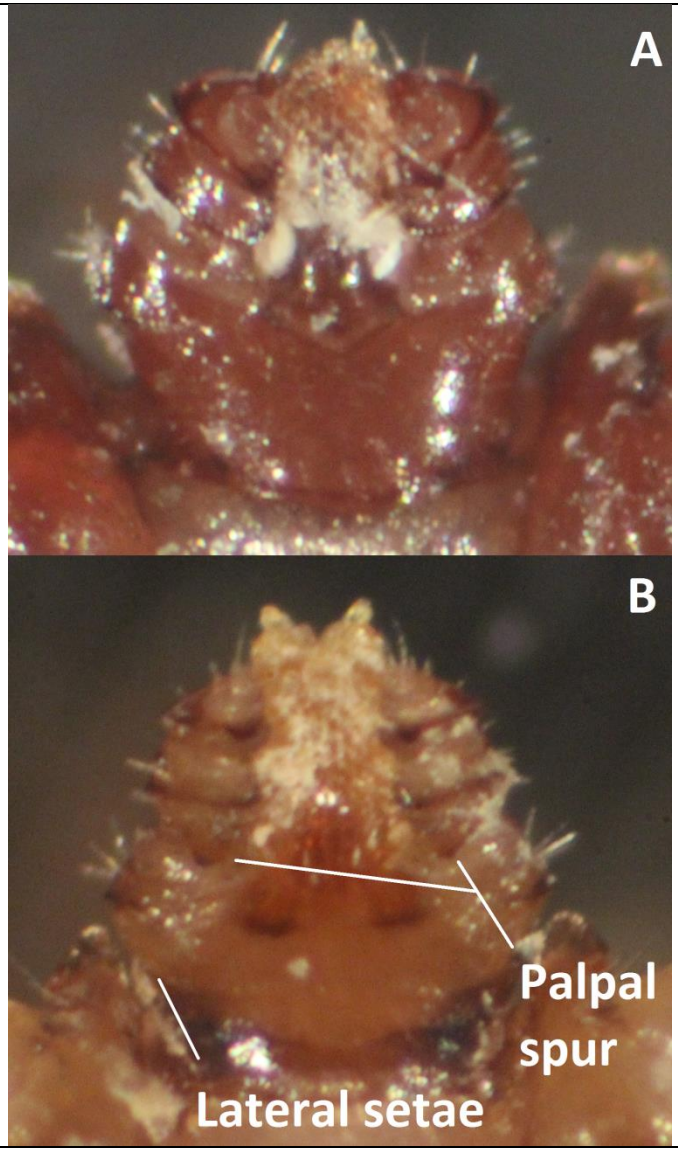
Figure 1.17: Morphological features of *R.(B) decoloratus* larvae; A: dorsal. B: ventral. Punctations on the scutum is indicated and appear as a 'wrinkling' and 'pitting' on the scutal surface. (Source: Authors own photograph).

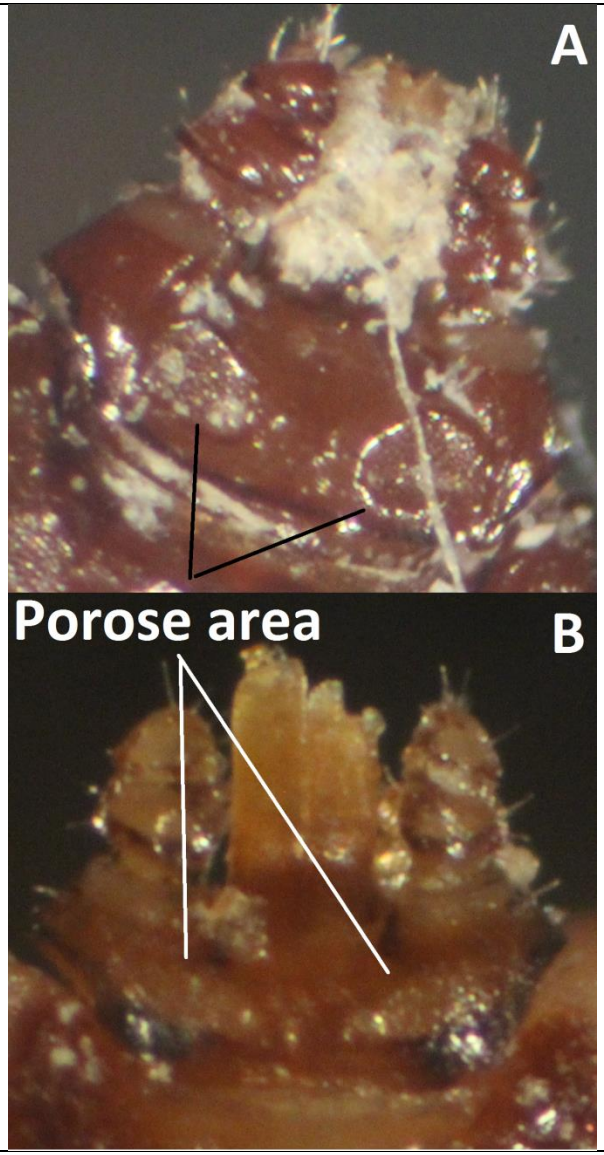
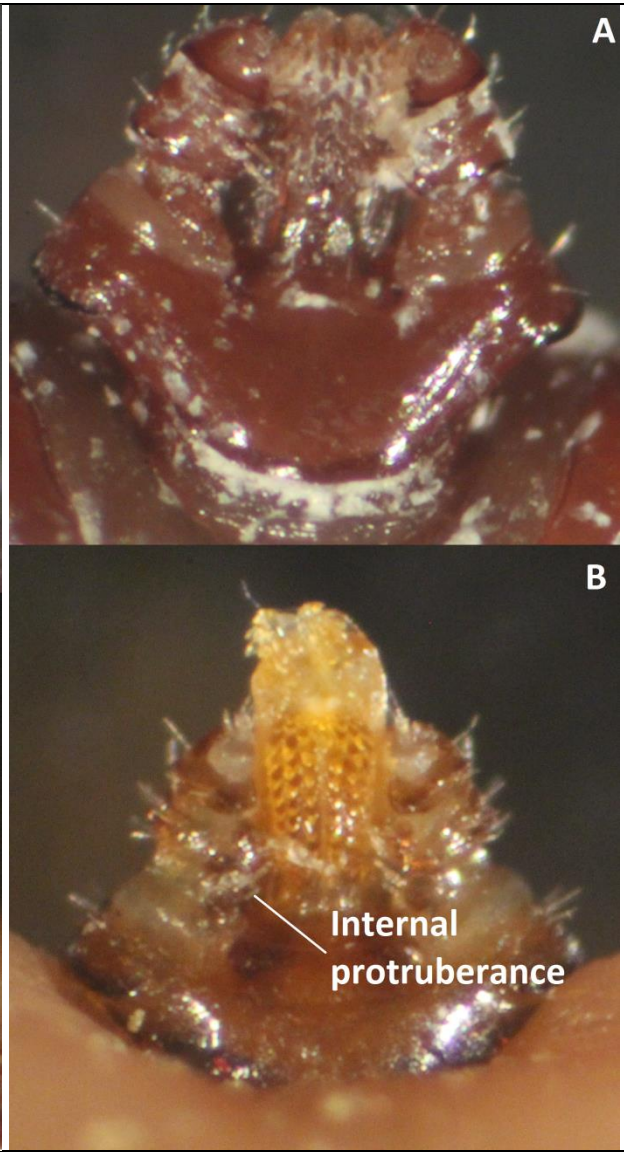
Distinguishing between the proposed species

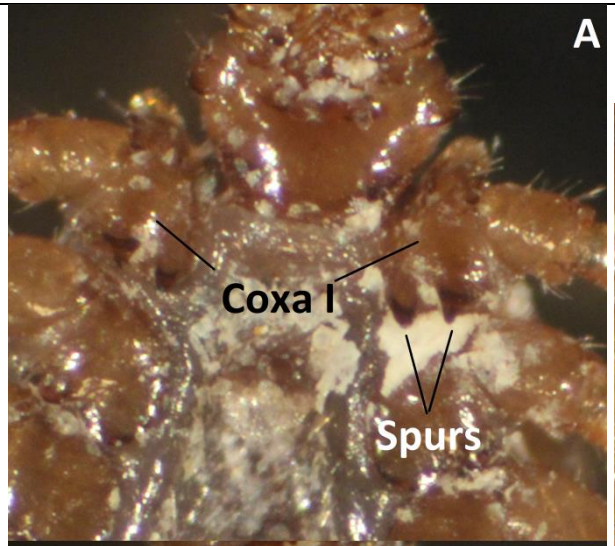
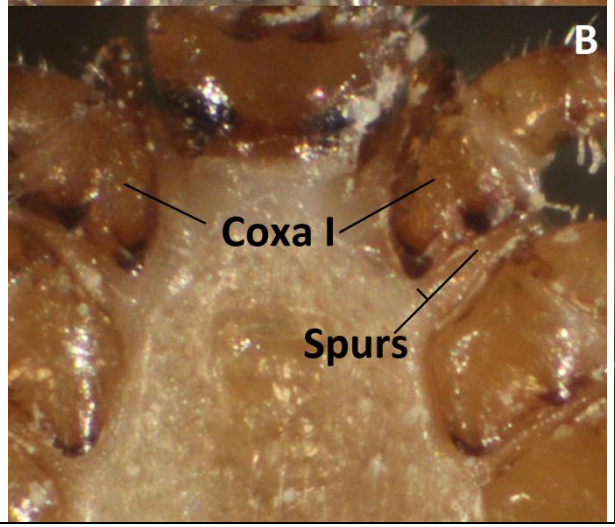
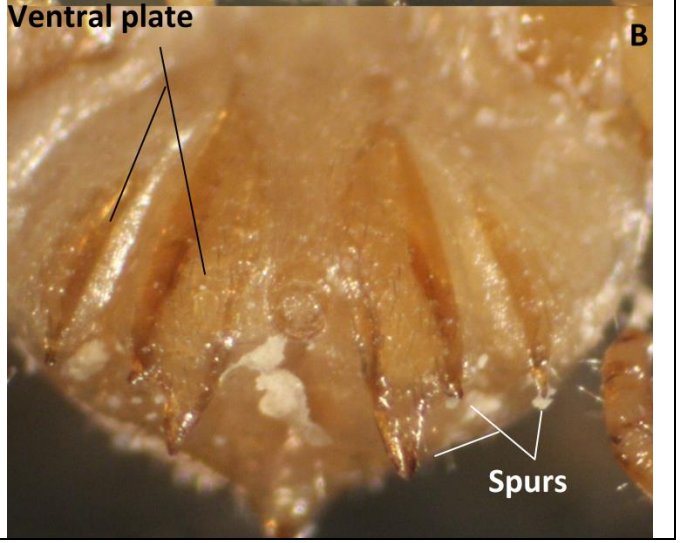
Below is a table summarizing the morphological differences between the four species described above based on the morphology outlined in the literature (Barker and Walker, 2014, Walker *et al.*, 2003) (table 1.5).

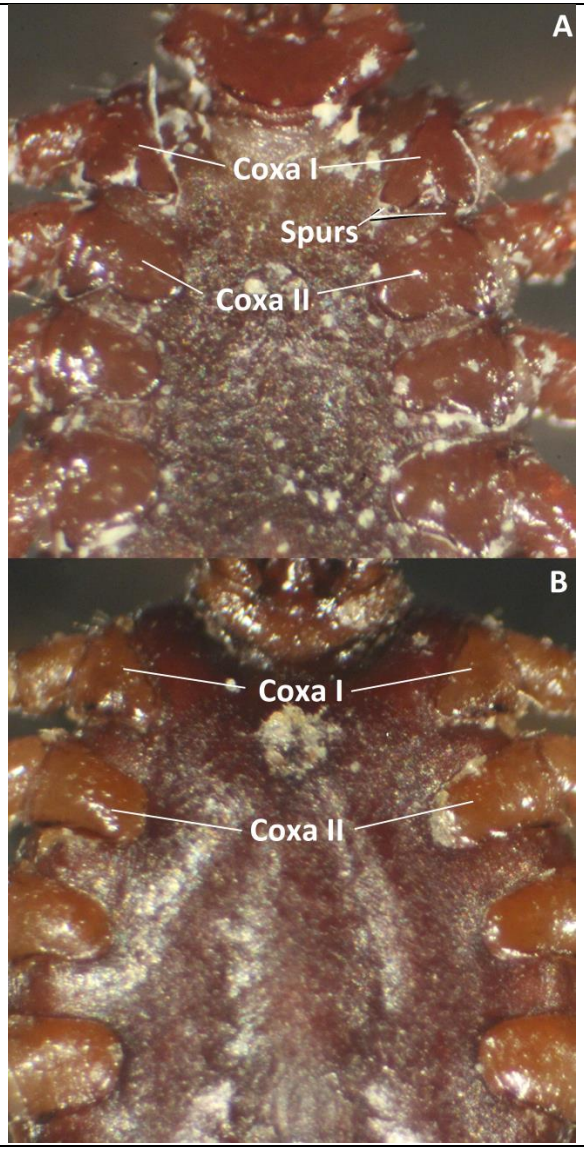
Table 1.5: A summary of the morphological difference between the four species of interest; *R.(B) microplus*, *R.(B) australis, R.(B) annulatus* and *R.(B) decoloratus.*

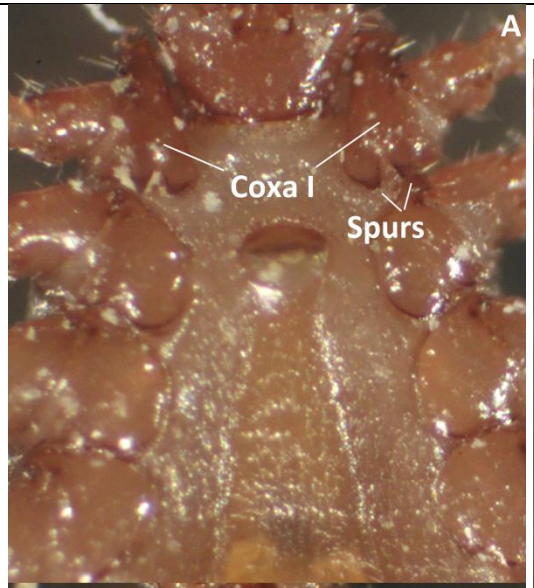
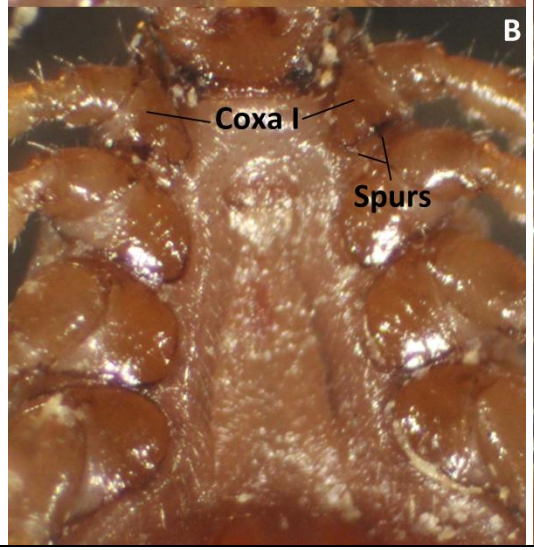
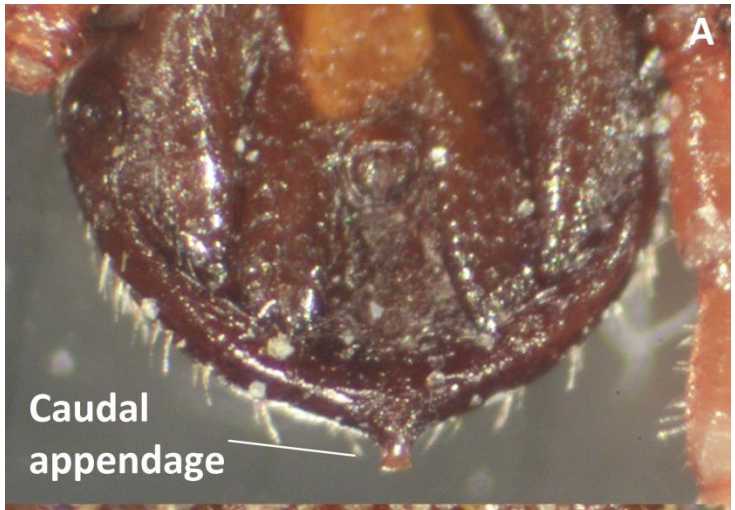
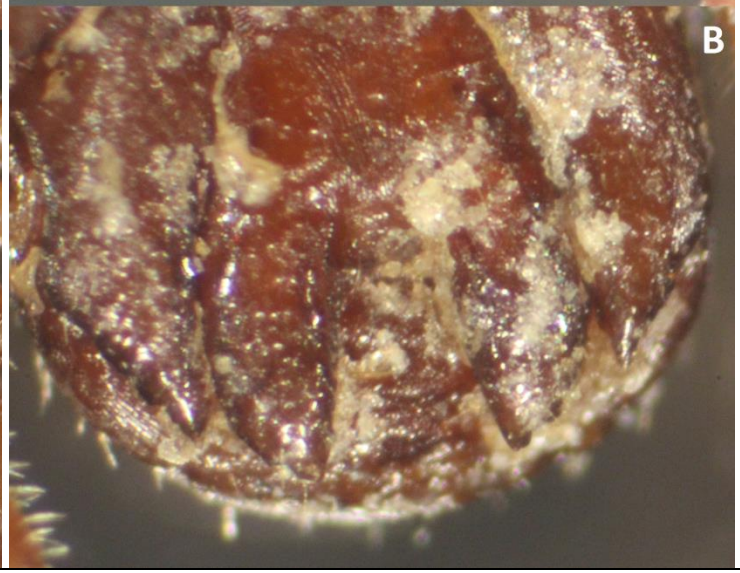
Sex	Comparison	Image
Female	***R.(B) microplus* features (A):** **>**Dorsal setae are shorter and slender. **>**Medial alloscutal setae form clusters of 2–3 rows. **>**Setae behind the eyes are unapparent/ absent. ***R.(B) australis* features (B):** **>**Dorsal setae abundant, long, and pale. **>**Medial alloscutal setae are in clusters of 4–6 rows. **>**Setae behind the eyes are clearly visible.	

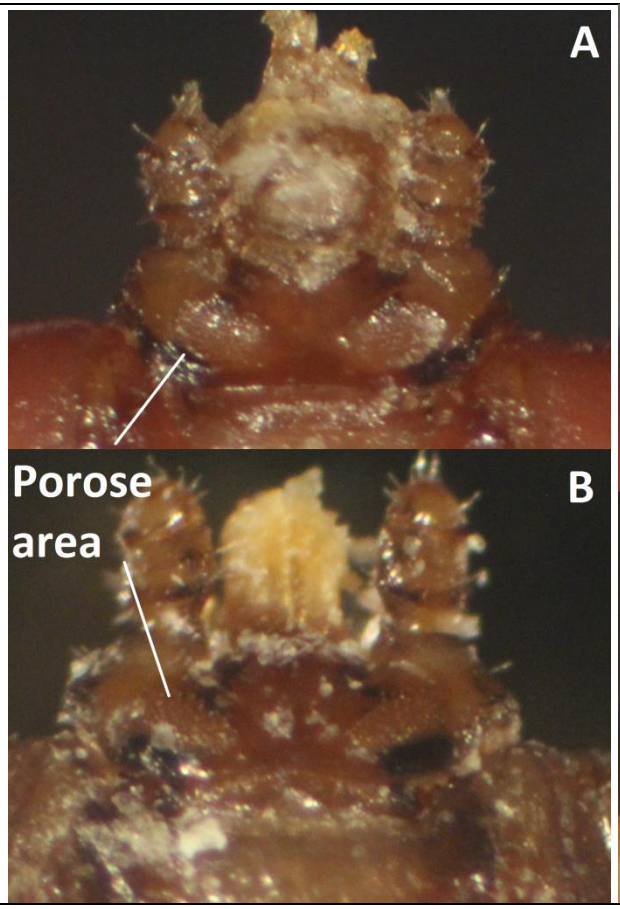
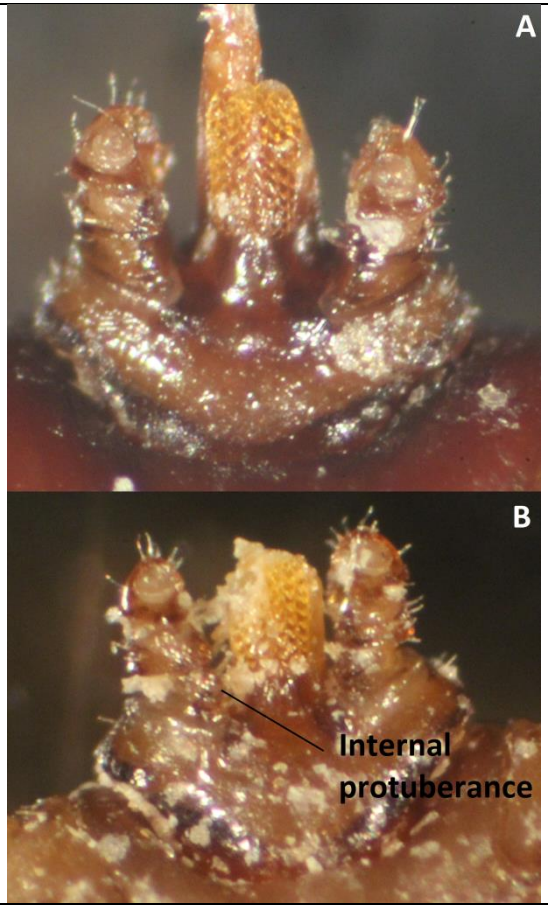
Male	***R.(B) microplus* features (A):** **>**Ventral spur absent from palp article i. **>**Several setae present on the lateral margins of the ventral surface of the capitulum. ***R.(B) australis* features (B):** **>**Spur present on the ventral surface of palp article i. **>**Setae on the lateral margins of the ventral surface of the capitulum either very short/absent.	

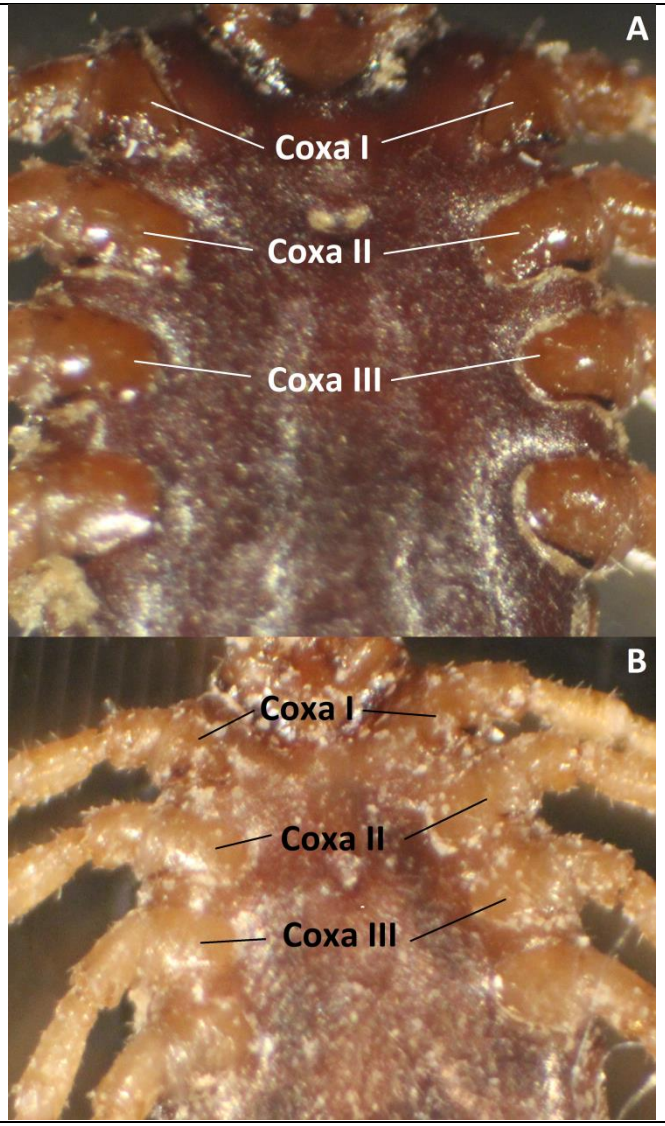
Female	***R.(B) microplus* features (A):** **>**Porose area broad oval. **>**Palp i internal protuberance absent. ***R.(B) decoloratus* features (B):** **>**Porose area narrow oval. **>**Palp i internal protuberance present	 

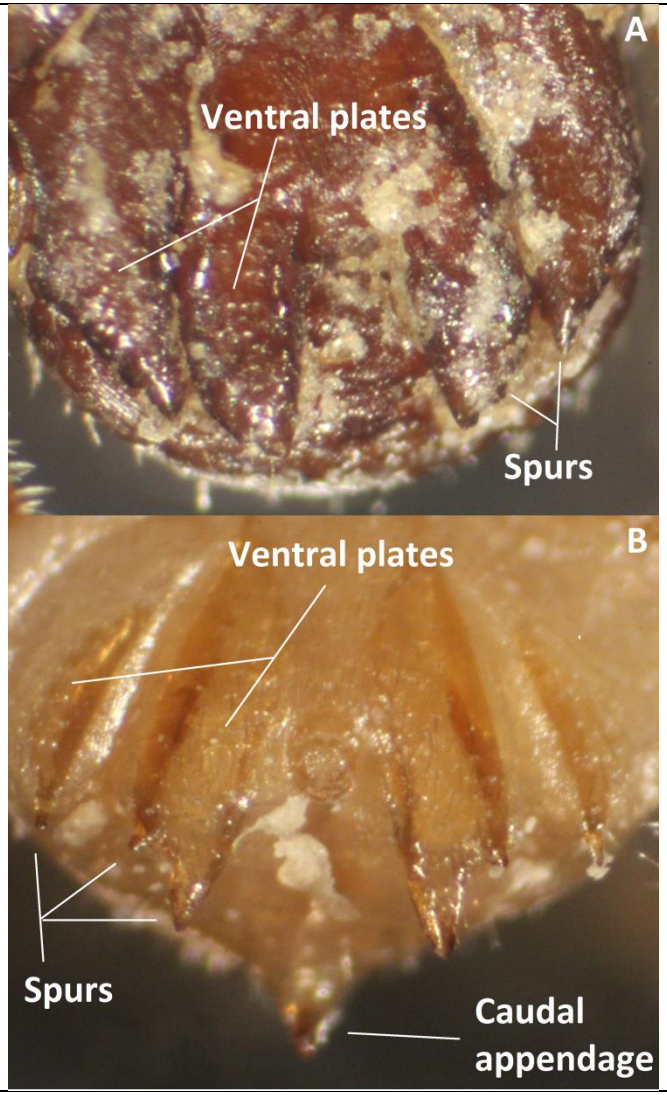
Male	***R.(B) microplus* features (A):** **>**Coxae I spur long. **>**Ventral plate spurs indistinct ***R.(B) decoloratus* features (B):** **>**Coxae I spur short. **>**Ventral plate spurs distinct	   

Female	***R.(B) microplus* features (A):** **>**Coxae I spurs distinct. **>**Coxae II + III spurs present ***R.(B) annulatus* features (B):** **>**Coxae I spurs indistinct. **>**Coxae II + III spurs absent.	

Male	***R.(B) microplus* features (A):** **>**Coxae I spurs long. **>**Caudal appendage present ***R.(B) annulatus* features (B):** **>**Coxae I spurs short. **>**Caudal appendage absent.	 	 

Female	***R.(B) annulatus* features (A):** **>**Porose area broad oval. **>**Palp i internal protuberance absent. **>**Coxae I spurs indistinct. **>**Coxae II + III spurs absent. ***R.(B) decoloratus* features (B):** **>**Porose area narrow oval. **>**Palp i internal protuberance present. **>**Coxae I spurs distinct. **>**Coxae II + III spurs present.	 


A
Coxa I
Coxa II
Coxa III
B
Coxa I
Coxa II
Coxa III

Male	***R.(B) annulatus* features (A):** **>**Ventral plate spurs indistinct. **>**Caudal appendage absent. ***R.(B) decoloratus* features (B):** **>**Ventral plate spurs distinct. **>**Caudal appendage present.		

Techniques used to quantify morphology

There are a number of approaches towards classifying morphology used in tick studies and they range from the more traditional to those that have been evolved alongside the development of image analysis software. The aim of these approaches is to quantify the phenotypic variation that result from genetic variation, excluding where possible, those changes in morphology that result from external factors.

Scoring

Scoring is a very simple method for comparing morphology. It does not require any special software and can be done by eye using a suitable microscope. This method allows for the comparison of morphology between species that are either distantly or closely related, however the morphological characteristics of at least one of the species type specimen of interest needs to have been documented previously. It is not a technique widely used in publication, as most studies favour morphometrics for a more in-depth insight into morphology; however some studies have used this approach, along with phylogenetic data to infer phylogeny (Klompen *et al*., 1997; Beati and Keirans 2001). Scoring is a technique used mainly in the construction of field guides or in the creation of identification keys for ticks and allows for easy sorting of tick specimens to then be used for morphometric analysis (Walker *et al*., 2003; Walker *et al*., 2005; Barker and Walker 2014; Hillyard 1996). In the case of identification keys, as seen in Hillyard, 1996, descriptive statements are used in the place of a binary system, for example 'spur on coxa I overlaps coxa II', in this case if the answer is yes it may either take you to a new set of questions or it might be enough to reach a conclusion on the species identification. If the answer is no then you are led to a new question and so on.

Scoring is the process of giving a feature a value of 1 or 0 depending on whether it meets a given set of criteria or not (Klompen *et al*., 1997; Beati and Keirans 2001). This can range from variances in the feature described to whether the feature is present or absent. Klompen *et al*., 1997 used this technique as part of the re-evaluation of relationships in the Metastriata, using 82 characters as points for comparison. As this study covered a large number of different species, some of

the features had more than two variations and were therefore given a score from 0-4. Results from this study mostly supported previous classifications based on morphology, with the exception of rejecting the previously proposed monophyly of *Aponomma* and *Amblyomma* when combined with phylogenetic data. Beati and Keirans, 2001 used a score matrix, along with phylogenetic data to explore the systematic relationships among *Rhipicephalus* and *Boophilus*. In this study they used 63 characters, giving a score of up to 0-4, again in the cases where features had more than two states of being. In this case the score matrix approach to morphology was found to be unsuccessful, where no character was found to be consistent. It was concluded that the high level of variability in the features tested with the matrix in Rhipicpehaline and Boophilid ticks made it impossible to reliable differentiate between females, however this approach did have a measure of success with males and larvae. The difficulties expressed by Beati and Keirans, 2001 are one that is still being expressed in more recent studies. Lempereur *et al*., 2010 have commented that due to the similarities in morphology between Boophilid species and the amount of variability observed in those features, differentiation is often unreliable, if not impossible.

Despite the potential problems with this technique, it is still a useful starting point for the gross sorting of tick specimens, particularly if they are from the field. However caution must be taken when drawing any conclusions of species identity based on features that can be observed as being highly variable.

Morphometrics

Morphometrics is the measurement of the distance from one anatomical landmark to another and is a widely used technique for quantifying phenotypic variation (Dujardin 2011). It can be used to compare different species or to look at intraspecies variation, it is also the most common technique seen in publications. Morphometrics as a statistical analysis of genetic variability through the variation of morphological features can be considered a measure of differences in a population and speciation (Sorensen and Footit 1992; Dujardin *et al*., 1999; Dujardin *et al*., 2000). The use of morphometrics in medical entomology has been used in studies of important vector species such as members of Triatominae and Phlebotominae (Dujardin 2011). Due to the importance of these insects as vectors of disease, the correct identification of any of these insects is critical in studies

(Dujardin and Slice 2007). Morphometrics is intended to be used as a tool to investigate variation of morphology within a population, however insect studies have used it more to describe species based upon a limited number of individuals (Dujardin and Slice 2007; Lent and Wygodzinsky 1979; Young and Duran 1994). The benefit of using this technique in medical entomology studies is that, unlike the score matrix, it does not require specific skills in entomological identification (Dujardin and Slice 2007). Dujardin *et al*., 1997, successfully used morphometrics as part of an entomological surveillance of sylvatic foci of *Triatoma infestans* in Bolivia. The successful identification of nymphs using seven head measurements enabled the researchers to ascertain wether reinfestation of domestic settlements was occurring by the same species and further used this information to suggest possible mechanisms of reinfestation.

The further use of morphometrics to monitor the adaptaptive process, as indicated by Dujardin *et al*., 1997 was done by Dujardin *et al*., 1998. In this study morphometric comparison between the allopatric sylvatic specimens, including a holotype, showed an overall reduction in size between sylvatic and domestic specimens. Morphometrics is useful at inferring phylogenetic relationships when combined with molecular data, Dujardin *et al*., 1999 used morphometric analysis of Rhododniini to derive phylogenetic patterns. The morphometric data for this organism was found to exhibit non-normality, with a large number of population variances, however despite these issues, when this data was combined with data obtained from a isoenzyme analysis, 3 novel species groups with the genus *Rhodnius* was detected. Morphometrics can also be used to investigate population dynamics, Patterson *et al*., 2001, used morphometrics to analyse features of the head of several species of *Triatoma* in order to reconstruct its evolutionary history. Eight head measurements which were then analysed used a canonical variate statistical analysis were used to infer the observed relationship between old world species and new world species, finding evidence of a possible new world ancestry.

In terms of tick research, morphometrics is widely used much in the same way as it is in the broader arthropod studies, ranging from making species identifications/descriptions (Abdel-Shafy *et al*., 2011), exploring morphological variation within populations to investigating population dynamics. Hutcheson *et*

al., 1995, used morphometrics to discriminate Nymph and adults of *Ixodes scapularis* from their northern and southern morphotypes from 6 geographically separate popluations. Morphology analysed varied between sex and stage; 17 from females, 25 from males and 28 from nymphs. A CVA was then carried out using these measurements, which found that discrimination between the populations based on morphological measurements varied between sex, stage and canonical variate score (CV). An example of this was in Nymphs, where CV1 discriminated the southern morphotype from all the other groups, but CV2 discriminated the remaining groups based on location, correlating to latitude. Ultimately this study supported previous conclusions that *I. scapularis* is a polytypic species with a large geographic distribution in North America. However, when this study was expanded by Hutcheson and Oliver Jr, 1996, the results were not consistent, alluding to the potential for irregularities in this method to quantifying morphology. Dietrich *et al*., 2013 used a combination of body size and shape, along with information from microsatellites to see if different populations of *I. uriae* have evolved significant morphological differences. In this study 255 unfed adults and nymphs were analysed. 31 morphological measurements were taken from both male and females adults and 29 from nymphs. A principal component analysis (PCA) was then conducted to convert the obserbations made into a set of values which could be used to emphasis variation and thus allow patterns in correlation to be seen more clearly. This study found that a large amount of variation seen in the morphology was associated with the size of individual ticks, but overall body size differed between the populations in adult ticks. It was concluded, along with the genetic data that differences seen between the populations reflect host-associated adaptations rather then phenotypic plasticity/drift.

With regard to the reinstatement of *R.(B) australis*, the morphology was re-described based on morphometric measurements (Estrada-Peña *et al*., 2012). *R.(B) microplus* and *R.(B) australis* larvae are stated to be distinguishable based on body length, body width, scutal width, length of certain setae, palpal length and tarsus I length (Estrada-Peña *et al*., 2012). Overall it was concluded that the larvae of *R. (B) microplus* are larger, with a wider anterior scutum than those of *R.(B) australis* (Estrada-Peña *et al*., 2012). With regard to the males and females, the presence or absence of certain features were concluded to be suitable for

differentiation. Although morphometrics is a tried and tested technique and size is a variable that should be taken into account when making identifications, it is subject to other factors apart from genetic influence. These factors can be hard to account for and greatly impact upon results obtained, so other techniques for understanding phenotypic variation must be explored in tick research.

As with all methods, morphometrics can be subject to errors. The main error causing factor in Morphometrics is metric variation (Dujardin and Slice 2007). Metric variation can be caused by a number of different factors including physiological status of the specimen, adaptive changes and lastly genetic differences (Durjardin 2011). The phenotypic expression of the genetic difference is the factor of interest; however, tick morphology can be greatly impacted upon by the physiological and adaptive influences (Dujardin and Slice 2007). One example of this is observed in adult females which may take up to fifteen times their body mass as a blood meal. Additionally, the health of the tick can affect morphometric measurements. In particular ticks that have fed well for a number of generations tend to be larger in size then those that have not fed as well, or have been fed on tick resistant cattle (Wilson *et al*., 1990, Wambura *et al*., 1998). In these situations, a biased conclusion may be reached on species differentiation. In order to remove the environmental impacts on metric variation, it is possible to consider taking a 'wild' population and lab-rear a colony for analysis; however this practice can be influenced by genetic elements such as genetic drift and may create a bias on the morphology (Dujardin and Slice 2007). A proposed method to consider the causes of metric variation separately is to consider size and shape separately, on the premise that shape would be less affected by environmental influences (Dujardin and Slice 2007).

Geometric morphometrics

Geometric morphometrics is the quantitative representation of shape using landmark coordinates instead of measurements (Dujardin, 2011, Dujardin and Slice, 2007). It aims to describe the shape of the feature, independent of size and so is useful for eliminating physiological changes as discussed above. The major goal of this type of approach is to measure the morphological similarity and differences of specimens, which can be done in either two or three dimensions. In this approach, linear measurements are not taken, instead a minimum of three

landmarks based on Cartesian coordinates (x,y,z) are placed on biologically or geometrically homologous points on a morphological feature (Dujardin and Slice, 2007, Dujardin, 2011). These landmarks are used to represent shape of the feature of interest and can be used to create an outline of the feature by using subsequent semi-landmarks to 'join the dots'. The advantage of this approach is that results can be visualized as change in shape, rather than a table of numbers and size is mathematically removed from the analysis, allowing the true shape to be considered (Dujardin and Slice, 2007, Dujardin, 2011). The shape change can then be analyzed by software to observe the variation in more than one plane, ultimately showing the complexity of difference between populations of the same species (Adams *et al*., 2004). In some cases however, the size difference may be biologically relevant and will be lost when using this technique and so must be taken into account.

This approach to morphology has been widely used in fish biology to compare phenotypic variations among different populations (O'Reilly and Horn 2004; Loy *et al*., 2000; Clabaut *et al*., 2007). As well as living organisms, geometric morphometrics has been used in the study of variation seen in skulls, particularly of dog breeds versus wolf breeds (Drake and Klingenberg 2010; Drake 2011). In terms of arthropods, variation in shape has been useful in successfully identifying many species of medical importance (Dujardin *et al*., 2014). However, this approach in arthropods has been found to be mostly useful in the exploration of variation, rather then a specific tool for identification (Dujardin *et al*., 2014). Often fly wings are used for comparison as they are flat structures which have features lends itself to the consistent placing of landmarks (Dujardin *et al*., 2014; Klingenberg, 2011).

This approach is relatively unused in tick research, with most work relying on the more traditional linear measurement approach. Where it has been used, geometric morphometrics has demonstrated potential in showing the similarities and differences between difficult to discriminate species of tick (Clarke and Pretorius, 2005, Pretorius and Clarke, 2000, Pretorius and Clarke, 2001). Pretorius and Clarke, 2000 used this approach to analyse the body shape of male and female *Hyalomma truncatum* and *H. marginatum rufipes*. Due to similarities observed in their morphology, difficulties in distinguishing between the two species

had been reported. By using 16, repeatable landmarks representing body shape, they were able to make observations on the differences in body shape between the sexes of the same species. In another study by Pretorius and Clarke, 2001, the same approach was taken to analyse the body shape of both male and female *Amblyomma gemma, A. variegatum* and *A. hebraeum*. In the case of these three species, differentiation can be difficult due to similarities in morphology, along with an overlap of distribution. In this study 17 landmarks were used to outline body shape, however the results from this study demonstrated the problem that can be observed with a landmarking approach, where errors in landmarking placing accounted for the shape variations observed. Overall it was concluded that the shape of the body could not be used to distinguish between these three species for either male or female ticks. Clarke and Pretorius, 2005 expanded on this study to use a combination of geometric morphometric analyses and cross-breeding studies to investigate the relatedness between the three *Amblyomma* species. Ventral body shape of both sexes for the three species was analysed, as well as that of F1 hybrids from the cross-breeding study. Results showed that *A. hebraeum* was the most different to the other two species, with *A. variegatum* and *A. gemma* appearing very similar in ventral body shape. This finding was not supported by the mating study, as *A. variegatum* appeared to not be genetically compatible to the other two species, where *A. gemma* and *A. hebraeum* were.

Although these studies indicate potential in this approach to understanding morphology, they also indicate potential disadvantages. Firstly, basing an identifcation on one factor alone is probably not going to resolve an identification issue. To overcome any potential bias it would be appropriate to select a number of different features to test. Secondly, the choice of position of landmarks must be repeatable as to avoid bias in the shape produced. The geometric morphometric software MorphoJ has the ability to test the repeatability of landmark placing and so can reduce this error's impact on the overall analysis (Klingenberg, 2011). Finally, the use of this approach may not be suitable in distinguishing between difficult to identify species; however it lends itself more to making an observation phenotypic variation observed in populations (Dujardin *et al*., 2014).

Proposed evolutionary pathway of ticks

Evolution of the Acari

The Acari are a subclass of Arachnida that contains mites and ticks. Three orders make up the Acari: Acariformes, Parasitiformes and Opilioacariformes. The Parasitiformes contains ticks and some species of mites and is split into Opilioacarida, Holothyrida, Ixodida and Mesostigmata (Nava *et al*., 2009). The hard tick family (Ixodidae), soft tick family (Argsidae) and Nuttalliellidae belong to Ixodida. Nuttalliellidae are considered to be the basal lineage of both Ixodidae and Argasidae (figure 1.18) (Nava *et al*., 2009, Barker and Murrell, 2004). The family Ixodidae is made up of the Prostriata and Metastriata which are further classified based on genus; Prostriata contain the genus *Ixodes* and Metastriata is composed of *Amblyomma, Haemaphysalis, Hyalomma, Rhipicephalus* (and *Boophilus*) (Nava *et al*., 2009, Barker and Murrell, 2004). Fossil records show that ticks originated in the pre-mid Cretaceous period with both Ixodidae and Argasidae becoming established in the mid Cretaceous period (Nava *et al*., 2009, Poinar Jr and Brown, 2003).

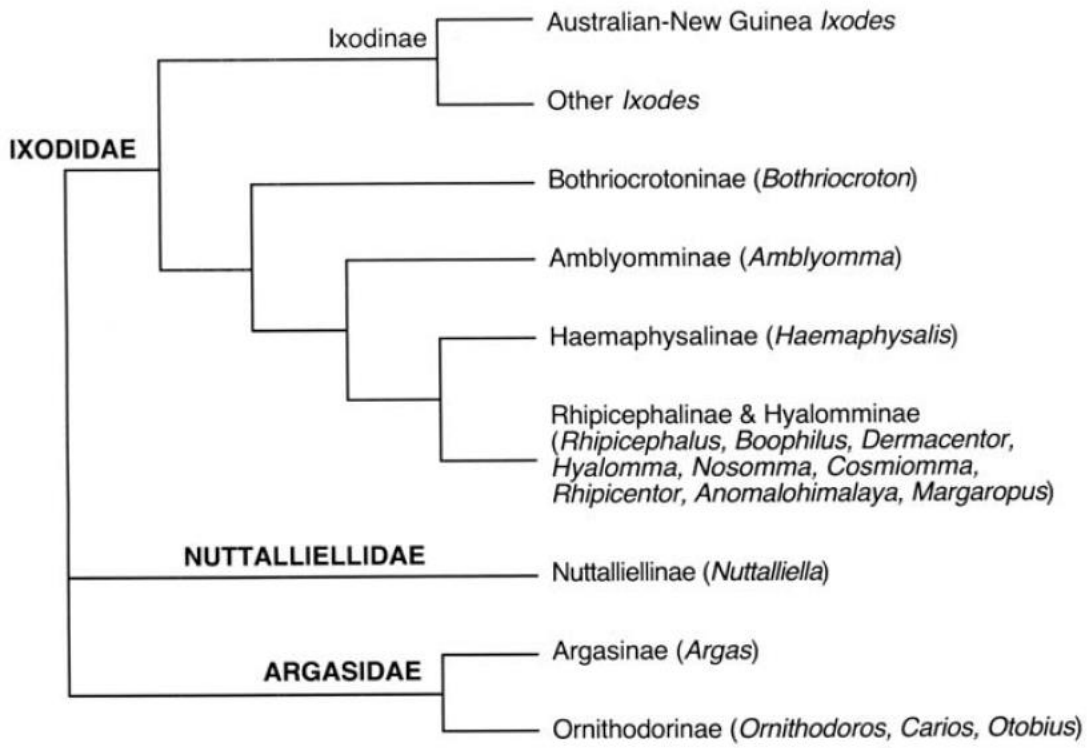


Figure 1.18: Proposed phylogeny of the subfamilies of ticks (From Barker and Murrell, 2004)

The actual evolutionary pathway adopted by these pre-historic ticks is impossible to deduce as there are few fossils available, however the main theory proposed indicated tick-host associations as the main driving force (Nava *et al*., 2009). It was suggested that the early ticks fed on reptiles and subsequently went through the first major part of their evolution during the late Paleozoic and early Mesozoic eras, during which time the climate became warm and humid (Nava *et al*., 2009). It was during this time that the Argasidae and members of the Ixodidae evolved, in particular the first species of the genera *Ixodes* and *Haemaphysalis*. This was closely followed by the development of *Amblyomma* and the later evolution of *Hyalomma* and *Rhipicephalus* found on mammals, in the late Cretaceous and Tertiary eras (Nava *et al*., 2009). This is one theory, however others suggest that instead of reptiles, ticks originally parasitized amphibians and originated from the Paleozoic era, further evolving in the Mesozoic, Triassic and Cretaceous periods (Nava *et al*., 2009, Dobson and Barker, 1999, Klompen, 2000, Oliver, 1989, Black and Piesman, 1994, Klompen *et al*., 1996b).

Evolution of Rhipicephaline ticks

There are currently 82 accepted species in the genus *Rhipicephalus*, 5 of which belonging to the *Boophilus* subgenus (Barker and Murrell, 2004, Guglielmone *et al*., 2010). As the phylogenies of this group have increased in accuracy, it has been possible to track certain traits and observe how and when they evolved. In particular, traits such as ornateness, life cycle and origins of ticks have been examined (Barker and Murrell, 2004, Murrell *et al*., 2001b). The historical zoogeography can also be mapped onto the phylogeny of ticks. Most species of the *Rhipicephalus* clade are thought to have originated in the Afrotropical region, however there are certain members of the clade that have origins in Palearctic and Oriental regions (Barker and Murrell, 2004, Murrell *et al*., 2000, Murrell *et al*., 2001b). This genus is proposed to have initiated in Africa when it was isolated from other regions (Palearctic and Oriental) (Barker and Murrell, 2004).

It is thought that ancestors of this genus developed and spread throughout Africa before the land bridge between Africa and Eurasia was formed, after which the Miocene period saw an influx of *Rhipicephalus* into Eurasia and Asia (Barker and Murrell, 2004). Some studies suggested that *Rhipicephalus (Boophilus)* originated within Europe; however other studies indicated the origins where also in Africa

(Barker and Murrell, 2004, Murrell *et al*., 2000, Murrell *et al*., 2001b). When looking at the genus, there are clear patterns in relationships that can be observed and have been confirmed by use of morphological and molecular approaches. *Rhipicephalus* itself appears to be made up of six main groups (figure 1.19); *R. pravus* group, *R. appendiculatus* group, *R. pulchellus* group, *R. simus* group, *R. capensis-longus* group and finally the *R. sanguineus* group (Barker and Murrell, 2004, Murrell *et al*., 2000, Murrell *et al*., 2001b). Additionally there are two other sub-genera, including *R. digineus* and the more recently accepted *R. Boophilus* (Barker and Murrell, 2004, Murrell *et al*., 2001b). There is also molecular and morphological evidence to suggest that certain members of *Rhipicephalus* are closely related to members of *Boophilus*, for examples *R. digineus*, containing the species *R. evertsi evertsi* appears to be phylogenetically closer to members of *Boophilus* (Barker and Murrell, 2004, Murrell *et al*., 2001b). Although in terms of evolution this relationship is not clear cut, especially when assessing the truncation of life cycles, i.e. 3-host to 2-host to 1-host. It can be observed that *R. evertsi evertsi*, a 2-host tick is more closely related to members of the *R. pravus* group (3-host ticks), than to members of *Boophilus* (1-host ticks) (Barker and Murrell, 2004).

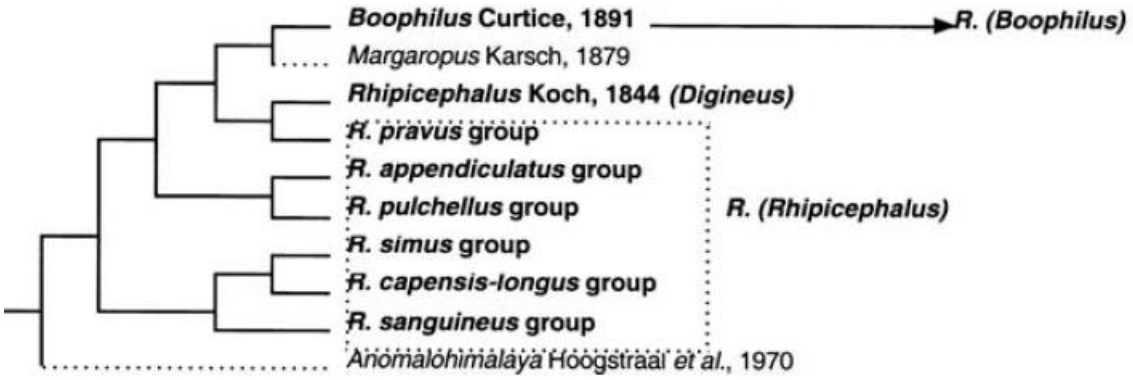


Figure 1.19: Proposed phylogeny of *Rhipicephalus* derived from both molecular and morphological data (from Barker and Murrell, 2004; Murrell *et al*., 2001b)

Historical overview of *R.(B) microplus*

R.(B) microplus was first described in 1888 by Canestrini, however at this time it was described as *Haemaphysalis micropla* from samples originating in Paraguay (Quinlan *et al*., 1980, Cooley, 1946). Since then, there has been debate regarding the taxonomic status of *R. (B) microplus*. In 1899 Fuller collected specimens from

Australia and South America and first described *R.(B) australis*, however *R.(B) australis* was not actively compared and distinguished from *R.(B) microplus*, but was separated based on comparison with African species including *R.(B) decoloratus* (Estrada-Peña *et al*., 2012). It has been suggested that this lack of separation of *R.(B) australis* and *R.(B) microplus* by Fuller and his conclusion that Australian and South American specimens were the same species lead to the merging of the two species into *R.(B) microplus* (Estrada-Peña *et al*., 2012). In 1901 discussion regarding the status of *R.(B) australis* and *R.(B) microplus* ensued with academics split between accepting Fuller's conclusions and considering *R.(B) australis* as a valid species based on observed differences in dentition on the hypostome (Estrada-Peña *et al*., 2012). It was during this time that Neumann brought forth the suggestion that *R.(B) australis* was neither a separate species nor *R.(B) microplus*, but a sub-species of *R.(B) microplus* (Estrada-Peña *et al*., 2012). In 1962, after observations based on specimens obtained from South America, Madagascar and Australia, Uilenberg concluded that there was not enough morphological difference to warrant separate species, and synonymized *R.(B) australis* with *R.(B) microplus* (Estrada-Peña *et al*., 2012). In 1975 *R.(B) australis* and *R.(B) microplus* from Australia and South Africa were compared again by Londt and Arthur and it was concluded that although morphological difference existed they were not prominent enough to support the separate species theory (Estrada-Peña *et al*., 2012). Up until 1978, all of the observations regarding the *R.(B) microplus* clade, used morphological identification only, however a mating study found that when South African female *R.(B) microplus* were crossed with males from Australia, 62% of progeny were viable. When Australian females were crossed with South African males this yield dropped to 1.82% thus supporting the theory of two species. It was concluded that South African and Australian populations of *R.(B) microplus* were in the process of diverging into separate species (Spickett and Malan, 1978).

It is unclear exactly where *R.(B) microplus* originates from, however it is clear that it has become one of the most successful invasive species, being introduced to novel geographic locations on imported cattle and establishing stable populations globally (Madder *et al*., 2011, Chevillon *et al*., 2013). From an evolutionary perspective, *R.(B) microplus* shows evidence of origins in the Afrotropical and Oriental regions, however with a lack of fossil evidence it is hard to determine their

exact evolutionary starting point (Barker and Murrell, 2004, Nava *et al*., 2009). The current distribution of *R.(B) microplus* is substantial including Australia, Africa, Asia, America and South America (Madder *et al*., 2011, Chevillon *et al*., 2013). It has been suggested, which has been further backed by mating studies that *R.(B) microplus* was introduced to America on imported cattle from Africa (Nava *et al*., 2009). However it is not clear whether these ticks originated in Africa or not, other studies have suggested that it originated in Asia and was introduced to Australia, Madagascar, South Africa, South America and USA on imported cattle during the mid to late 19th century (Madder *et al*., 2011, Hoogstraal, 1956, Temeyer *et al*., 2004). Australian records have indicated that *R.(B) microplus* was probably introduced to Australia on Banteng cattle imported from Timor and Bali to the Northern Territory, in the period 1850-1870 (Angus, 1996). Since this time it has become endemic in northern parts of Western Australia, the Northern territory, Queensland and areas of New South Wales (Cutulle *et al*., 2009). Infested cattle were exported from Australia to New Caledonia during the second world war resulting in the establishment of populations there (Labruna *et al*., 2009, Estrada-Peña *et al*., 2012). Other studies suggest that *R.(B) microplus* originated in India (as well as Indonesia), and was introduced into Africa, and possibly South America via Indian cattle (Chevillon *et al*., 2013, Labruna *et al*., 2009). The exact origin and path of migration is not known, however the general consensus is that *R.(B) microplus* was present in India and Indonesia initially, the evolutionary starting point however, is impossible to deduce (Angus, 1996, Chevillon *et al*., 2013, Estrada-Peña *et al*., 2012). The distinction of *R.(B) microplus* and *R.(B) australis* as separate species however would be more consistent with the existence of two geographically distinct origins. What is clear, is the adaptability of *R.(B) microplus* to new locations and new climates, it demonstrates a successful ability to colonize new areas, even when closely related species are present (Estrada-Peña *et al*., 2006c, Estrada-Peña *et al*., 2006a, Estrada-Pena *et al*., 2006b). An example of this is in Western Africa, where introduction of *R.(B) microplus* on imported cattle have not only successfully established populations but has also caused a decline in native species such as *R.(B) decoloratus* in a relatively short period, in areas previously free of it (Tonnesen *et al*., 2004).

The Rhipicephaline genomes

Genome size of the Ixodidae has been found to vary among the genera (Mirsky and Ris, 1951, Gregory, 2005). In general, tick genomes are large, with the smallest estimated at being in excess of 1 Gbp (Palmer *et al*., 1994, Ullmann *et al*., 2005, Geraci *et al*., 2007, Roe *et al*., 2014) up to the largest documented of *R.(B) microplus* with an estimated genome size of 7.1 Gigabase pairs (Gbp) (Bellgard *et al*., 2012). These large genomic sizes have resulted in problems in sequencing the entire genome which limits the amount of information that can be deduced. Additional to the overall size of the genome, reported variable rates of sequence evolution have negatively impacted the development of molecular tools for phylogenetic analysis (Klompen *et al*., 2007, Roe *et al*., 2014, Meyer and Hill, 2014). Studies into mapping out the Rhipicephaline genome have also encountered problems with repetitive DNA, this has caused a particular problem in the progress of sequencing *R.(B) microplus*, which is still not fully annotated (Bellgard *et al*., 2012). The *R.(B) microplus* genome consists 0.82% foldback, 31% highly repetitive, 38% moderately repetitve and only 30% of unique DNA (Ullmann *et al*., 2005). This means that the genome is made up of around 70% repetitve DNA. The arrangmenet of this repetitive DNA is similar across Ixodidae species, occuring as a mixture of both long and short period conbinations, however the majority of the DNA follows a patterns of short periods of repetitive DNA (Ullmann *et al.,* 2005). Despite the lack of information available on Rhipiecphaline genomes,and infact any of the Ixodidae genomes, what is clear is their size and arrangement are different from other arthropods, particularly in the case of the amount of moderately repetitive DNA and a lower amount of highly repetitive DNA (Ullmann *et al.,* 2005). Moderately repetitive DNA consists of families of sequences that can occur as tandem or dispersed repeats from 1000 to over 100,000 copies, they can also include transposable elements and members of multigene families (Ullmann *et al.,* 2005). The diffculty found in fully sequencing the *R.(B) microplus* genome could be attributed to both this higher proportion of moderately repetitve DNA and the overall size of the genome.

In terms of genetic variation, DNA can be separated into two categories; the nuclear DNA (chromosomes) and mitochondrial DNA.

Nuclear genome

The nuclear genome is complex, consisting of coding as well as a large amount of non-coding DNA. Nuclear genes are organized on chromosomes in linear arrangements, these chromosomes act as a scaffold for replication and regulation of gene expression. In the Ixodidae the chromosome number can range from 12-35. Chromosomal determination of sex has been observed to differ among ticks (Oliver, 1989, Oliver Jr, 1977, Oliver Jr, 1981, Oliver, 1982a, Oliver, 1982b, Oliver, 1983). Many ticks have a typical XX, XY system, however there is a similar number of tick species that have an XX, XO system, in which phenotypic sex is determined by the level of expression of genes located on the X chromosome (Marın and Baker, 1998).

In the case of *R.(B) microplus* the XX, XO arrangement is seen, with an autosomal (non-sex) chromosome number of 20; diploid number of 22 for females and 21 for males (Gunn *et al*., 1993, Newton *et al*., 1972, Oliver and Bremner, 1968). Autosomes are easily distinguishable from the X chromosomes, which are distinctly larger (Gunn *et al*., 1993). Based on this, it has been possible to distinguish between different members of the sub-genus *Boophilus* using differential staining. However for observing the amount of variation within populations of the same species, this renchique would not be useful.

Nuclear ribosomal genes are mainly used for uncovering hidden relationships within a species (Cruickshank, 2002). The main locus is composed of three genes encoding the 18S rDNA, 5.8S rDNA and 28S rDNA subunits and unlike the mitochondrial genes, are separated by two non-coding internal transcribed spacers; 18S - *ITS1* - 5.8S - *ITS2* - 28S (Navajas and Fenton, 2000, Hillis and Dixon, 1991). There are often hundreds of copies of the ribosomal genes on a single chromosome and may additionally be found on more than one chromosome, resulting in the easy detection of these genes over the single copy genes (Navajas and Fenton, 2000).

Mitochondrial genome

The mitochondrial DNA, located in the mitochondria, makes up a small proportion of an organism's total DNA. In the Ixodidae the mitochondrial genome is approximately 14-16 kb in length, circular in formation and is composed of 37

genes including, 13 protein-coding genes, 2 ribosomal RNA genes, 22 transfer RNA genes and a non-coding region (Shao and Barker, 2007).

The mitochondrial genome, in general, has been found to be variable with regard to the content and gene arrangement (Meyer and Hill, 2014, Shao *et al*., 2005, Jeyaprakash and Hoy, 2007). Mitochondrial genes are widely used in molecular studies and demonstrate a number of distinct advantages over nuclear genes. Firstly the mitochondrial genes have a high copy number, which makes them easier to work with as there is more DNA to amplify (Cruickshank, 2002). Secondly, mitochondrial genes are inherited only through the maternal parent, and along with the lack of recombination observed, can be useful for infraspecific discrimination (Cruickshank, 2002, Latrofa *et al*., 2013, Erster *et al*., 2013, Navajas and Fenton, 2000).

Sequencing status and state of knowledge including databases

Genomic databases allow researchers from around the world to combine efforts in mapping out genomes of key tick species and have proven invaluable in the process of mapping genomes of the ixodidae. There are a number of different databases currently available that allow the user to search well defined genomes for an individual species (Lawson *et al*., 2009). The majority of these projects have focused on tick expressed sequence tags (ESTs) (small fragments of mRNA resulting by sequencing performed on randomly selected clones from cDNA libraries), particularly from genes transcribed in the salivary glands of species such as *Ixodes scapularis* (Ribeiro *et al*., 2006), *Rhipicephalus appendiculatus* (Nene *et al*., 2004) and *Amblyomma variegatum* (Nene *et al*., 2002, Wang *et al*., 2007). However, additional ESTs have been isolated from ovaries, salivary glands and hemocytes in *R.(B) microplus*. There are two databases that have focused on the acquisition of data from *R.(B) microplus*; BmiGI and CattleTickBase. It should be noted that *R.(B) australis* had not yet been reinstated around the time of the database's launch and CattleTickBase was conducted using the Australian *R.(B) microplus* (currently reinstated as *R.(B) australis)*.

BmiGI is a database containing information on ESTs derived from different tissues of different stages and isolates of *R.(B) microplus*. In 2005 the first version of the BmiGI database was launched containing a library of complementary DNA (cDNA)

synthesized from RNA taken from larvae that had undergone different acaricide treatment, as well heat/cold shock, exposure to host odor and infection with *Babesia bovis* (Guerrero *et al*., 2005). In the case of the acaricide treatment, tick isolates were chosen with variable susceptibility to three main compounds; amitraz, pyrethroid and organophosphate. RNA from eggs, nymphs, adults and dissected organs were also included, as well as plasmid DNA taken from 11,520 cDNA clones (Guerrero *et al*., 2005). The first version of the database yielded 8,270 sequences with a varying degree of associated putative functional assignments, based upon available information (Guerrero *et al*., 2005) 53 of these sequences were identified as having a potential role in acaricide resistance (Wang *et al*., 2007). In the second version of the database, updated 2007 resulted in 5,373 new additions to the library (Wang *et al*., 2007). BmiGI contains a significant amount of information on the *R.(B) microplus* genome, covering a large amount of the coding regions.

CattleTickBase was launched in 2012 as a platform for international collaborators to work together to sequence the *R.(B) microplus* genome. There are nine available datasets on the CattleTickBase website, made up of acquired and assembled genomic DNA (gDNA) representing approximately 1.8 Gigabase pairs of DNA, which along with the transcriptomic sequence data, represents around 0.9x coverage of the gene-coding regions (Bellgard *et al*., 2012). Ticks used for this database were collected from two main areas; USA and Australia. DNA was extracted from eggs of the USA isolate ‘Deutch’ which originates from a lab colony formed from a few engorged females taken from a Southern Texas outbreak in 2001 (Bellgard *et al*., 2012). It has been shown that although inbred, members of this colony are not genetically homogeneous (Bellgard *et al*., 2012). The Australian ticks used (larvae and engorged females) were from the ‘N’ strain kept by the Department of Employment, Economic Development and Innovation in Queensland, Australia (Bellgard *et al*., 2012, Stewart *et al*., 1982).

Genetic markers and tools

The understanding of population dynamics and taxonomic relationships within the Acari has come a long way since the discovery of tick species by the likes of Latreille, Koch and Canestrini. It has become clear that the differentiation of ticks based on morphology alone does not fully encompass the level of diversity now observed within and between Ixodidae species. There are now a large number of molecular markers available that can resolve population dynamics at different levels, i.e. relationships within genera down to variation between members of the same species (Barker and Murrell, 2004). The difference in the evolution rates of markers is what allows for such a diverse toolbox, and there are a number of genes used in molecular evolution studies located within mitochondrial and nuclear ribosomal DNA (Navajas and Fenton, 2000).

Mitochondrial genes as markers

There are three main mitochondrial genes used in phylogenetic studies, these ribosomal genes are not separated by internal transcribed spacers, and include 16S rDNA and 12S rDNA (Cruickshank, 2002). The third gene comprises mitochondrial cytochrome oxidase subunits.

16S has been found to be useful in studies that are attempting to resolve phylogeny at or below the family level, however it is not a good marker for resolving phylogenetic relationships between distantly related taxa (Cruickshank, 2002, Mangold *et al*., 1998, Norris *et al*., 1999). With regard to populations of *R. (B) microplus*, 16 different isolates from America, Africa, Asia and Oceania were analyzed using the 16S rDNA gene (Labruna *et al*., 2009). This study found that isolates from America and Africa were not genetically divergent, however these were more divergent from samples obtained from Oceania and Asia (Labruna *et al*., 2009). Based upon this finding, it was proposed that three separate clades were formed; America, Africa and Taiwan formed the first clade, the second included Australia, New Caledonia and Indonesia and the final clade clustered an Indian strain with members of *R.(B) annulatus* (Labruna *et al*., 2009). This study concluded that *R.(B) microplus* from Australia is different to those from Asia, Africa and America. However, when the 16S sequences were analyzed, independent of any other gene, the phylogeny was not resolved. The conclusion that the

Australian population was a separate species was further supported when published 16S sequences were analyzed. This led to the call for the reinstatement of *R.(B) australis* as a separate species (Estrada-Peña *et al*., 2012). 16S was later found to successfully resolve phylogenetic relationships within *R.(B)* microplus, with an 84% bootstrap support for monophyly of the *R.(B) microplus* complex with evidence for two potential clades separating ticks from Asia, Africa, South America and Australia from India and China (Burger *et al*., 2014). Support for the further monophyly of the Australian and Indian ticks within each clade with 63% and 84% bootstrap support, respectively was also observed (Burger *et al*., 2014). It was concluded however that 16S alone cannot resolve *R.(B) microplus*.

As with 16S, 12S has been suggested to be useful for resolution of relationships within genera (Norris *et al*., 1999). 12S has shown evidence that *Rhipicephalus* and *Boophilus* are not monophyletic, however it was concluded that this gene alone could not resolve the relationship further (Murrell *et al*., 2000). Subsequent studies used 12S to show that *Boophilus* was monophyletic and arose from within the *Rhipicephalus* genus, grouping closely with species from the *Rhipicephalus evertsi* group (Beati and Keirans, 2001). The use of 12S, along with other genes resulted in the conclusion that *Boophilus* should be synonymized with *Rhipicephalus* (Murrell and Barker, 2003b, Murrell *et al*., 2001b). 12S has been found to have evolved slightly faster than 16S; however, despite this, studies that have used 12S in conjunction with 16S have demonstrated little resolving power difference between the two, and in the case of understanding the relationships within the *R.(B) microplus* clade, both genes revealed that they do not have the power alone or combined to resolve *R.(B) microplus* (Estrada-Peña *et al*., 2012, Labruna *et al*., 2009, Burger *et al*., 2014).

Mitochondrial cytochrome oxidase subunit I gene (*COX1*) is another gene that has proved useful in phylogenetic analysis. In many studies, *COX1* has been used along with other mitochondrial genes and has been able to resolve *Rhipicephalus* ticks at genus and species level (Latrofa *et al*., 2013, Murrell and Barker, 2003b, Murrell *et al*., 2000, Murrell *et al*., 2001b). With regard to *R.(B) microplus, COX1* has shown its ability to clarify complex region groupings that are not as clear when using other mitochondrial genes, as well as being proposed as the main gene for differentiating between *R.(B) microplus* and *R.(B) australis* (Burger *et al*., 2014).

Nuclear ribosomal genes as markers

Studies have found that 18S is more conserved then 16S and so is a suitable tool for resolving phylogenies at the family and subfamily levels, with other studies showing it to be more suitable than 12S and 16S at this level of analysis (Cruickshank, 2002, Klompen, 2000, Norris *et al*., 1999). 28S has been suggested as a gene for resolving phylogenies at a deeper level (Black and Piesman, 1994, Black *et al*., 1997, Crampton *et al*., 1996, Mangold *et al*., 1998).

Although the ribosomal genes have proven effective when differentiating unrelated species (Navajas and Fenton, 2000, Kaliszewski *et al*., 2009), the more rapid evolution of the ITS regions have been proposed to be useful when identifying between closely related taxa (Navajas and Fenton, 2000, Hillis and Dixon, 1991). Despite this, more recent studies have brought to light the unsuitability of using the ITS approach to species identification. It was found that using an *ITS2* sequence for identification between members of the *Rhipicephalus* spp ultimately yielded conflicting results when compared to that of mitochondrial gene-based results (Latrofa *et al*., 2013). This result was further supported by attempts to resolve the *R.(B) microplus* clade, where *ITS2* was found to show little phylogenetic structure within the clade, which was revealed when using mitochondrial markers (Burger *et al*., 2014). Regardless, the use of the *ITS2* gene is still considered effective when distinguishing between more distinct species (Latrofa *et al*., 2013, Barker, 1998, Burger *et al*., 2014).

Many phylogenetic studies have generated data based on mitochondrial 16S rDNA, 18S and 28S ribosomal genes (partial and whole), as well as *COX1* (Navajas and Fenton, 2000, Cruickshank, 2002). However with regard to phylogenetic analysis of closely related species, studies have shown that the use of *ITS2* and *COX1* act as a powerful combination, similarly when investigating species that are not closely related 18S rDNA and 28S rDNA can be used (Cruickshank, 2002). These findings are generalized across the Acari, with regard to the status of *R.(B) microplus*, it is clear that the strongest tool for resolution is the mitochondrial *COX1* gene, which has been suggested to be able to distinguish between *R.(B) microplus* and *R.(B) australis* (Barker and Murrell, 2004, Burger *et al*., 2014). It is also clear that the nuclear genes that are currently available are

not suitable to resolve this issue and so development of novel nuclear markers is required (Burger *et al*., 2014).

Tick Control

Ticks and tick-borne diseases cause billions of dollars of economic loss globally. The impact of members of the *Rhipicephalus* genus on the cattle industry makes up a large portion of that loss, through damage to hides from tick feeding activity, disease transmission and the consequences of blood-feeding on cattle productivity (Estrada-Peña and Salman, 2013, Jonsson, 2006). Tick control is important to limit the impact of existing species through eradication programs but is also important with regard to controlling the spread of different species into a novel area and subsequently establishing. The introduction of *R.(B) microplus* into Australia from Java in the 1870's demonstrated the essential need for tick control programs as tick fever infected the introduced, British breed, cattle populations (Pegram *et al*., 2000). Initial attempts at control were the creation of tick quarantine lines which demonstrated little effect on the spread of tick borne disease (Angus, 1996). The next step was to introduce cattle dips, in which, 300 proposed acaricides were trialed including tobacco, soap, soda, Sulphur and kerosene, however these first dips proved more effective at killing off the cattle being treated than the *R.(B) microplus* infesting them (Angus, 1996). Nearly 30 years after the introduction of *R.(B) microplus* the first arsenic-based cattle dips were introduced, and proved to be an effective method of control (Angus, 1996). These arsenic-based dips were subsequently adopted as a control method in South Africa and Cuba, however *R.(B) microplus* resistance to arsenic was recognized in Australia in the mid 1900's (Angus, 1996, Hitchcock and Roulston, 1955). Subsequent acaricides were used; DDT, BHC, Diazinon, Dioxathion, Coumaphos and Chlorpyrifos, however towards the end of the 1900's, *R.(B) microplus* became resistant to all (Angus, 1996, Utech *et al*., 1978a, Wharton and Roulston, 1970). As well as Australia, acaricide resistance of *R.(B) microplus* to different acaricide groups has been reported all over the world (table 1.6).

Table 1.6: Overview of year of introduction and reported resistance for the main acaricide groups used globally to control *R.(B) microplus*, reproduced from (Roe *et al*., 2014)

Acaricide	Year Introduced	Country	Reported acaricide resistance
Arsenic	1893	Australia	1936
		Argentina	1936
		Brazil	1948
		Colombia	1948
		Uruguay	1953
		Venezuela	1966
DDT	1946	Australia	1953
		Argentina	1953
		Brazil	1953
		Venezuela	1966
Organophosphates/ carbamates	1944	Australia	1963
		Argentina	1964
		Brazil	1963
		Colombia	1967
		Venezuela	1967
		Uruguay	1983
		Mexico	1986
Formamidine	1975	Australia	1978
		Brazil	1989
		Venezuela	1995
		Colombia	1997
		Argentina	2000
		Mexico	2002
Pyrethroids	1975	Australia	1981
		Mexico	1993
		Brazil	1995
		Colombia	2000
Macrocyclic lactones	1981	Brazil	2001
		Mexico	2010

Despite the development of resistance, the following acaricides are still applied to cattle to kill *R.(B) microplus*; organophosphates (targets acetylcholinesterase) (Fournier and Mutero, 1994), synthetic pyrethroids (targets voltage-gated sodium channels) (Soderlund *et al*., 1995), amidines (targets octopamine receptors) (Corley *et al*., 2013), macrocyclic lactones (targets glutamate-gated chloride channels) (Kane *et al*., 2000), spinosyns (targets nicotinic acetylcholine receptors) (Snyder *et al*., 2007), benzoylphenylureas (targets chitin synthesis) (Oliveira *et al*., 2007) and phenylpyrazoles (gamma-aminobutyric acid (GABA)-gated chloride channel) (Sammelson *et al*., 2004). There have been efforts to develop alternatives to synthetic acaricides, however currently, most successful controls are in the development of vaccines for cattle against the ticks (Angus, 1996, Johnston *et al*., 1986, Trager, 1939, Galun, 1978, Allen and Humphreys, 1979).

Genes relevant to control

A large amount of work has been undertaken into functional genes for tick control. The most successful of which was the production of the vaccines Tick-Guard^{PLUS} and Gavac™, both based on the tick mid-gut protein *Bm86*. A number of other genes have been considered for the development of novel vaccines against ticks but will not be considered here. It should be noted that these genes are not suitable as taxonomic markers, this is because these genes are subject to selection pressure based on treatment with the vaccine in the case of *Bm86* and the class of acaricdes being used in the case of *βAOR*. In terms of the ideal taxonomic marker, the substitution rate should be optimum so that it provides enough sites to be informative, the problem with genes that evolve too fast is that they may undergoe multiple substitutions and thus become saturated (Cruickshank 2002). The mutation rate of genes associated with acaricide resistance ahs been previously documented to be very fast (Roe *et al*., 2014) which does not make them suitable for phylogenetic studies.

Bm86

Bm86 is an epidermal growth factor (EGF)-like membrane glycoprotein that was the basis of the first effective and commercialised vaccine against an arthropod parasite, *R.(B) microplus*.

Bm86 is described as a concealed antigen because it is located inside the tick and is not secreted in the saliva of the tick in the course of feeding. Hence, most hosts are not exposed to the antigen (Ag) and do not develop an anamnestic response to it. Consequently, frequently repeated vaccinations are required to maintain titres and efficacy. It was developed from the Yeerongpilly strain of *R.(B) microplus* based in Australia. *Bm86* acts as a protective antigen that was extracted from the plasma membrane of gut epithelial cells from partially fed female *R.(B) microplus* (Gough and Kemp, 1993, Rodríguez *et al*., 1994). Antibodies to *Bm86* in the blood meal from vaccinated cattle bind with complement in the blood to cause lysis of the tick gut epithelial cells, with subsequent leakage of gut content into the haemocoel, resulting in some mortalities, reduced digestive efficiency and impaired reproductive performance in the ticks (Rodríguez *et al*., 1994, Willadsen *et al*., 1989, Johnston *et al*., 1986, Opdebeeck *et al*., 1988, Kemp *et al*., 1989, Willadsen *et al*., 1988).

The efficacy of the vaccine was measured in terms of effect on number of engorged females, number of eggs produced by females and the number of successfully hatched larvae. Early results showed a 20-30% reduction in tick engorgement, 30% decrease in engorged tick weight and 60-80% decrease in subsequent egg weight. This equated to an overall reduction of around 90% in tick reproductive performance (Willadsen *et al*., 1995). The Australian version of the vaccine TickGARD^{PLUS} was further tested on lactating Holstein-Friesian dairy cows (Jonsson *et al*., 2000). This study found that when comparing vaccinated to non-vaccinated cattle, there was a 73% reduction in the reproductive index (reduction in ticks engorging × reduction in ticks producing eggs × reduction in eggs hatching) of ticks on vaccinated cattle compared with controls (Jonsson *et al*., 2000). Ultimately this resulted in a 56% reduction in the density of ticks in the pastures in which vaccinated cattle were held. The South American version of the vaccine Gavac^{TM} was found to have variable efficacy when tested on different breeds of cattle, where Jersey cattle were found to have a smaller reduction of 42% in tick burden when compared to the 64% reduction of tick burden in mixed breed cattle (Rodríguez *et al*., 1995a). In another study using Gavac^{TM} a 75% reduction in tick burden was observed (Rodriguez *et al*., 1995b).

Regional variation in the responsiveness of *R.(B) microplus* to the *Bm86* vaccine has been documented in a number of studies (García-García *et al*., 1999, Willadsen *et al*., 1995, García-García *et al*., 2000). *Bm86* was sequenced and the divergence of the sequences from the Yeerongpilly strain was compared to the level of protection obtained from a number of strains of *R.(B) microplus* (García-García *et al*., 1999). It was found that vaccination of cattle against the Argentinean strain A had a protective efficacy of only 10-15% (García-García *et al*., 1999). From this finding the Argentinean strain A gene, homologous for Bm86, was cloned and expressed in *Pichia pastoris* and named *Bm95*. Testing of this vaccine on the herds that had shown previous resistance to *Bm86* yielded a great improvement in efficacy of the vaccine (García-García *et al*., 2000).

Beta-adrenergic octopamine receptor (*βAOR*)

G protein-coupled receptors (GPCRs) are receptor proteins belonging to the seven trans-membrane group, located in the membrane of cells. Their general role is to convert physical and chemical extracellular signals into physiological responses (Broeck, 2001). In arthropods, the GPCR family is quite extensive with around 270 having been described in both *Drosophila melanogaster* and *Anopheles gambiae* (Brody and Cravchik, 2000, Hill *et al*., 2002). With regard to ticks and in particular *R.(B) microplus*, what is known about GPCRs is limited in comparison, however research that has been conducted thus far has demonstrated the potential for GPCRs as novel acaricide candidates; octopamine-like receptors (Baxter and Barker, 1999b), myokinin receptors (Holmes *et al*., 2003) and serotonin receptors (Chen *et al*., 2004, Corley *et al*., 2012, Corley *et al*., 2013).

Additional research has demonstrated the potential of using genomic databases such as the *Ixodes* genome project (IGP) (Van Zee *et al*., 2007) for further investigation into possible GPCR sequences for a number of tick species (Guerrero and Dowd, 2010). With regard to this bioinformatic study, an additional 30 possible GPCR candidates were discovered with another 232 indicating GPCR activity, thus demonstrating the potential for further investigation into GPCR receptors present in the Ixodidae (Guerrero and Dowd, 2010). More recently, Corley *et al*. (2012), differentiated among some of the putative functions of GPCR from *R.(B) microplus* using degenerate primers created from aligned amino acid sequences from similar receptors found in other arthropods. When

phylogenetically analyzed, the results showed that different types of receptors had been generated; $5HT_7$, Muscarinic, $GABA_B$, Dopamine D_2 like, Dopamine D_1 like, Octopamine β-Adrenergic like, Dopamine lNDR and Octopamine α-Adrenergic like (Corley *et al*., 2012).

Beta-adrenergic octopamine receptor (*βAOR*) is a GPCR located on the neuronal cell surface. Its function is to activate signal transduction pathways within the neuronal cells in the presence of neurotransmitters. When the ligand binds to *βAOR* it causes a conformational change in the shape, this change then triggers the activation of associated G-proteins, and signal transduction. Amitraz resistance has been suggested to be associated to a mutation in the *βAOR* gene (Corley *et al*., 2013). Expression of *βAOR* has been found in six tick cell lines, originating from four different strains of *R.(B) microplus*, each with a different acaricide resistance (Koh-Tan *et al*., 2016). This study also found the potential for three different variants in the *βAOR* gene, one of which was not successfully sequenced (Koh-Tan *et al*., 2016).

Summary of current taxonomic views on the *Rhipicephalus (Boophilus)* clade using molecular and morphological evidence

Up until 13 years ago *Boophilus* was considered a genus separate to *Rhipicephalus*; however with further morphological and molecular studies, evidence has been suggested to support the theory that *Boophilus* is synonymous with *Rhipicephalus* (Murrell and Barker, 2003b). Using a study of 12S and cytochrome oxidase I (*COX1*) mitochondrial DNA sequences, it was found that the three species *B. microplus*, *B. decoloratus* and *B. annulatus* formed a clade with the Rhipicephaline ticks *R. evertsi* and members of the species group *R. pravus*, with 93% bootstrap support (Murrell *et al*., 2000). However previous studies had only shown weak evidence of paraphyly of *Rhipicephalus* with *Boophilus* when using 12S rDNA (Murrell *et al*., 1999, Beati and Keirans, 2001), 16S rDNA (Mangold *et al*., 1998) and internal transcribed spacer 2 (*ITS2*) (Murrell *et al*., 2001a). Additional research that used more than one molecular tool; 12S rDNA, *COX1*, *ITS2* and 18S (combined with morphology) found a 99% bootstrap support that all five members of the *Boophilus* genus formed a monophyletic group within *Rhipicephalus* (Murrell *et al*., 2001b). With regard to morphological similarities studies that have used around 30 morphological features and a simple scoring

technique e.g. ornamentation of scutum present: 1 absent: 0 concluded that many of the species-defining features found in *Boophilus* are also shared by *Rhipicephalus* (Murrell and Barker, 2003b). Conversely, other research has indicated that the combination of phenotypic and genotypic data supports the genus status of *Boophilus* and that while the molecular findings irrefutably validate the close association of the two genera, the clear morphological differences, as well as differences in life cycle should not be discounted (Uilenberg *et al*., 2004).

Morphological and molecular differences among different *R.(B) microplus* populations have been suggested by a number of different studies (Labruna *et al*., 2009, García-García *et al*., 1999, de la Fuente *et al*., 2000a) that has led to the proposal of separate species. Molecular and fertility studies have been carried out in order to determine whether there are in fact separate species within *R.(B) microplus*, a number of which have eluded to the possible separation of the Australian strain from the rest of the world (Labruna *et al*., 2009, Spickett and Malan, 1978, Guglielmone *et al*., 2010). Molecular analysis using the ribosomal *ITS2* demonstrated similarities between the Australian strains and the South African, Brazilian and Kenyan (Barker, 1998); however in other studies using the mitochondrial 12S rDNA, divergence has been observed to separate the Australian populations from the South African and Mexican (Campbell and Barker, 1999). One study in particular has been the driving force behind the suggestion of divergence between Australian isolates and the rest of the geographic strains (Labruna *et al*., 2009); this work was split into two main sections; a mating study and the genetic analysis. The mating study was set up using three geographically separate strains of *R.(B) microplus*; Australian, Argentinean and Mozambican. The results from this study indicated that when the Australian strain was crossed with the other two strains the offspring that were produced were infertile, but when the ticks from Mozambique and Argentina were crossed their offspring were found to be fertile. Therefore concluding that the Australian strain is in fact a separate species (Labruna *et al*., 2009). The genetic analysis part of this investigation, which used 16S rDNA, 12S rDNA and microsatellites yielded no conclusive answers with regard to Australian strains being a separate species, never the less it was concluded that when combined with the mating study it did provide evidence for a separate Australian species (Labruna *et al*., 2009).

The reinstatement of *R.(B) australis* was subsequently proposed (Estrada-Peña *et al*., 2012) with a more recent study into the morphology paired with 16S rDNA and 12S rDNA molecular analysis. In this study larvae and adults obtained from Australia were re-described and differentiated from *R.(B) microplus* using measurements from unfed specimens and previously sourced neotype material. This study found that although *R.(B) australis* and *R.(B) microplus* are clearly related, there are distinct morphological differences. For the males the presence of a spur on the ventral surface of article i of the palp is a feature that is found on *R.(B) australis* males only. Divergent patterns of distribution of setae between the two species was also reported. The study dismissed the importance of the size and shape of the adanal shields and coxal spurs due to their high variability and potential for incorrect identification (Estrada-Peña *et al*., 2012). In addition to the morphology, the evolutionary history of *R.(B) australis*, *R.(B) microplus*, *R.(B) annulatus* and *R.(B) decoloratus* was inferred using DNA extractions from 10 male and 10 female Australian specimens and sequences already available on GenBank. It was deduced from these findings that the 16S rDNA and 12S rDNA phylogenetic analysis supported the re-description of *R.(B) australis* and when paired with previous mating studies demonstrated irrefutable proof for the divergence of the Australian strain (Estrada-Peña *et al*., 2012).

Project aims

There is conflicting evidence regarding the taxonomic status of *R.(B) microplus*, however the most recent published research has been in support of the reinstatement of *R.(B) australis* as a species distinct from *R.(B) microplus*. This thesis aims to apply genetic and morphological studies of tick populations from numerous countries, to provide a comprehensive phylogenetic analysis of the former *Boophilus microplus* and its nearest relations. As a result it aims to propose a credible evolutionary framework and determine the extent to which genetic divergence within the former species, among geographically distinct populations, is likely to lead to functional consequences for vaccine and acaricide development.

The quantification and analysis of the variation within *Boophilus* clade can be separated into two main studies: Morphology: this study consists of three main approaches to analysing tick morphology (scoring based on published criteria

(Estrada-Peña *et al*., 2012), morphometric and geometric morphometric). Molecular genetics: This study consists of investigating the amount of variation in three genes: *COX1*, a mitochondrial gene, and *Bm86* and *βAOR*, both nuclear genes. *COX1* has been used to successfully distinguish between members of the *Boophilus* clade previously (Barker and Walker, 2014, Burger *et al*., 2014). *Bm86* is a gene of importance, as it is the target gene of current tick vaccines (Kemp *et al*., 1989, Willadsen *et al*., 1988, Willadsen *et al*., 1989). Furthermore regional variation of the *Bm86* sequence has been documented and linked with the variability of the vaccines efficacy (Penichet *et al*., 1994). *βAOR* has been suggested to be involved in amitraz resistance and so is a functional gene of interest in terms of understanding acaricide resistance (Koh-Tan *et al*., 2016, Corley *et al*., 2013).

CHAPTER 2

General Materials and Methods

Tick specimens

Tick specimens from all locations were assembled; approximately 1,650 individual ticks for the morphology study (50 ticks per stage, per sex for each isolate/country) and enough individual ticks for 20 DNA extractions per isolate per country (220 ticks) (table 2.1). Stages that were used in this study were adults (males and females) and larvae were obtained by single time point collections on either naturally infested cattle or cattle that were artificially infested in the course of maintaining colonies of reference strains. Nymphs were not included because the collection of good quality nymph specimens is usually achieved by artificial infestation and collection of fully engorged larvae at the point of moulting, followed by incubation *in vitro* for one or two days. Given the dependence on wild-sourced ticks, this was not possible to achieve for the number of samples required for this study. In the cases of the 'wild' sourced ticks, adult females were in varying stages of engorgement.

Table 2.1: Summary of information regarding the origin of ticks samples, including information on how and when they were collected.

Country of origin	Region of origin	Isolate	Species	Date collected	Collected by	Method of collection	Transportation method
Australia	Townsville, Queensland	Field sample	*R.(B) australis*	December, 2014	Constantin Constantinoiu and Robert Hedlefs	Direct from naturally infested cattle	70% ethanol sealed tubes
North America	Mexico	Yucatan	*R.(B) microplus*		Dr Robert J Miller	Lab colony (originally from Mexico from red deer)	70% ethanol sealed tubes
North America	Texas	Deutch	*R.(B) microplus* *R.(B) annulatus*		Dr Robert J Miller	Lab colony (originally from cattle) (*R.(B) annulatus* from outbreak in Texas on cattle)	70% ethanol sealed tubes
South Africa	Soto village camps, Soto village, Eastern cape province	Field sample	*R.(B) decoloratus*	November/ December 2013	Nkululeko Nyangiwe	Direct from naturally infested cattle/ field drag	70% ethanol sealed tubes
	Bathhurst research station, Eastern province	Field sample	*R.(B) microplus*	November/ December 2013	Nkululeko Nyangiwe	Direct from naturally infested cattle/ field drag	70% ethanol sealed tubes

Brazil	Eldorado do Sul, Rio Grande do Sul (Colony established in 2010)	Jaguar	*R.(B) microplus*	September 2014	Guilherme Klafke	Collected after natural detachment from bovine hosts	70% ethanol sealed tubes
Brazil	Jacareí, São Paulo, Brazil (colony established in 2012)	Juarez	*R.(B) microplus*	September 2014	Guilherme Klafke	Collected after its natural detachment from bovine hosts	70% ethanol sealed tubes
Uruguay	DIlave Miguel Rubino Laboratories	Mozo	*R.(B) microplus*	September 2014	Guilherme Klafke	Collected after its natural detachment from bovine hosts	70% ethanol sealed tubes
Brazil	São Gabriel, Rio Grande do Sul (field derived strain Research Station)	Sao Gabriel	*R.(B) microplus*	September 2014	Guilherme Klafke	Collected after its natural detachment from bovine hosts	70% ethanol sealed tubes
Thailand	Kasetsart University	Field sample	*R.(B) microplus*	August 2014	Sathaporn Jittapalapong	Direct from artificially infested project cattle	70% ethanol sealed tubes

DNA extraction protocol

DNA was extracted from 180 putative *R.(B) microplus* from 9 locations (table 2.1), 20 *R.(B) decoloratus* from South Africa and 20 *R.(B) annulatus* from Texas. The process of DNA extraction from the ethanol-fixed tick specimens was challenging with multiple attempts resulting in failure. The first DNA extraction technique used was the Qiagen purification of total DNA from ticks using the DNeasy® blood and tissue kit protocol. Individual tick specimens were placed into separate 1.5 ml eppendorf tubes, containing 180 µl buffer ATL (a Qiagen tissue lysis buffer for use in purification of nucleic acids) and vortexed thoroughly. Specimens were then cut up in order to allow full lysis (this step is particularly important with regard to the tick specimens, as the highly scleretorized integument may prevent release of DNA). The samples were centrifuged briefly before 20 µl of proteinase K was added, followed by vortexing and finally by incubation overnight at 56ºC in a temperature-controlled shaker. Samples were then subjected to the DNAeasy kit protocol, resulting in a final eluate volume of approximately 60 µl.

The Qiagen technique, although successfully used on *Ixodes ricinus* in a previous study was found to extract the DNA of *R.(B) microplus* inconsistently, however when it did work, the DNA was found to be of a high quality (A260/280 and A260/230 ratios, see DNA quantification). The inefficacy of the Qiagen protocol was thought to arise from the tough nature of integument of *R.(B) microplus*. In order to compensate for this, different cutting techniques were used to prepare the specimen for extraction; crushing the specimen against the tube using a scalpel, tearing the specimens apart using two needles and chopping the tick up using a scalpel. It was found that none of these techniques noticeably affected the protocol, and were difficult as the ticks were often quite soft. Based on this a drying step was added where ticks were placed into separate 1.5 ml eppendorf tubes and dried at 95ºC for 15 minutes in order to remove any ethanol contaminating the specimen. This additional step made it easier to crush the specimen against the side of the tube, thus breaking it up better than previously; however it did not greatly increase the yield in DNA. The final stage of optimization taken with this protocol was to crush the tick specimen using Qiagen stainless steel 5mm beads in a Qiagen TissueLyser II at 30 rpm for 2 minutes; this converted the specimen into a fine powder and slightly improved the protocol's

consistency. Nonetheless a large number of ticks subjected to this technique failed to yield DNA.

The second extraction method was based on a method developed in the Animal Genetics Laboratory of University of Queensland by Sean Corley. This method compromises on purification in favour of yield. In preparation for extraction a “Hair Lysis Buffer” (10 ml) was made up using 1 ml 10x PCR buffer, 50 µl $MgCl_2$ (50 mM), 50 µl Tween20 and 8.9 ml sterlised water. Individual tick specimens were placed into separate 1.5 ml Eppendorf tubes and crushed. To each of the tubes, 50 µl of the hair lysis buffer and 0.25 µl Proteinase-K (20 mg/ml) was added. Samples were then incubated at 60ºC for 45 minutes then 95ºC for 45 minutes.

This technique, along with using the drying and ball-bearing stage, was found to work far more predictably than the Qiagen protocol; however, the DNA yielded, although high in concentration was assessed as being low in quality. Despite the low quality, the second protocol was used for the majority of the specimens.

DNA quantification

Extracted DNA was quantified using a Thermo Scientific NanoDrop 1000 Spectrophotometer. The first value noted was the concentration (ng/µl), samples that yielded very low values, in some cases negative values were deemed unsuitable for use in PCR and binned. Samples that yielded a high concentration of DNA were checked for quality by looking at the A260/280 and A260/230 ratios. The A260/280 ratio is used to determine protein contamination in the sample; aromatic proteins have a strong UV absorbance around 280 nm. For high quality DNA, the A260/280 ratio should fall between 2.1 and 1.8, in samples where the ratio is lower than this, protein contamination is indicated. The A260/230 ratio can be used to indicate the presence of other organic contaminants, samples with a ratio lower than 1.8 are considered highly contaminated, where samples with a ratio close to 2.0 are considered of a high quality.

Sequencing protocol

PCR products were then cleaned using the AMPure XP kit before being used in the sequencing reaction. 27 µl of AMPure XP was added to 15 µl of PCR product and mixed by pipetting. After 5 minutes incubation at room temperature, samples

were placed onto a magnetic plate and left for 2 minutes, until the solution had cleared. The liquid was then removed and 200 µl of 70% ethanol was added and vortexed until mixed thoroughly. The reaction plate was placed again on the magnetic plate and left for 2 minutes, until the solution cleared. The ethanol was then discarded and the steps of adding and removing the ethanol were repeated. The plate was then allowed to air dry for approximately 20 minutes before 22 µl of nuclease-free water was added. A final check using a 1% agarose gel with 4 µl of product was then run to check that the cleanup had not destroyed the PCR product.

Cleaned PCR product was then set up in a new 96-well plate, alternating each column with forward and reverse primers (figure 2.1).

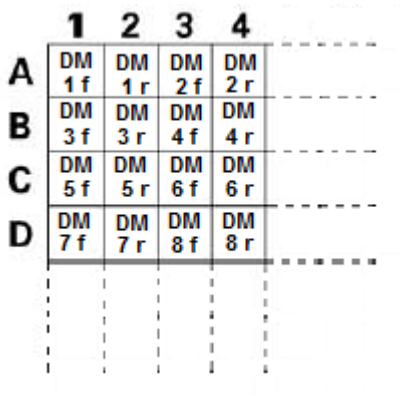


Figure 2.1: An excerpt taken from the plate map for plate 1 used in the sequencing of *COX1*. (Source: Authors own drawing)

A reaction master mix was then set up using the Applied Biosystems kit and the following recipe; 10 µl cleaned PCR product, 0.32 µl sequencing primer (10 µM), 0.5 µl Ready Reaction Mix = BigDye® Terminator v3.1 Cycle Sequencing Kit (ABGene #4336917), 3.5 µl 5X Sequencing Buffer (ABGene #4336697) and 5.68 µl sterile water (to a final volume of 20 µl). The following protocol was then set up on the thermocycler; 10 seconds at 96ºC, 10 seconds at 55ºC, 3 minutes at 60ºC, 25 cycles of steps 1-3, forever at 10ºC.

Once complete the sequencing product was cleaned using CleanSEQ. 10 µl of CleanSEQ and 85% ethanol was added to each reaction according to table 2.2.

Table 2.2: Amount of 85% ethanol used based on the volume of sequence reaction used, in the case of the following works 20 µl of sequence reaction was used resulting in 62 µl of ethanol being used.

Sequence Reaction Vol (µl)	85% ethanol (µl)
5	31
10	42
20	62

The plate was then sealed, vortexed, and pulsed at 1000 rpm for 1 s. The plate was then placed on a SPRI magnet for at least 3 minutes, until the solutions became clear. With the reaction plates still on the magnet, the solution was discarded by tipping upside down onto paper towel. 150 µl of 85% ethanol was then added to each well and incubated for 30 seconds. With the reaction plate still on the magnetic plate, the ethanol was carefully aspirated, avoiding touching the ring of magnetic beads and discarded. The plate was then air-dried for 10-20 minutes. Once all the ethanol had evaporated 40 µl of nuclease-free water was added to each well, sealed with a new adhesive seal, vortexed and pulse span at 1000 rpm for 1 second. Cleaned plate was then placed on a SPRI magnet and 20 µl of cleaned product was pipetted into a new semi-skirted plate for use in the sequencer. Empty wells were filled with 20 µl of nuclease-free water. Plates were analyzed using the Applied Biosystems 3130XL Genetic Analyzer.

CHAPTER 3

A three-way approach to the analysis of the morphology of *R.(B) microplus* with a view to resolve the validity of the proposed species *R.(B) australis*

Introduction

Up until recently *Boophilus* was considered a genus separate to *Rhipicephalus*; however with further morphological and molecular studies, evidence has been suggested to support the theory that *Boophilus* is synonymous with *Rhipicephalus* (Murrell and Barker, 2003b; Murrell *et al*., 2001b). With regard to morphological similarities studies that have used around 30 morphological features and a simple scoring technique e.g. ornamentation of scutum present: 1 absent: 0 concluded that many of the species defining features found in *Boophilus* are also shared by *Rhipicephalus* (Murrell and Barker, 2003b).

R.(B) microplus was first described in 1888 by Canestrini, however at this time it was described as *Haemaphysalis micropla* from samples originating in Paraguay (Quinlan *et al*., 1980, Cooley, 1946). Since then, there has been debate regarding the taxonomic status of *R.(B) microplus*. Up until 1978, all of the observations regarding the *R.(B) microplus* clade used morphological identification only. Differences among *R.(B) microplus* populations have been suggested by a number of different studies that combined both morphological and molecular analyses (Labruna *et al*., 2009, García-García *et al*., 1999, de la Fuente *et al*., 2000a).

The reinstatement of *R.(B) australis* was proposed and supported by a study using 16S rDNA and 12S rDNA molecular analysis as well as morphology (Estrada-Peña *et al*., 2012). In this study larvae and adults obtained from Australia were re-described and differentiated from *R.(B) microplus* using measurements from unfed specimens and previously sourced neotype material. This study found that although *R.(B) australis* and *R.(B) microplus* are clearly related, there are distinct morphological differences. For males the presence of a spur on the ventral surface of article I of the palp is a feature is found on *R.(B) australis* males only. Differences in setae patterns between the two species was also reported, which although is a valid feature to compare, can be damaged easily during the removal

process from the host, not to mention damage occurring while under alcohol. This study goes on further to dismiss the importance of the size and shape of the adanal shields and coxal spurs (features that are well documented to be key in morphological based identification) due to their high variability and potential for incorrect identification (Estrada-Peña *et al*., 2012).

Despite the call for the reinstatement of *R.(B) australis* based upon the morphological differences outlined above, it has subsequently been suggested that it is impossible to make an unambiguous distinction between *R.(B) australis* and *R.(B) microplus* based upon morphological criteria alone (Barker and Walker, 2014). Morphological features of *R.(B) australis* and *R.(B) microplus* were found to be too variable to allow for successful differentiation. They additionally commented on the difficulty of distinguishing between these two species and *R.(B) annulatus*, stating that once again the variability in the morphology makes all three very difficult to separate (Barker and Walker, 2014). Other researchers have also commented on the difficulties in distinguishing between *R.(B) microplus*, *R.(B) annulatus* and *R.(B) decoloratus* due to the lack of morphological differences and the variability observed in the features that are used for differentiation (Lempereur *et al*., 2010, Uilenberg, 1962).

The re-described morphology of *R.(B) australis* (Estrada-Peña *et al*., 2012) as well as morphological descriptions of *R.(B) microplus, R.(B) annulatus* and *R.(B) decoloratus* (Barker and Walker, 2014, Walker *et al.*, 2003) will be used as a morphological foundation for three studies that are intended to test the validity of the reinstatement of *R.(B) australis*, and explore the variability within and among populations.

This study comprises three approaches to the analysis of tick morphology: 3.1 classification of ticks according to binary scores based on criteria that have been proposed previously to enable differentiation of *R.(B) microplus* and closely related species; 3.2 a complete morphometric analysis of features that have been stated to or might have a value for discriminating among species or subspecies; 3.3 a geometric morphometric analysis that enables a similar approach to morphometrics, however removing the strong effect of size.

Materials and Methods

Samples

One thousand six hindred and fifty individual ticks were photographed, including adult males, females and larvae from South Africa, South America, North America, Thailand and Australia (table 3.1). In study 3.1 only the females and males were used, due to the lack of annotation on the larval morphology and the difficulty in obtaining nymphs. In the rest of the studies (3.2-3.3) both adults and larvae were used.

Table 3.1: Summary of the tick specimens photographed for this study. Country of origin including information on the isolate/strain where available in brackets, abbreviation used in this study, number of specimens photographed for each sex/stage.

Country of origin (isolate/strain)	Species	Number of ticks photographed		
		Females	Males	Larvae
South Africa	*R.(B) microplus*	49	50	49
	R.(B) decoloratus	50	50	49
Brazil (Jaguar)	*R.(B) microplus*	50	10	50
Brazil (Juarez)	*R.(B) microplus*	44	38	50
Brazil (Sao Gabriel)	*R.(B) microplus*	45	28	50
Uruguay (Mozo)	*R.(B) microplus*	40	21	50
USA (Deutch)	*R.(B) microplus*	50	50	50
	R.(B) annulatus	48	49	49
Mexico (Yucatan)	*R.(B) microplus*	49	22	50
Thailand	*R.(B) microplus*	50	50	50
Australia	*R.(B) microplus*	50	50	50

All ticks collected from the field (Australia and South Africa) were initially identified to genus using morphological criteria outlined by Walker *et al* (2003) for adults and Nuttall *et al* (1926) for larvae. As a result one African female, previously assigned to *R.(B) microplus*, was re-assigned to the genus *Dermacentor* and subsequently removed from the study. Two south African larvae, one previously assigned to *R.(B) microplus* and the other *R.(B) decoloratus*, were also removed from the study after being re-assigned to the genus *Haemaphysalis*. Any discrepancies at species level were addressed in study 3.1.

Microscopy and image capture

The microscope set up used in this project was a Zeiss Stemi 2000-C with zoom objective lens magnification ranging from 0.65- 5.0. Images were taken using a Canon 60D DSLR digital camera, modified for use on the microscope using a C-mount adapter on the phototube section of the trinocular microscope. Ticks were removed from tubes containing 70% ethanol using needle forceps and dried on a paper towel to remove any excess ethanol. Once ticks were dry they were placed onto a S1 stage micrometer with a graticule range of 0.1mm to 10mm. Lighting was provided by the microscope's own above-platform light as well as a bifurcated gooseneck light box, which enabled maximum illumination of the specimen for imaging. Images were taken using remote shutter control to reduce shake and improve clarity of the images. Up to 50 individual tick specimens from each population for each stage was photographed, and images were then stored in file-folders corresponding to the morphological feature imaged, sex/stage and geographical location.

3.1 Differentiation between adult members of the Boophilid clade based on a morphological scoring system

Materials and methods

Binary scoring systems

The scoring system was based upon the morphological criteria outlined (Barker and Walker, 2014, Walker *et al.*, 2003, Estrada-Peña *et al*., 2012) (table 3.2). Four two-way analyses were set up; *R.(B) australis* versus *R.(B) microplus*, *R.(B) microplus* versus *R.(B) annulatus*, *R.(B) microplus* versus *R.(B) decoloratus* and *R.(B) annulatus* versus *R.(B) decoloratus.* For each morphological feature that was assessed a score of either 0 or 1 was given, representing whether the feature was absent or present respectively (figure 3.1). This method was not used on larvae, as the suggested features enabling differentiation between *R.(B) australis* and *R.(B) microplus* are based on measurements of features rather than presence or absence of features.

Table 3.2: Summary of identifying features used in the score analysis for *R. (B) australis, R. (B) microplus, R.(B) annulatus* and *R.(B) decoloratus.*

Species/sex	Feature
R.(B) australis (Female)	Dorsal setae are abundant, long, and pale Medial alloscutal setae are in clusters of 4–6 rows Setae behind the eyes are clearly visible
R.(B) microplus (Female)	Dorsal setae in *R. microplus* are short and slender Medial alloscutal setae form clusters of 2–3 rows Setae behind the eyes are unapparent/absent Coxa I spur is distinct Spurs present on coxae II and III Porose area is broad oval Internal protuberance is absent on palp article I
R.(B) annulatus (Female)	Coxa I spur is indistinct Coxae II and III spurs are absent Porose area is broad oval Internal protuberance is absent on palp article I
R.(B) decoloratus (Female)	Coxa I spur is distinct Spurs present on coxae II and III Porose area is a narrow oval Internal protuberance is present on palp article I
R.(B) australis (Male)	Spur present on the ventral surface of palp article I Setae on the lateral margins of the ventral surface of the capitulum either very short or appear to be absent
R.(B) microplus (Male)	Ventral spur absent from palp article I Several setae present on the lateral margins of the ventral surface of the capitulum Coxa I spur is long Caudal appendage is present Ventral plate spurs are indistinct
R.(B) annulatus (Male)	Coxa I spur is short Caudal appendage is absent Ventral plate spurs are indistinct
R.(B) decoloratus (Male)	Coxa I spur is short Caudal appendage is present Ventral plate spurs are distinct

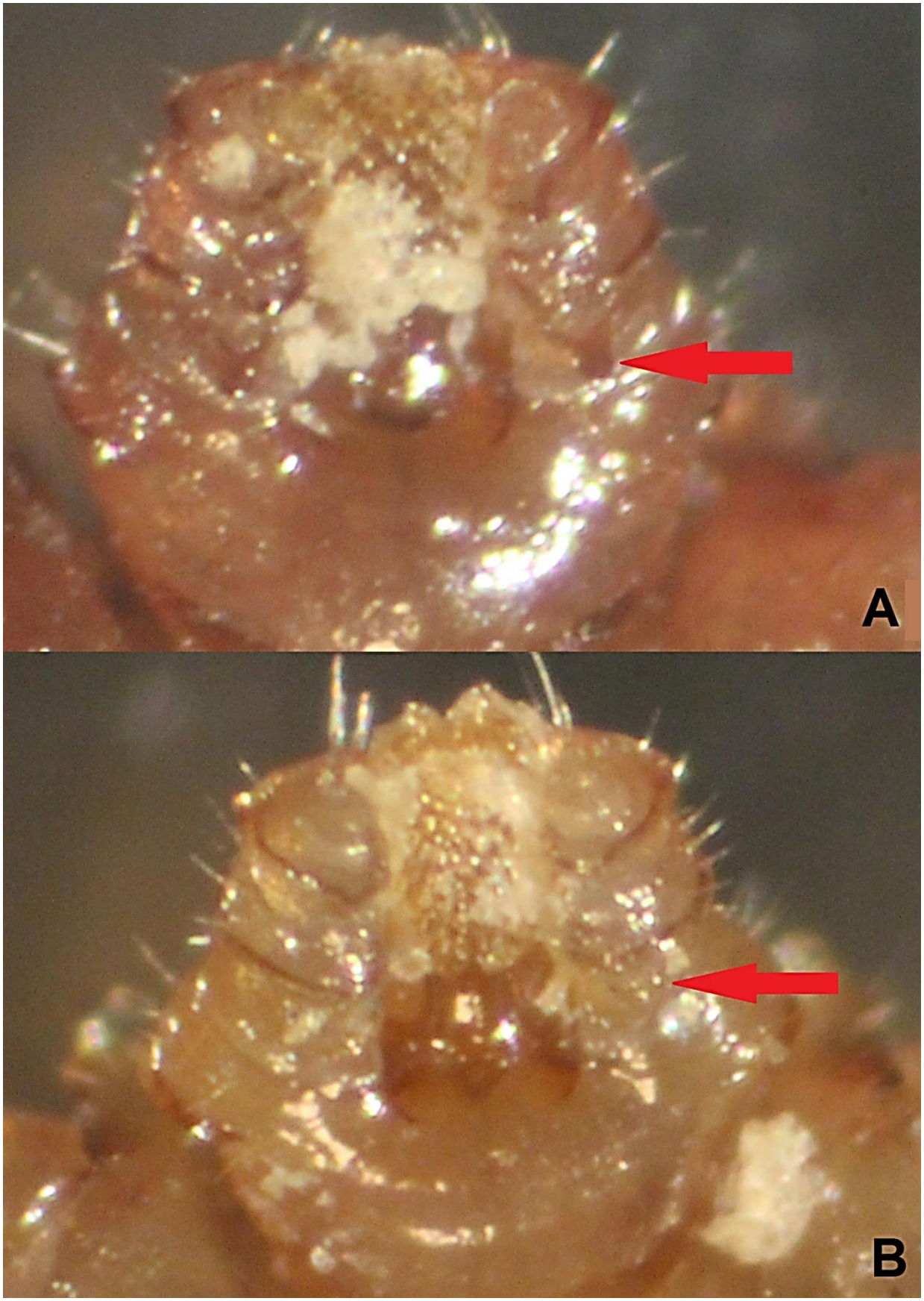


Figure 3.1: Example of scoring of presence of the ventral spur on palp article I (both A and B are Thai males); Red arrow shows the location of the ventral spur: A = feature present (score = 1), B = feature absent (score = 1). (Source: Authors own photograph).

Results

Table 3.3 shows the result of applying the scoring system criteria to the samples of female ticks collected as and believed to be *R.(B) annulatus* and *R.(B) decoloratus.* There was complete agreement in all cases and none of the samples examined showed features of more than one species. Among male ticks, however, samples that were collected as being *R.(B) decoloratus* had features that were not consistent with either species, nor with any of the other species for which the criteria were defined (table 3.4).

Table 3.3: Number of female ticks collected as *R.(B) annulatus* and *R.(B) decoloratus* assigned according to the features listed in Table 3.2: Number of ticks assigned unambiguously: 98, ambiguous or incorrect: 0; 100% of ticks unambiguously assigned to presumed correct species.

Population (females)	Number of population classed		
	R.(B) annulatus	***R.(B) decoloratus***	**Mixed**
R.(B) annulatus	48	0	0
R.(B) decoloratus	0	50	0

Table 3.4: Number of male ticks collected as *R.(B) annulatus* and *R.(B) decoloratus* assigned according to the features listed in table 3.2: Number of ticks assigned unambiguously: 94, ambiguous or incorrect: 0; 100% of ticks unambiguously assigned to presumed correct species.

Population (males)	Number of population classed			
	R.(B) annulatus	***R.(B) decoloratus***	**Mixed**	**Other *R* spp.**
R.(B) annulatus	49	0	0	0
R.(B) decoloratus	0	45	0	5

When the criteria for differentiating *R.(B) decoloratus* from *R.(B) microplus* and *R.(B) australis* were applied to the entirety of the presumed *R.(B) microplus* and *R.(B) australis* sample set, there was a clear distinction, with the single exception of one male from the African sample set, which was not fully consistent with the criteria of either *R.(B) microplus*/*R.(B) australis* or *R.(B) decoloratus* (tables 3.5 and 3.6).

Table 3.5: Number of female ticks collected as *R.(B) microplus/R.(B) australis* scored based on the features listed in table 3.2: Number of ticks assigned unambiguously: 427, incorrect or ambiguous: 0; 100% of ticks unambiguously assigned to presumed correct species.

Population (females)	Number of population classed		
	R.(B) microplus/ R.(B) australis	***R.(B) decoloratus***	**Mixed**
Australian	50	0	0
African	49	0	0
Yucatan	49	0	0
Deutch	50	0	0
Jaguar	50	0	0
Juarez	44	0	0
Mozo	40	0	0
Sao Gabriel	45	0	0
Thailand	50	0	0

Table 3.6: Number of male ticks collected as *R.(B) microplus/R.(B) australis* scored based on the features listed in table 3.2: Number of ticks assigned unambiguously: 319, incorrect or ambiguous: 1; 99.7% of ticks correctly assigned to species.

Population (males)	Number of population classed			
	R.(B) microplus/ R.(B) australis	***R.(B) decoloratus***	**Mixed**	**Other *R* spp.**
Australian	50	0	0	0
African	49	0	0	1
Yucatan	22	0	0	0
Deutch	50	0	0	0
Jaguar	10	0	0	0
Juarez	38	0	0	0
Mozo	21	0	0	0
Sao Gabriel	28	0	0	0
Thailand	50	0	0	0

As above, it was also found that *R.(B) microplus/ R.(B) australis* could be distinguished from *R.(B) annulatus* 100% of the time across all geographical regions for the females (table 3.7) using the published criteria. For 3 males of the Yucatan sample the caudal appendage was absent (a *R.(B) annulatus* feature) however the spur of coxa I was long (a *R.(B) microplus* feature), and so they would be classified on the basis of the criteria as likely hybrids of both species. The one male that did not fit under either *R.(B) microplus/ R.(B) australis* or *R.(B) decoloratus* mentioned above, was also found to not be *R.(B) annulatus* according to the criteria outlaid by Walker *et al*., 2003, and so was hypothesized to belong to a different Rhipicephaline species, (table 3.8). If it were assumed that hybrids are

not possible due to non-overlapping ranges, then the classification system would be estimated to give a correct and unambiguous assignment for 99.1% of cases.

Table 3.7: Number of female ticks collected as *R.(B) microplus* and *R.(B) australis* scored based on the features listed in table 3.2: Number of ticks scored correctly: 427, incorrectly: 0; 100% of ticks unambiguously assigned to presumed correct species.

Population (females)	Number of population classed		
	R.(B) microplus/ R.(B) australis	***R.(B) annulatus***	**Mixed**
Australian	50	0	0
African	49	0	0
Yucatan	49	0	0
Deutch	50	0	0
Jaguar	50	0	0
Juarez	44	0	0
Mozo	40	0	0
Sao Gabriel	45	0	0
Thailand	50	0	0

Table 3.8: Number of male ticks collected as *R.(B) microplus* and *R.(B) australis* scored based on the features listed in table 3.2: Number of ticks scored correctly: 316, incorrectly: 3; 99.1% of ticks correctly assigned to species.

Population (males)	Number of population classed			
	R.(B) microplus/ R.(B) australis	***R.(B) annulatus***	**Mixed**	**Other *R* spp.**
Australian	50	0	0	0
African	49	0	0	1
Yucatan	19	0	3	0
Deutch	50	0	0	0
Jaguar	10	0	0	0
Juarez	38	0	0	0
Mozo	21	0	0	0
Sao Gabriel	28	0	0	0
Thailand	50	0	0	0

When using the criteria to differentiate between *R.(B) microplus* and *R.(B) australis*, in the case of the Australian population of female ticks, 23/50 were classified as *R.(B) australis*, however 27/50 were classified either as *R.(B) microplus* or a mixture of both. The rest of the populations scored with the majority as *R.(B) microplus,* however several females presented features of both species in all populations except Mozo, which was assigned to *R.(B) microplus*. A small number of individual females in the South African and Juarez populations

also scored as *R.(B) australis* (table 3.9). The male ticks demonstrated a different overall trend, with the majority of all populations assigned either to *R.(B) australis* or having a mixture of both *R.(B) australis* and *R.(B) microplus* features (table 3.10).

Table 3.9: Number of female ticks collected as *R.(B) microplus*/*R.(B) australis* scored based on the features listed in table 3.2: Number of ticks scored correctly: 341, incorrectly: 86; 79.86% of ticks correctly assigned to species.

Population (females)	Number of population classed		
	R.(B) microplus	***R.(B) australis***	**Mixed**
Australian	19	23	8
African	33	6	10
Yucatan	41	0	8
Deutch	43	0	7
Jaguar	47	0	3
Juarez	33	4	7
Mozo	40	0	0
Sao Gabriel	44	0	1
Thailand	37	0	13

Table 3.10: Number of male ticks collected as *R.(B) microplus*/*R.(B) australis* scored based on the features listed in table 3.2: Number of ticks scored correctly: 52, incorrectly: 267; 16.3% of ticks correctly assigned to species.

Population (males)	Number of population classed		
	R.(B) microplus	***R.(B) australis***	**Mixed**
Australian	1	21	28
African	9	20	21
Yucatan	5	5	12
Deutch	7	17	26
Jaguar	0	2	8
Juarez	2	3	33
Mozo	3	3	15
Sao Gabriel	2	5	21
Thailand	3	29	18

3.2 Differentiation between members of the Boophilid clade based on morphometric analysis

Materials and methods

Samples

Specimens used in this study are listed in table 3.1. Unique abbreviations used in this study are summarized in table 3.11.

Table 3.11: List of abbreviations used in this study.

Name	Abbreviation
R.(B) microplus	MIC
R.(B) annulatus	ANN
R.(B) decoloratus	DEC
R.(B) australis	AUS
Population	POP

Morphometric measurements taken

For the morphometric part of the study, twelve anatomical features used in previous morphometric studies were used (Abdel-Shafy *et al*., 2011). Some measurements were unable to be taken due to the condition of the specimens. In the case of the females the measurements that were greatly impacted upon were the overall body length as well as the idiosoma length and width. This was due to a large number of females being fully or partially engorged, which drastically alters the dimensions of the tick's body. Measurements were taken of body length, idiosoma length, idiosoma width, scutum length, scutum width, width of basis capituli, palpal length (left), palpal length (right), length of dorsal basis capituli, hypostome length, hypostome width and length of ventral basis capituli (figure 3.2). The same measurements were taken from the males with the exception of scutal measurements because in males this is the conscutum, which corresponds with the idiosoma in females. Mechanical damage incurred during the collection of the specimens, particularly to parts of the mouthparts (palp left and/or right and hypostome) was seen in 16% of females, 31% of larvae and 1% of males. For these specimens measurements of the damaged palps and/or hypostome were not taken. Measurements were made using ImageJ software and recorded in an

Excel spreadsheet. In all specimens the limbs were not easily visible and would require removal for photographing in detail. This was deemed impractical due to the large number of specimens and so limb measurements were not included.

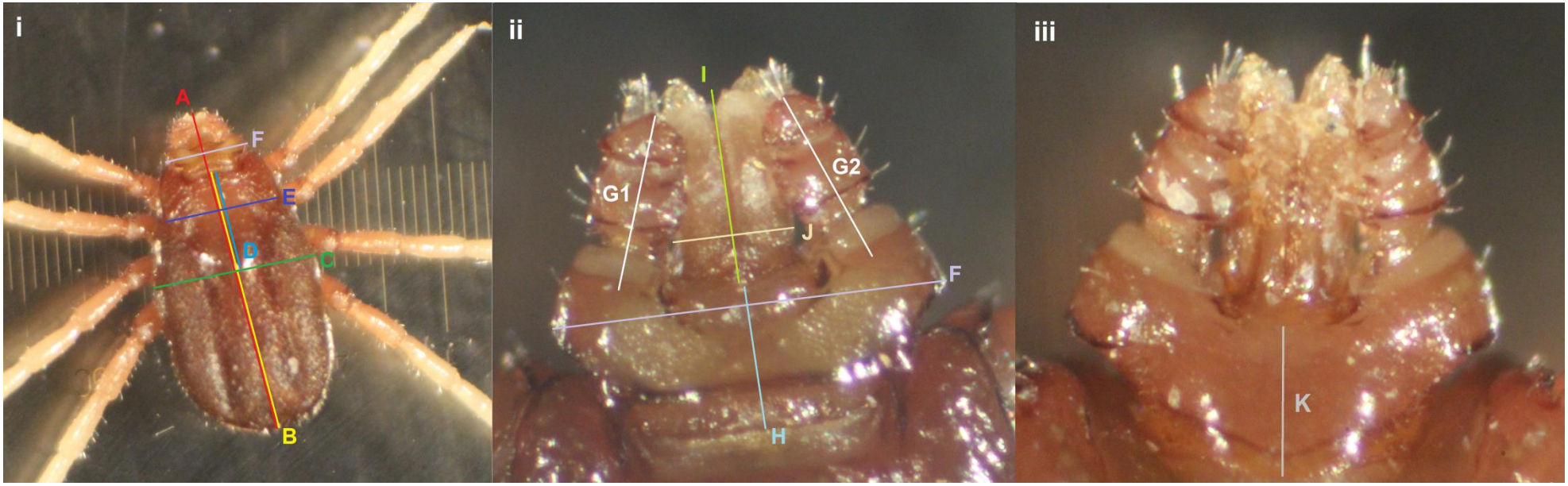


Figure 3.2: Summary of the measurements made on specimens for the morphometric study; i: body length (A), idiosoma length (B), idiosoma width (C), scutum length (D), scutum width (E), width of basis capituli (F); ii: width of basis capituli (F), palpal length (left) (G1), palpal length (right) (G2), length of dorsal basis capituli (H), hypostome length (I), hypostome width (J); iii: length of ventral basis capituli (K). (Source: Authors own photograph).

Statistics

Statistical analysis was performed using Minitab 17 Statistical software. The first stage in the statistical analysis was to perform Dixon's test to check for outliers in the three data sets.

Anderson-Darling normality tests were performed for all variables. For each of the variables, the variance was determined and a test for equal variances for each population was applied and overlapping confidence intervals were noted to show which variances were similar in each of the populations. Tests of equal variance were not applied across species because the numbers of samples in *R.(B) microplus* were several-fold higher than either *R.(B) annulatus*, *R.(B) decoloratus* or *R.(B) australis*. A test for equal variance of feature versus population was undertaken. This was further demonstrated through box plots of feature against population showing median confidence intervals, interquartile range, outlier and mean. A one-way anova was then performed to determine whether the features were similar in different populations. A Fisher Pairwise comparison was conducted to assess the statistical significane of differences between menas using 95% confidence interval. Groups generated are assigned a letter based on the distribution of the means, indicating which groups are significantly different. A principal component analysis (PCA) was used to explore the amount of variation between the morphological features and then observe how populations and species group based on variation.

Differentiation between larvae of the Boophilid clade using morphometric measurements

Body length, idiosoma width, width of basis capituli, scutum width and the length of the right palp were all normally distributed (p=>0.05). The remaining features were not normally distributed according to the Anderson-Darling test. In all cases, however, deviation from normality could be attributed to a small number of individuals at the extreme ends of the distribution. In the absence of any serious deviations from normality, given the number of samples used (544) and in the expectation that the dimensions of the anatomical features measured would be normally distributed, it was considered that parametric statistical analyses would be effective.

The standard deviation of each population was determined using Bonferroni confidence intervals. Overlapping confidence intervals showed that the variances were similar in each of the populations. From this Levene's method was applied to test if populations demonstrated equal variances for each morphological feature. Bartlett's test is very accurate for normally distributed data, which also means any deviations from normal distribution can cause errors, whereas the multiple comparisons and Levene's method is less sensitive to these deviations. It is for this reason that features that had a p-value <0.05 were tested for equal variances using the multiple comparisons and Levene's method.

Table 3.12: Test for equal variances for each morphological feature using the Bartlett method.

Morphological feature	Test Statistic	P-value
Body length	11.31	0.334
Idiosoma width	26.30	0.003
Scutum width	38.12	0.000
Width of basis capituli	17.22	0.070
Palpal length (right)	11.36	0.330

All morphological features in table 3.12 were tested for equal variances against population using the Bartlett method as they showed normal distribution. Body length, width of basis capituli and palp length (right) all showed equal variances with p-values >0.05. Idiosoma width and scutum width were not equally variable (table 3.13).

Table 3.13: Test for equal variances: morphological feature versus the population (POP) using the multiple comparisons method.

Morphological feature	P-value
Idiosoma length	0.036
Scutum length	0.001
Palpal length (left)	0.321
Length of dorsal basis capituli	0.108
Hypostome length	0.067
Hypostome width	0.408
Length of ventral basis capituli	0.193

Table 3.14: Test for equal variances: morphological feature versus the population (POP) using the Levene's method.

Morphological feature	Test Statistic	P-value
Idiosoma length	2.48	0.007
Scutum length	4.31	0.000
Palpal length (left)	1.04	0.406
Length of dorsal basis capituli	1.52	0.129
Hypostome length	2.00	0.032
Hypostome width	0.98	0.460
Length of ventral basis capituli	1.37	0.193

The rest of the morphological features were tested for equal variances using multiple comparisons (table 3.13) and Levene's method (table 3.14). For most features the significance didn't change between the two tests, however for hypostome length equal variance was observed when using the multiple comparisons method but not observed with the Levene's method. In this case the result from the multiple comparison method was taken over the Levene's, as the multiple comparison method is more powerful and the distribution for this feature had heavy tails on the normal distribution curve. Results showed that Idiosoma length and scutum length were not equally variable, however the rest of the features did show equal variance (table 3.14; 3.15). All features that showed equal variance (p=>0.05) were then tested using a one-way ANOVA (table 3.15). Features with a p-value <0.05 were tested using Welch's ANOVA instead, as this test does not assume equal variances (table 3.14).

Table 3.15: Results from the one-way ANOVA for each morphological feature versus population (POP). Analysis of variance is shown.

Morphological feature	Adj SS	Adj MS	F value	P value
Body length	0.1026	0.010257	10.86	0.000
Palpal length (left)	0.003528	0.000353	4.85	0.000
Palpal length (right)	0.004131	0.000413	4.49	0.000
Length of dorsal basis capituli	0.001947	0.000195	2.99	0.001
Hypostome length	0.004433	0.000443	2.91	0.002
Hypostome width	0.000417	0.000042	1.28	0.241
Length of ventral basis capituli	0.004688	0.000469	15.44	0.000

Table 3.16: Results from the Welch's ANOVA for each morphological feature versus population (POP). Analysis of variance is shown.

Morphological feature	DF Den	F value	P value
Idiosoma length	201.790	16.50	0.000
Idiosoma width	212.025	13.19	0.000
Scutum length	213.273	12.39	0.000
Scutum width	213.137	34.78	0.000
Width of basis capituli	213.382	10.00	0.000

Hypostome width was the only morphological feature that did not differ between populations (p=>0.05) (table 3.16). The rest of the features, however do vary between populations, with a strong significance (all around p-value= 0.000). The means for each population for each morphological feature was compared using a Fisher Pairwise comparison (table 3.17).

Table 3.17: Fisher Pairwise comparisons, grouping information using the Fisher least significant difference (LSD) method and 95% confidence interval to assess the statistical significance of differences between means. Means that do not share a letter are significantly different.

Morphological feature	Population	Mean	Grouping
Body length	Jaguar	0.59500	A
	Mozo	0.58807	A
	R.(B) decoloratus	0.57202	B
	R.(B) annulatus	0.57141	B
	Yucatan	0.56923	B C
	South Africa	0.56140	B C D
	Thailand	0.55561	C D
	Sao Gabriel	0.55383	D
	Juarez	0.55237	D
	Deutch	0.55079	D
	Australia	0.53074	E
Idiosoma length	Jaguar	0.45184	A
	Mozo	0.43943	B
	R.(B) annulatus	0.43478	B
	R.(B) decoloratus	0.42991	B C
	Yucatan	0.42232	C D

	Juarez	0.41538	D
	Deutch	0.41500	D
	Sao Gabriel	0.41338	D
	South Africa	0.41233	D
	Thailand	0.41176	D
	Australia	0.39106	E
Idiosoma width	*R.(B) annulatus*	0.42553	A
	South Africa	0.41837	A B
	Juarez	0.41640	B C
	R.(B) decoloratus	0.41406	B C
	Mozo	0.40792	C D
	Australia	0.40474	D
	Deutch	0.40361	D E
	Jaguar	0.40152	D E F
	Sao Gabriel	0.39488	E F
	Thailand	0.39438	F G
	Yucatan	0.38594	G
Scutum length	Juarez	0.26036	A
	R.(B) decoloratus	0.25086	B
	R.(B) annulatus	0.25016	B
	Sao Gabriel	0.24940	B C
	Deutch	0.24676	B C
	South Africa	0.24613	B C
	Australia	0.24451	B C
	Yucatan	0.24126	C
	Thailand	0.23272	D
	Mozo	0.23168	D E
	Jaguar	0.22432	E
Scutum width	*R.(B) annulatus*	0.36853	A
	Juarez	0.36434	A
	Thailand	0.35442	B
	Mozo	0.35172	B C
	Sao Gabriel	0.35080	B C D
	R.(B) decoloratus	0.34304	C D E

	Jaguar	0.34254	D E
	Deutch	0.34168	E
	Yucatan	0.33838	E
	South Africa	0.32390	F
	Australia	0.30280	G
Width of basis capituli	South Africa	0.15565	A
	Yucatan	0.15466	A
	Juarez	0.15188	A B
	R.(B) annulatus	0.15010	B C
	Sao Gabriel	0.15004	B C
	Jaguar	0.14954	B C
	Deutch	0.14938	B C
	Australia	0.14937	B C
	Thailand	0.14792	C
	Mozo	0.14248	D
	R.(B) decoloratus	0.14010	D
Palpal length (left)	Deutch	0.07584	A
	R.(B) annulatus	0.07473	A B
	South Africa	0.07225	A B C
	Jaguar	0.07066	B C D
	Mozo	0.06997	C D
	Australia	0.06922	C D
	Sao Gabriel	0.06857	C D E
	R.(B) decoloratus	0.06829	D E
	Yucatan	0.06774	D E
	Juarez	0.06703	D E
	Thailand	0.06503	E
Palpal length (right)	South Africa	0.07395	A
	R.(B) annulatus	0.07290	A B
	Deutch	0.07242	A B
	Mozo	0.06852	B C
	Jaguar	0.06841	B C
	Sao Gabriel	0.06757	C
	Yucatan	0.06750	C D

	R.(B) decoloratus	0.06680	C D
	Juarez	0.06621	C D
	Australia	0.06541	C D
	Thailand	0.06314	D
Length of dorsal basis capituli	South Africa	0.05314	A
	R.(B) annulatus	0.05247	A
	Mozo	0.05082	A B
	Sao Gabriel	0.05024	A B
	Deutch	0.05016	A B
	Australia	0.05011	A B
	Juarez	0.05003	A B
	Jaguar	0.04948	A B
	Thailand	0.04835	B C
	Yucatan	0.04803	B C
	R.(B) decoloratus	0.04507	C
Hypostome length	Mozo	0.08230	A
	Deutch	0.07840	A B
	South Africa	0.07730	A B
	Jaguar	0.07686	A B
	Juarez	0.07534	B C
	Sao Gabriel	0.07438	B C
	Yucatan	0.07358	B C
	R.(B) decoloratus	0.07318	B C
	Thailand	0.07311	B C
	Australia	0.07054	B C
	R.(B) annulatus	0.06953	C
Hypostome width	*R.(B) annulatus*	0.04130	A
	South Africa	0.04057	A B
	R.(B) decoloratus	0.039889	A B
	Yucatan	0.039818	A B C
	Jaguar	0.039571	A B C
	Juarez	0.039448	A B C
	Sao Gabriel	0.039378	A B C
	Australia	0.03884	A B C

	Thailand	0.038432	B C
	Mozo	0.038364	B C
	Deutch	0.037167	C
Length of ventral basis capituli	*R.(B) annulatus*	0.050755	A
	Deutch	0.049740	A B
	South Africa	0.049104	A B
	Juarez	0.048480	B C
	Yucatan	0.047940	B C
	Australia	0.047816	B C
	Sao Gabriel	0.046460	C
	R.(B) decoloratus	0.044265	D
	Jaguar	0.043060	D
	Thailand	0.042531	D
	Mozo	0.042120	D

The Fisher pairwise comparison grouped populations based on the comparison of the means and assigned groups with a letter. Means that do not share a letter are significantly different. The population pairwise comparisons that differed significantly were inconsistent by feature. Populations that were significantly different for each feature (table 3.17) included; body length: Jaguar and Mozo ticks from Australian ticks; idiosoma length: Jaguar ticks from Australian ticks; idiosoma width: *R.(B) annulatus* from *R.(B) microplus* ticks from Yucatan; scutum length: Juarez ticks from Jaguar ticks; scutum width: *R.(B) annulatus* from Australian ticks; width of basis capituli: South African and Yucatan ticks from Mozo and *R.(B) decoloratus*; palpal length (left): ticks from Deutch from Thai ticks; palpal length (right): South African ticks from Thai ticks; length of dorsal basis capituli: South African ticks and *R.(B) annulatus* from *R.(B) decoloratus*; hypostome length: ticks from Mozo from *R.(B) annulatus*; hypostome width: *R.(B) annulatus* from Deutch ticks; length of ventral basis capituli: *R.(B) annulatus* from *R.(B) decoloratus*, as well as ticks from Jaguar, Thailand and Mozo.

A principal component analysis (PCA) was used to explore the amount of variation between the morphological features and then observe how populations and species group based on variation.

Table 3.18: Eigenvalues (along with proportion and cumulative values) for the first two principal components.

	PC1	PC2
Eigenvalue	3.8241	2.0414
Proportion	0.319	0.170
Cumulative	0.319	0.489

Table 3.19: Principal components values (PC1 and PC2) for each morphological variable.

Variable	PC1	PC2
Body length	0.327	0.388
Idiosoma length	0.195	0.547
Idiosoma width	0.325	0.191
Scutum length	0.337	0.092
Scutum width	0.292	0.264
Width of basis capituli	0.284	0.032
Palpal length (left)	0.307	-0.389
Palpal length (right)	0.331	-0.366
Length of dorsal basis capituli	0.292	-0.271
Hypostome length	0.337	-0.256
Hypostome width	0.227	0.029
Length of ventral basis capituli	0.125	-0.107

In the first component (PC1), the greatest amount of variation can be observed between scutum and hypostome length (both 0.337) and the length of ventral basis capituli (table 3.19; figure 3.3). In the second component (PC2), the greatest amount of variation is between idiosoma length and palpal length (left) (table 3.19; figure 3.3). From the loading plot it also clear that there are some groupings based on a close correlation, particularly obvious for PC2, where hypostome length, length of dorsal basis capituli and both palpal lengths show a close correlation (figure 3.3). This means that in PC1 the strongest features to use to distinguish between the populations are scutum length and hypostome length. In PC2 the idiosoma length would be used todifferentiate populations.

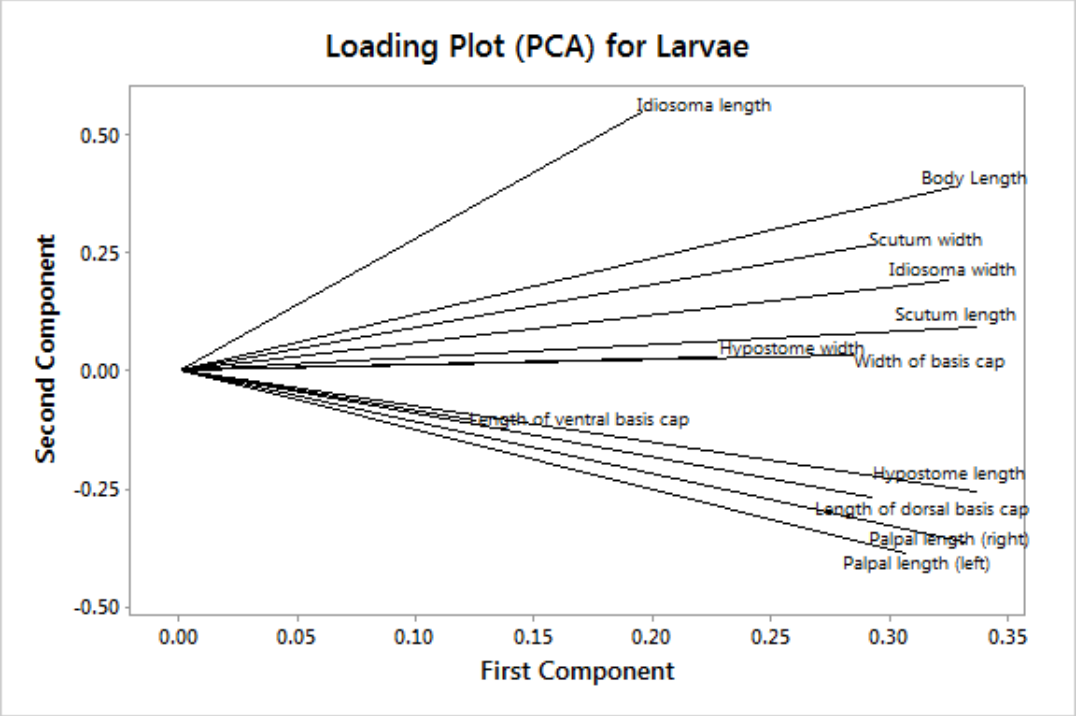


Figure 3.3: Loading plot output from principal component analysis (PCA) for morphological features of larvae. The plot shows the loadings of the first two components, presenting the correlations between the variable and the largest two components. Loadings have been multiplied by the standard deviation of the component which makes it viewable as a distance. Variables that appear closer together on the plot are more highly correlated.

A score plot for larvae based on population indicated that it was not possible to differentiate the separate populations based on the morphological criteria (figure 3.4).

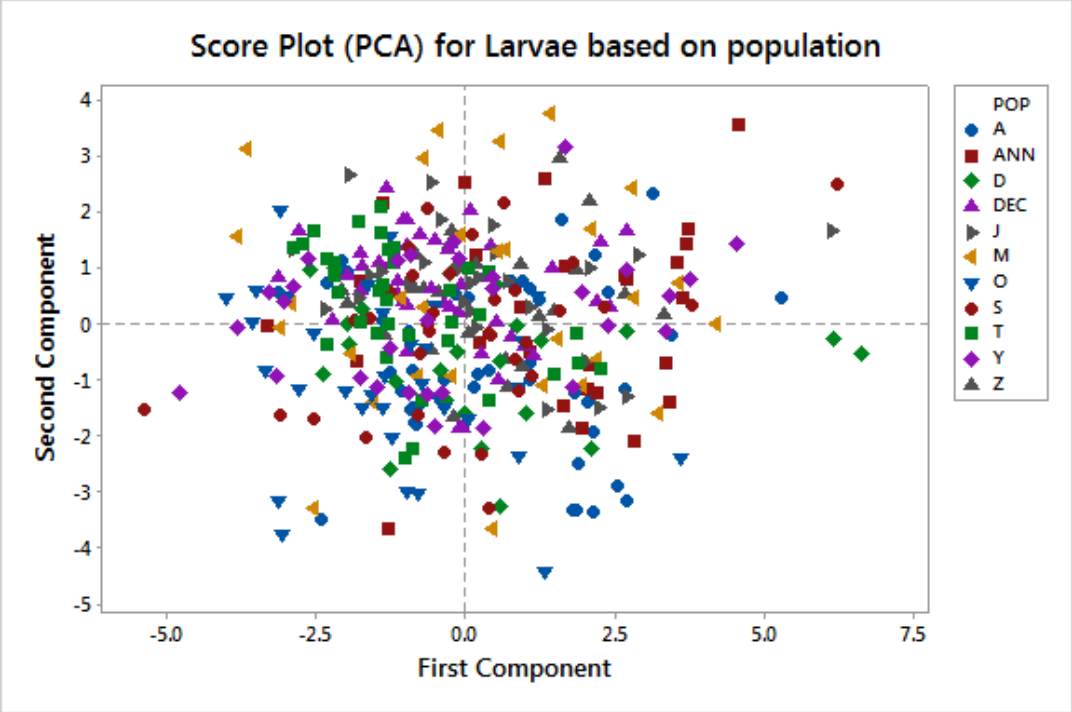


Figure 3.4: Score plot from principal component analysis (PCA) for larvae based on population. A: South Africa, ANN: *R.(B) annulatus*, D: Deutch, DEC: *R.(B) decoloratus*, J: Jaguar, M: Mozo, O: Australia, S: Sao Gabriel, T: Thailand, Y: Yucatan, Z: Juarez.

The score plot for larvae based on species showed a trend for *R.(B) australis* to group toward the negative values, indicating that they tend to be shorter (figure 3.5). With regard to the rest of the species, no discernable pattern emerged.

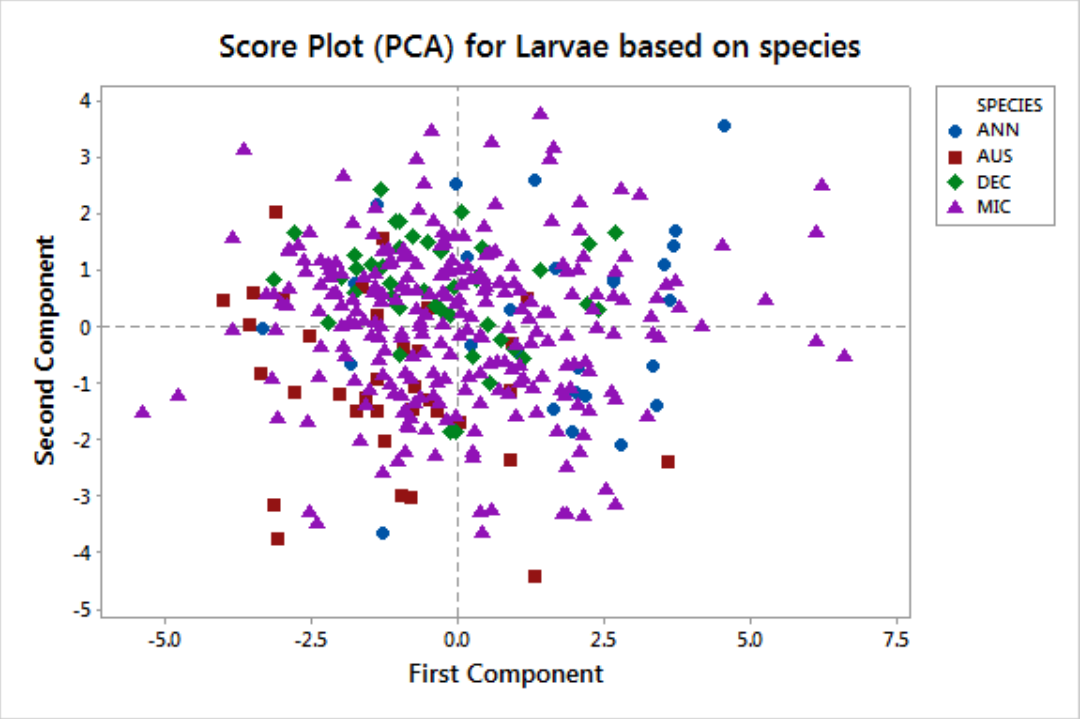


Figure 3.5: Score plot from principal component analysis (PCA) for larvae based on species. ANN: *R.(B) annulatus*, AUS: *R.(B) australis*, DEC: *R.(B) decoloratus*, MIC: *R.(B) microplus*.

Differentiation between males of the Boophilid clade using morphometric measurements

Body length and idiosoma length were not normally distributed (p=<0.05) and so were not used in the rest of the analysis. In the case of the larvae, non-normally distributed features remained in the rest of the analysis because the data followed the normality line, except for a few individuals at the extreme ends. For body length and idiosoma length, this was not the case as a lack of normal distribution was seen throughout the graph.

The standard deviation of each population was tested using the Bonferroni confidence intervals. Overlapping confidence intervals showed that the variances were similar in each of the populations. From this a test for equal variances was used to test the equality of variances between the morphological features within each population. Bartlett's test was used for features that had a p=>0.05, whereas the multiple comparisons and Levene's method was used on features that had a p=<0.05.

Table 3.20: Test for equal variances: morphological feature versus the population (POP) using the Bartlett method.

Morphological feature	Test Statistic	P-value
Idiosoma width	103.38	0.000
Width of basis capituli	55.58	0.000
Palpal length (right)	39.22	0.000
Length of dorsal basis capituli	26.47	0.003
Length of ventral basis capituli	26.36	0.003

Features tested with the Bartlett method were shown to not be equally variable (p=<0.05) (figure 3.20).

Table 3.21: Test for equal variances: morphological feature versus the population (POP) using the multiple comparisons method.

Morphological feature	P-value
Palpal length (left)	0.007
Hypostome length	0.001
Hypostome width	0.001

Table 3.22: Test for equal variances: morphological feature versus the population (POP) using the Levene's method.

Morphological feature	Test Statistic	P-value
Palpal length (left)	3.08	0.001
Hypostome length	4.89	0.000
Hypostome width	3.68	0.000

The rest of the morphological features were tested for equal variances using multiple comparisons (table 3.21) and Levene's method (table 3.22). Equal variance was not observed for the rest of the morphological features, for either test. None of the features showed equal variance, so Welch's ANOVA was used, as this test does not assume equal variances (table 3.23).

Table 3.23: Results from the Welch's ANOVA for each morphological feature versus population (POP). Analysis of variance is shown.

Morphological feature	DF Den	F value	P value
Idiosoma width	116.899	52.37	0.000
Width of basis capituli	116.651	60.22	0.000
Palpal length (left)	117.216	43.31	0.000
Palpal length (right)	117.757	33.47	0.000
Length of dorsal basis capituli	120.062	25.51	0.000
Hypostome length	116.694	35.10	0.000
Hypostome width	114.204	38.24	0.000
Length of ventral basis capituli	115.751	24.17	0.000

Welch's ANOVA showed that all of the features do vary between populations, with a strong significance (p-value= 0.000) (table 3.23). The means for each population for each morphological feature was then compared using a Fisher Pairwise comparison (table 3.24).

Table 3.24: Fisher Pairwise comparisons, grouping information using the Fisher least significant difference (LSD) method and 95% confidence interval to assess the statistical significance of differences between means. Means that do not share a letter are significantly different.

Morphological feature	Population	Mean	Grouping
Idiosoma width	DEC	1.4444	A
	ANN	1.4175	A B
	Z	1.4003	A B
	J	1.3903	A B C
	T	1.3844	B
	S	1.2891	C D
	A	1.2746	D
	M	1.2609	D E
	Y	1.2004	E
	D	1.1985	E
	O	1.0143	F
Width of basis capituli	T	0.53122	A
	J	0.5028	A B

	ANN	0.49892	B
	Z	0.49418	B
	S	0.45896	C
	A	0.44278	C D
	Y	0.43886	C D
	DEC	0.43532	D
	M	0.43410	C D
	D	0.42544	D
	O	0.35104	E
Palpal length (left)	Z	0.22784	A
	T	0.21982	A B
	S	0.21286	B C
	J	0.20980	B C D
	ANN	0.20373	C D
	DEC	0.20080	D
	M	0.19676	D E
	Y	0.19291	D E F
	D	0.18992	E F
	A	0.18294	F
	O	0.14096	G
Palpal length (right)	Z	0.21592	A
	T	0.21440	A
	J	0.20440	A B
	S	0.19864	B
	DEC	0.19708	B
	ANN	0.19114	B
	Y	0.18536	B C
	M	0.18519	B C
	D	0.17906	C
	A	0.17350	C
	O	0.13766	D
Length of dorsal basis capituli	T	0.20000	A
	ANN	0.19708	A B
	J	0.19690	A B C D

	Z	0.19453	A B C
	S	0.18700	B C D
	Y	0.18250	C D E
	M	0.17790	D E F
	A	0.17202	E F
	D	0.16682	F
	DEC	0.16614	F
	O	0.13112	G
Hypostome length	Z	0.31524	A
	J	0.2842	B C D
	T	0.28052	B
	DEC	0.27582	B C D
	S	0.27143	B C D
	M	0.26857	B C D
	A	0.26398	D
	ANN	0.26202	C D
	Y	0.22964	E
	D	0.22454	E
	O	0.18732	F
Hypostome width	Z	0.14268	A
	J	0.13090	B
	S	0.12593	B
	A	0.12473	B
	ANN	0.12452	B
	T	0.12382	B
	M	0.12338	B
	DEC	0.12108	B
	Y	0.10168	C
	D	0.10142	C
	O	0.09808	C
Length of ventral basis capituli	J	0.16610	A
	Z	0.15984	A B
	Y	0.15523	A B C
	A	0.15426	B C

	T	0.14918	C D
	D	0.14812	C D
	ANN	0.14633	D
	O	0.13730	E
	DEC	0.13518	E
	M	0.12614	F
	S	0.11668	G

The Fisher pairwise comparison grouped populations based on the comparison of the means and assigned groups with a letter. Means that do not share a letter are significantly different. The Australian population was significantly different for all features apart from the length of ventral basis capituli and hypostome width, favoring the smaller mean values (table 3.24). For hypostome width, although the Australian population has the smallest mean, it still grouped with ticks from Yucatan and Deutch ticks. The populations that Australian ticks differed from varied depending on the feature. *R.(B) decoloratus* was significantly different to the Australian population for idiosoma width. For palpal lengths, hypostome length and width ticks from Australia were significantly different to ticks from Juarez. For the width of the basis capituli and the length of the dorsal basis capituli, ticks from Thailand were significantly different to the Australian populations. Ticks from Juarez were significantly different to ticks from Sao Gabriel for the length of the ventral basis capituli.

A principal component analysis (PCA) was used to explore the amount of variation between the morphological features and then observe how populations and species group based on variation.

Table 3.25: Eigenvalues (along with proportion and cumulative values) for the first two principal components.

	PC1	**PC2**
Eigenvalue	5.1482	0.9085
Proportion	0.644	0.114
Cumulative	0.644	0.757

PC1 had an Eigenvalues above 1 and so was accepted, PC2 was also used even though it was below 1, as it was not far below. This meant that the first two eigenvalues were considered significant for each morphological feature (table 3.25).

Table 3.26: Principal components values (PC1 and PC2) for each morphological variable.

Variable	PC1	PC2
Idiosoma width	0.370	0.047
Width of basis capituli	0.393	0.066
Palpal length (left)	0.398	-0.147
Palpal length (right)	0.392	-0.149
Length of dorsal basis capituli	0.348	-0.047
Hypostome length	0.373	-0.198
Hypostome width	0.328	0.005
Length of ventral basis capituli	0.170	0.953

In PC1, the greatest amount of variation can be observed between Palpal length (left) and the length of ventral basis capituli (table 3.26; figure 3.6). In PC2, the greatest amount of variation is between the length of the ventral basis capituli and hypostome length (table 3.26; figure 3.6). The loading plot indicates that in PC2 most features, apart from the length of the ventral basis capituli, group (figure 3.6). This means that in PC1 the strongest features to use to distinguish the populations are both palpal lengths. In PC2 however, the length of the ventral basis capituli would be used.

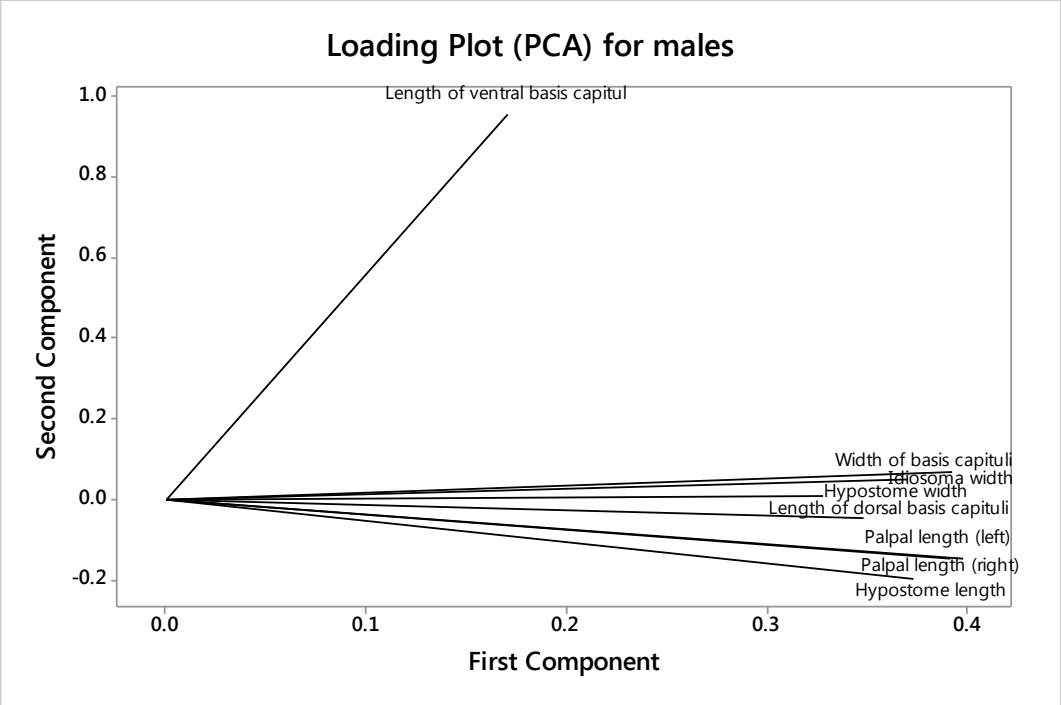


Figure 3.6: Loading plot output from principal component analysis for morphological features of males. The plot shows the loadings of the first two components, presenting the correlations between the variable and the largest two components. Loadings have been multiplied by the standard deviation of the component which makes it viewable as a distance. Variables that appear closer together on the plot are more highly correlated.

A score plot for males based on population indicated differentiation of the Australian population from the others, however no other differentiation of populations could be seen (figure 3.7).

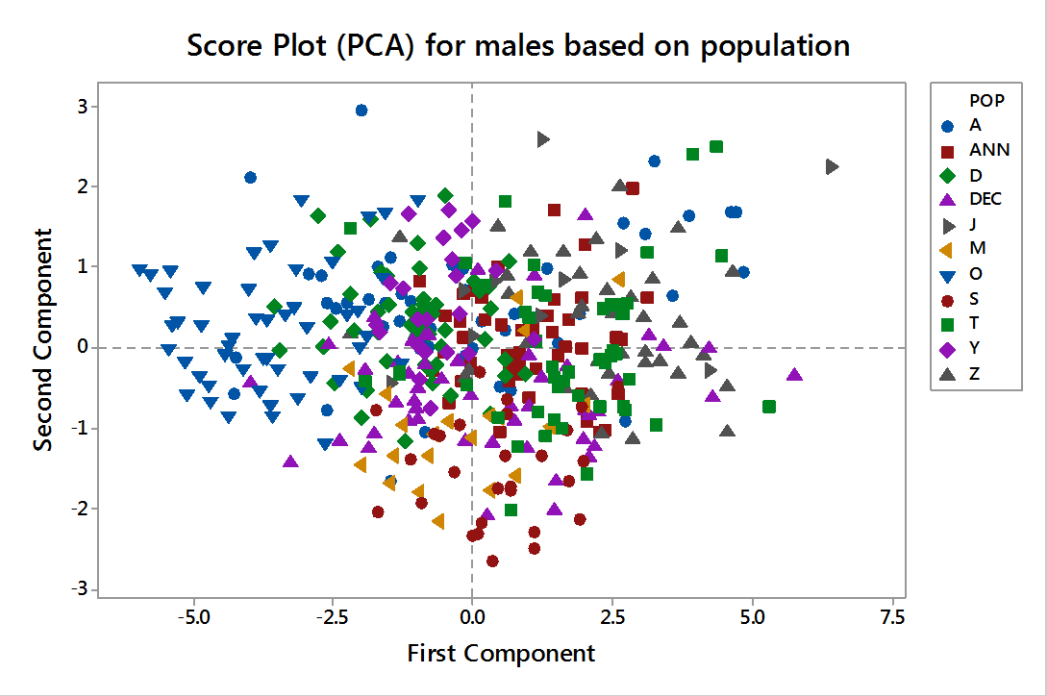


Figure 3.7: Score plot from principal component analysis (PCA) for males based on population. A: South Africa, ANN: *R.(B) annulatus*, D: Deutch, DEC: *R.(B) decoloratus*, J: Jaguar, M: Mozo, O: Australia, S: Sao Gabriel, T: Thailand, Y: Yucatan, Z: Juarez.

The score plot for males based on species yielded the same result as for population, with the grouping of the Australian ticks clearer than in the score plot based on population. No pattern can be clearly observed for the other three species (figure 3.8).

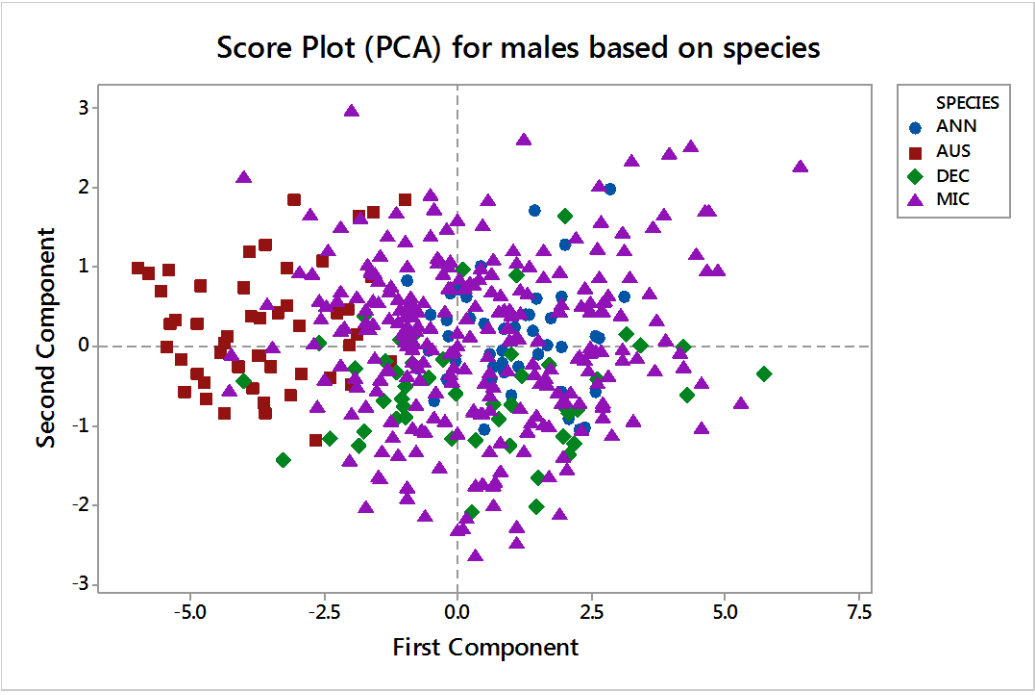


Figure 3.8: Score plot from principal component analysis (PCA) for males based on species. ANN: *R.(B) annulatus*, AUS: *R.(B) australis*, DEC: *R.(B) decoloratus*, MIC: *R.(B) microplus*.

Differentiation between females of the Boophilid clade using morphometric measurements

Body length, idiosoma length/width and scutum length/width were not normally distributed (p=<0.05) consistently along the line of normal distribution and so were not used in the rest of the analysis. The rest of the features, apart from hypostome width were also not normally distributed (p=<0.05), however this effect was created by a small number of individuals at the extreme values and was not a large deviation, so the decision was made to stick with a parametric analysis, without removing the individuals that did not fit on the normal distribution, as this would make the results bias. Hypostome width was normally distributed (p=>0.05).

The standard deviation of each population was tested using the Bonferroni confidence intervals. Overlapping confidence intervals showed that the variances were similar in each of the populations. From this a test for equal variances was used to test the equality of variances between the morphological features within each population. Bartlett's test was used on any feature that was p=>0.05, which was only this hypostome width (table 3.27). The rest of the features with p=<0.05 were tested for equal variances using the multiple comparisons and Levene's method (table 3.28).

Table 3.27: Test for equal variances: morphological feature versus the population (POP) using the Bartlett method.

Morphological feature	Test Statistic	P-value
Hypostome width	18.11	0.053

When tested with Bartlett's method, the hypostome width did show equal variance (p=>0.05), however it was borderline with p=0.053 (table 3.27).

Table 3.28: Test for equal variances: morphological feature versus the population (POP) using the multiple comparisons method.

Morphological feature	P-value
Width of basis capituli	0.000
Palpal length (left)	0.032
Palpal length (right)	0.116
Length of dorsal basis capituli	0.002
Hypostome length	0.000
Length of ventral basis capituli	0.000

Table 3.29: Test for equal variances: morphological feature versus the population (POP) using the Levene's method.

Morphological feature	Test Statistic	P-value
Width of basis capituli	6.03	0.000
Palpal length (left)	1.96	0.036
Palpal length (right)	1.56	0.116
Length of dorsal basis capituli	2.59	0.005
Hypostome length	3.75	0.000
Length of ventral basis capituli	1.31	0.230

The rest of the morphological features were tested for equal variances using multiple comparisons (table 3.28) and Levene's method (table 3.29). For most features the significance didn't change between the two tests, however for the length of the ventral basis capituli, equal variance was not observed for the multiple comparisons method but was observed with Levene's method. In this case the result from the multiple comparison method was taken over the Levene's, as the multiple comparison method is more powerful and the distribution for this feature had heavy tails on the normal distribution curve. Palpal length (right) showed equal variance for both multiple comparison and Levene's methods (p=>0.05). All other features showed no equal variance (p=<0.05) (table 3.28; 3.29). All features that showed equal variance (p=>0.05) were then tested using a one-way ANOVA (table 3.30). Features with p= <0.05 were tested using Welch's ANOVA instead, as this test does not assume equal variances (table 3.31).

Table 3.30: Results from the one-way ANOVA for each morphological feature versus population (POP). Analysis of variance is shown.

Morphological feature	Adj SS	Adj MS	F value	P value
Palpal length (right)	0.3200	0.032002	24.04	0.000

Table 3.31: Results from the Welch's ANOVA for each morphological feature versus population (POP). Analysis of variance is shown.

Morphological feature	DF Den	F value	P value
Width of basis capituli	199.791	41.28	0.000
Palpal length (left)	195.385	32.24	0.000
Length of dorsal basis capituli	201.146	53.71	0.000
Hypostome length	156.332	24.80	0.000
Hypostome width	159.100	25.55	0.000
Length of ventral basis capituli	14.6031	4.95	0.004

Results from both the ANOVA (table 3.28) and Welch's ANOVA (table 3.29) showed that all morphological features differed between populations ($p=<0.05$). The means for each population for each morphological feature was then compared using a Fisher Pairwise comparison (table 3.32).

Table 3.32: Fisher Pairwise comparisons, grouping information using the Fisher least significant difference (LSD) method and 95% confidence interval to assess the statistical significance of differences between means. Means that do not share a letter are significantly different.

Morphological feature	Population	Mean	Grouping
Palpal length (left)	Y	0.33673	A
	ANN	0.31775	B
	D	0.31176	B
	M	0.29284	C
	J	0.29165	C
	S	0.28730	C
	Z	0.28317	C
	T	0.25906	D
	O	0.25711	D E
	A	0.25704	D E
	DEC	0.24359	E
Palpal length (right)	Y	0.32771	A
	ANN	0.30544	B
	D	0.29688	B C
	M	0.28269	C D
	J	0.28035	D E
	S	0.27970	D E
	Z	0.26525	E F
	A	0.25751	F G
	O	0.25720	F G
	T	0.24844	G
	DEC	0.24389	G
Length of dorsal basis capituli	Y	0.31337	A
	D	0.26540	B
	ANN	0.25868	B
	M	0.24167	C
	J	0.23839	C
	S	0.22739	C D
	Z	0.21528	D
	T	0.19570	E

	A	0.19263	E
	O	0.18533	E F
	DEC	0.17196	F
Hypostome length	J	0.4464	A
	Z	0.4445	A
	M	0.4397	A
	S	0.43549	A
	O	0.4330	A B
	ANN	0.4218	A B
	A	0.4052	B C
	Y	0.37957	C D
	DEC	0.3594	D E
	T	0.34984	E
	D	0.34390	E
Hypostome width	Y	0.20806	A
	S	0.20107	A
	M	0.18982	B
	J	0.18652	B
	A	0.18263	B C
	O	0.17521	C D
	D	0.17135	D
	ANN	0.17038	D
	Z	0.16950	D
	T	0.16869	D
	DEC	0.15150	E
Length of ventral basis capituli	O	0.2543	A B C D
	Y	0.25396	A
	ANN	0.2520	A B C D E F
	D	0.24324	A B C
	J	0.24265	A B C D
	M	0.23850	A B C D E F
	T	0.23126	D F
	S	0.23004	C D E F
	Z	0.21400	E F

	A	0.2065	B C D E F

When means were compared, the greatest difference was seen between ticks from Yucatan and *R.(B) decoloratus* for both palpal lengths, the length of the dorsal basis capituli and hypostome width (table 3.32). The feature that had the least differences between the populations was the length of the ventral basis capituli. For hypostome length ticks from Juarez and Deutch were significantly different, however they both grouped with other populations.

A principal component analysis (PCA) was used to explore the amount of variation between the morphological features and then observe how populations and species group based on variation.

Table 3.33: Eigenvalues (along with proportion and cumulative values) for the first two principal components.

	PC1	PC2
Eigenvalue	3.9533	1.2091
Proportion	0.565	0.173
Cumulative	0.565	0.737

Eigenvalues above 1 were accepted, with anything below 1 being omitted. This meant that the first two eigenvalues were considered significant for each morphological feature (table 3.33).

Table 3.34: Principal components values (PC1 and PC2) for each morphological variable.

Variable	PC1	PC2
Width of basis capituli	0.448	-0.123
Palpal length (left)	0.455	0.149
Palpal length (right)	0.453	0.168
Length of dorsal basis capituli	0.438	0.243
Hypostome length	0.262	-0.544
Hypostome width	0.352	-0.260
Length of ventral basis capituli	0.053	0.715

In the first component (PC1), the greatest amount of variation can be observed between palpal length (left) and the length of the ventral basis capituli (table 3.34; figure 3.9). In the second component (PC2), the greatest amount of variation is between the length of the ventral basis capituli and hypostome length (table 3.34; figure 3.9). From the loading plot it also clear that there is a grouping based on a close correlation, observed in PC2, where the length of dorsal basis capituli and both palpal lengths show a close correlation (figure 3.14). This close correlation is also in PC1; however the width of the basis capituli also groups. This means that in PC1 the strongest features to use to distinguish the populations are both palpal lengths, and potentially the width of the basis capituli as well as the length of the dorsal basis capituli. In PC2 however, the length of the ventral basis capituli is the strongest feature by a large amount.

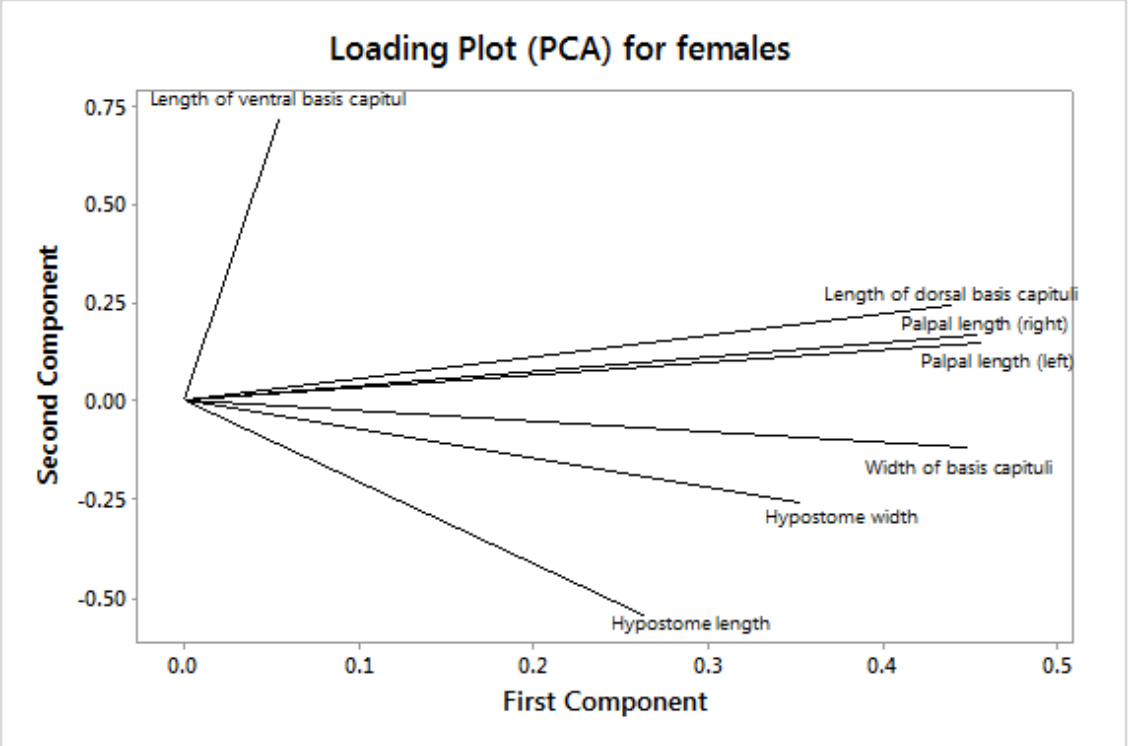


Figure 3.9: Loading plot output from principal component analysis for morphological features of females. The plot shows the loadings of the first two components, presenting the correlations between the variable and the largest two components. Loadings have been multiplied by the standard deviation of the component which makes it viewable as a distance. Variables that appear closer together on the plot are more highly correlated.

A score plot for females based on population indicated some differentiation of the Australian isolates from other populations, however out of the 50 Australian ticks obtained; only two were suitably unfed for measuring (figure 3.10). Two clusters of

ticks from Yucatan can also be observed, however the rest of the populations appear to group closely together.

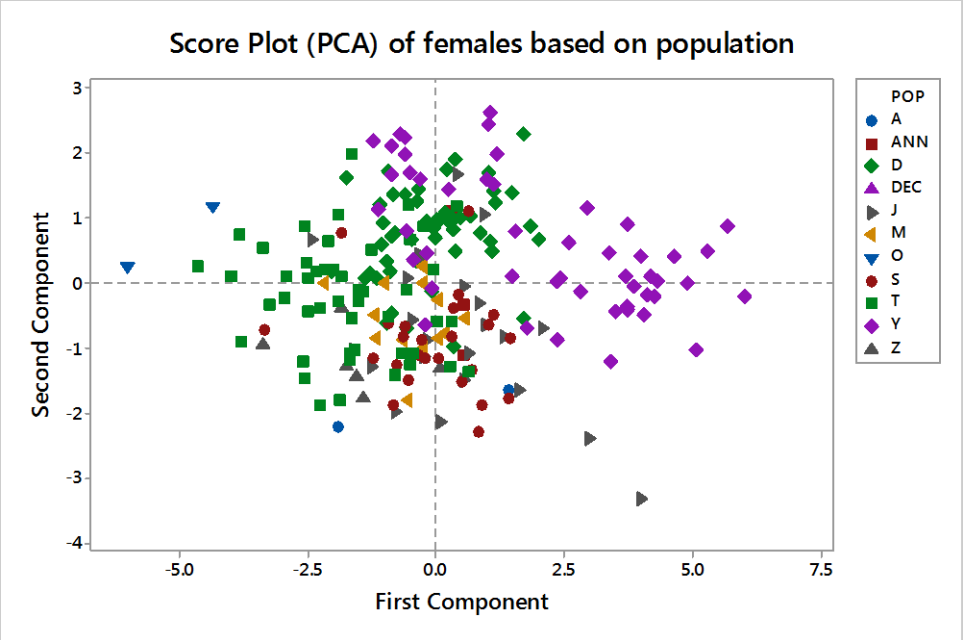


Figure 3.10: Score plot from principal component analysis (PCA) for females based on population. A: South Africa, ANN: *R.(B) annulatus*, D: Deutch, DEC: *R.(B) decoloratus*, J: Jaguar, M: Mozo, O: Australia, S: Sao Gabriel, T: Thailand, Y: Yucatan, Z: Juarez. *R.(B) decoloratus* appears absent in this scatter plot sue to the lack of unfed females available for measuring. Numbers of *R.(B) australis* and *R.(B) annulatus* were also low for the same reason.

The score plot for females based on species yielded a similar result as for population, with the separation of Australian ticks from the other species clearer than in the score plot based on population. No pattern can be clearly observed for the other three species (figure 3.11).

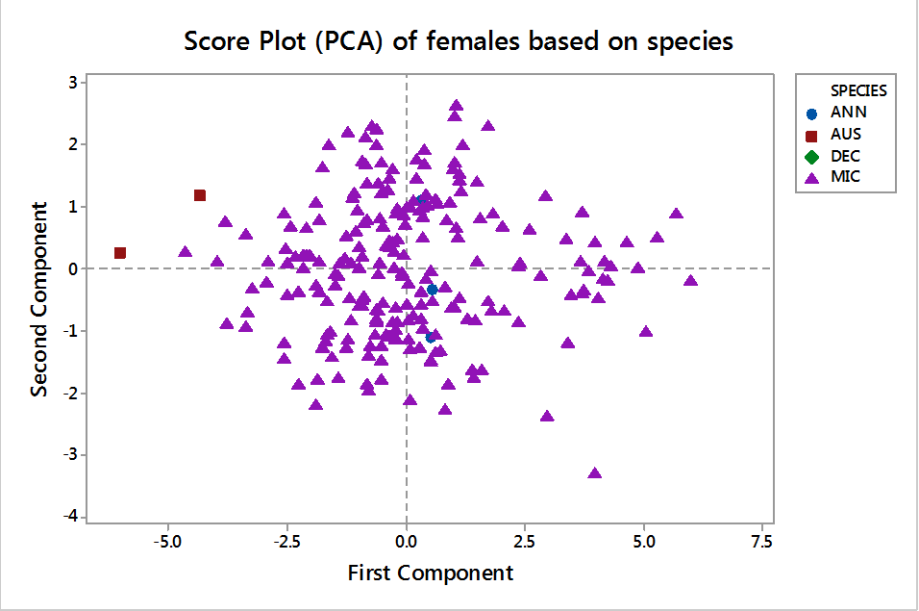
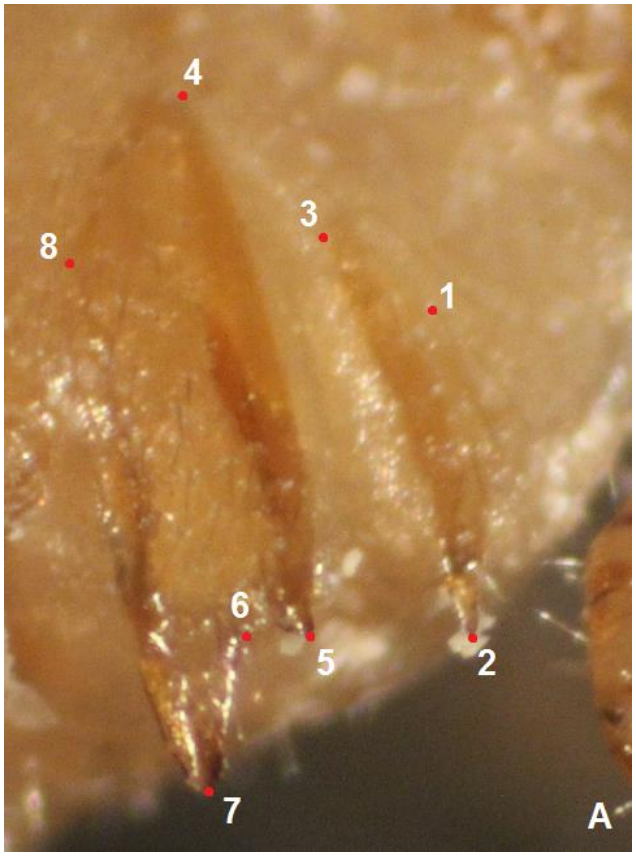


Figure 3.11: Score plot from principal component analysis (PCA) for females based on species. ANN: *R.(B) annulatus*, AUS: *R.(B) australis*, DEC: *R.(B) decoloratus*, MIC: *R.(B) microplus*.

3.3 Differentiation between members of the Boophilid clade based on Geometric morphometric analysis

Materials and methods

Morphological features were selected for this study such that they would not be affected by the feeding status of the specimen. This resulted in concentration on the shape of highly sclerotized areas; coxae, basis capituli, scutum/conscutum and porose area shape in females as well as adanal shield shape in males. Scutal shape was the only feature measured in the larvae, as the boundaries for the other features were not clear enough for accurate land marking. Images were grouped based on feature, and where necessary stage/sex and converted to tps files using tpsUtil64 software so that landmarks could be placed using tpsDIG264 software. Prior to landmarking, scale was set using the S1 stage micrometer with a graticule range of 10mm/0.1mm. Landmarks were selected based on points on the specimen that could be located precisely and consistently from one specimen to another (figure 3.12).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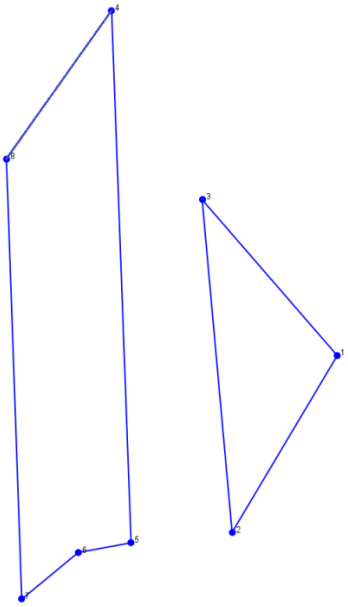

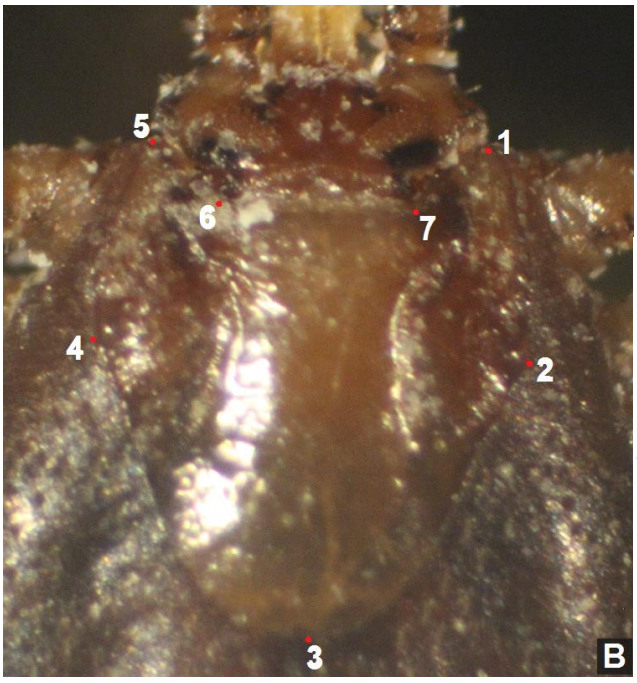
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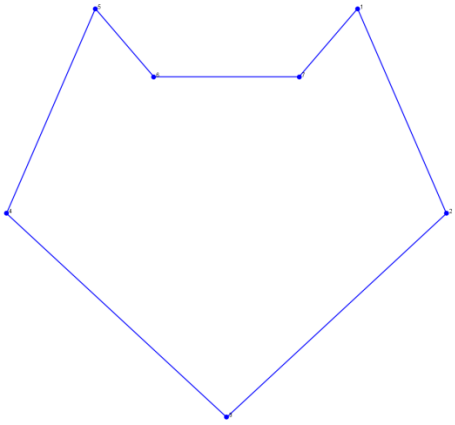


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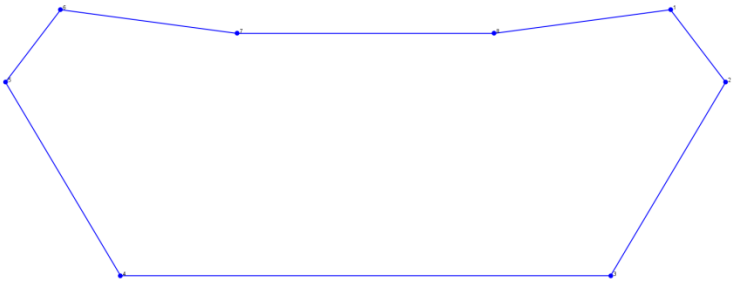

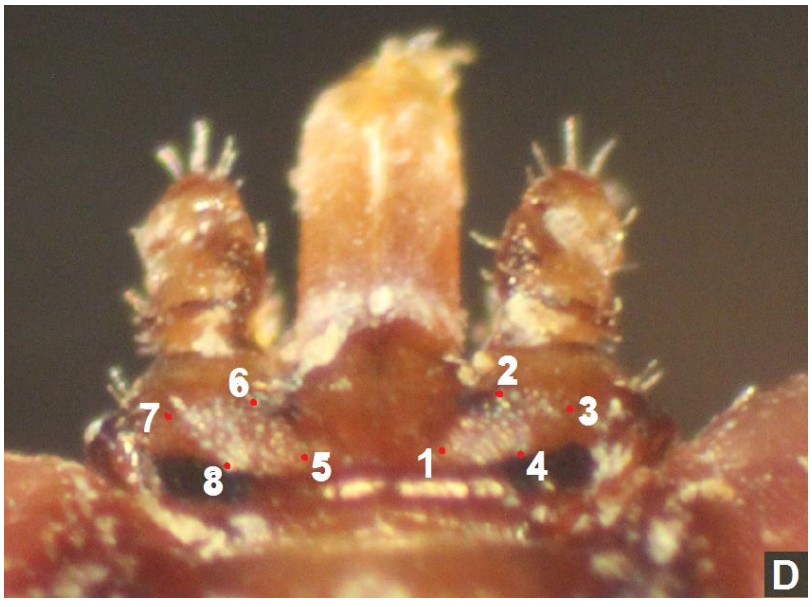
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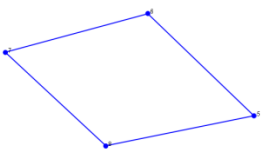
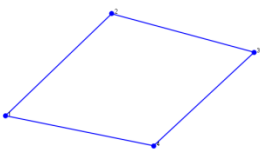




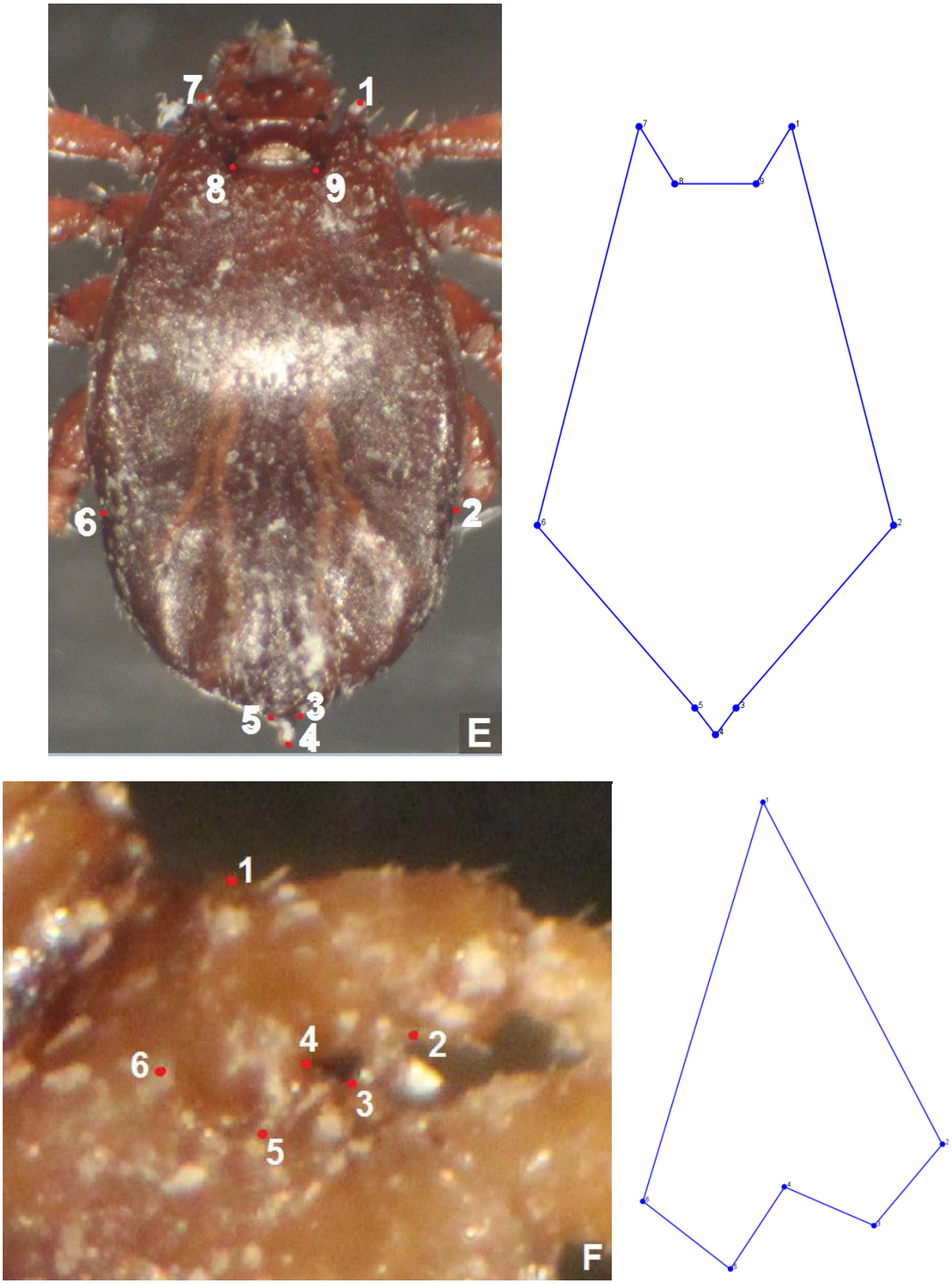


Figure 3.12: Layout of land marking used on the photographs and the standardized wireframe for each morphological feature; A: adanal shield shape (males only); B: scutum shape (females and larvae only); C: basis capituli shape; D: porose shape (females only); E: conscutum shape (males only); F: Coxae I shape. (Source: Authors own photograph).

Statistics

TPS files were imported into MorphoJ 1.06d where a canonical variate analysis (CVA) was carried out with a permutation test. CVA is one of the more common types of analysis used in morphometrics, it can be used to identify features that best differentiate known groups within multiple groups of specimens. From the CVA output, shape change for region and species could be observed. The type of shape change was based on canonical variate (CV) values (the axes along which groups are best discriminated, Zelditch *et al* 2012) with a cumulative value below 90% which resulted in CV1 and CV2 shape change used for all morphological features. Mahalanobis distances among groups were also used to distinguish which groups were most different and least different from one another. Mahalanobis distance is the measure of the distance between a point and the distribution, measuring how many standard deviations away the point is from the mean of the distribution.

Landmark repeatability

A study was carried out in order to determine the level of repeatability when placing the landmark on the specimens. For this study 20 photos were selected from the main data set, duplicated and the converted into two TPS files to be landmarked using tpsDIG264 software. By land marking the same 20 photos twice, it was possible to then run a CVA in MorphoJ. Repeatability was judged on the distance between the two points for each sample, where each point represents the repeated land marking (figure 3.13). Data points that lay closely to one another signified a good level of repeatability, specimens whose data points were found to be far apart where checked for correct landmark positioning. The results for this study indicated that the repeatability of land marking for all features was consistent and could therefore be applied to the main study.

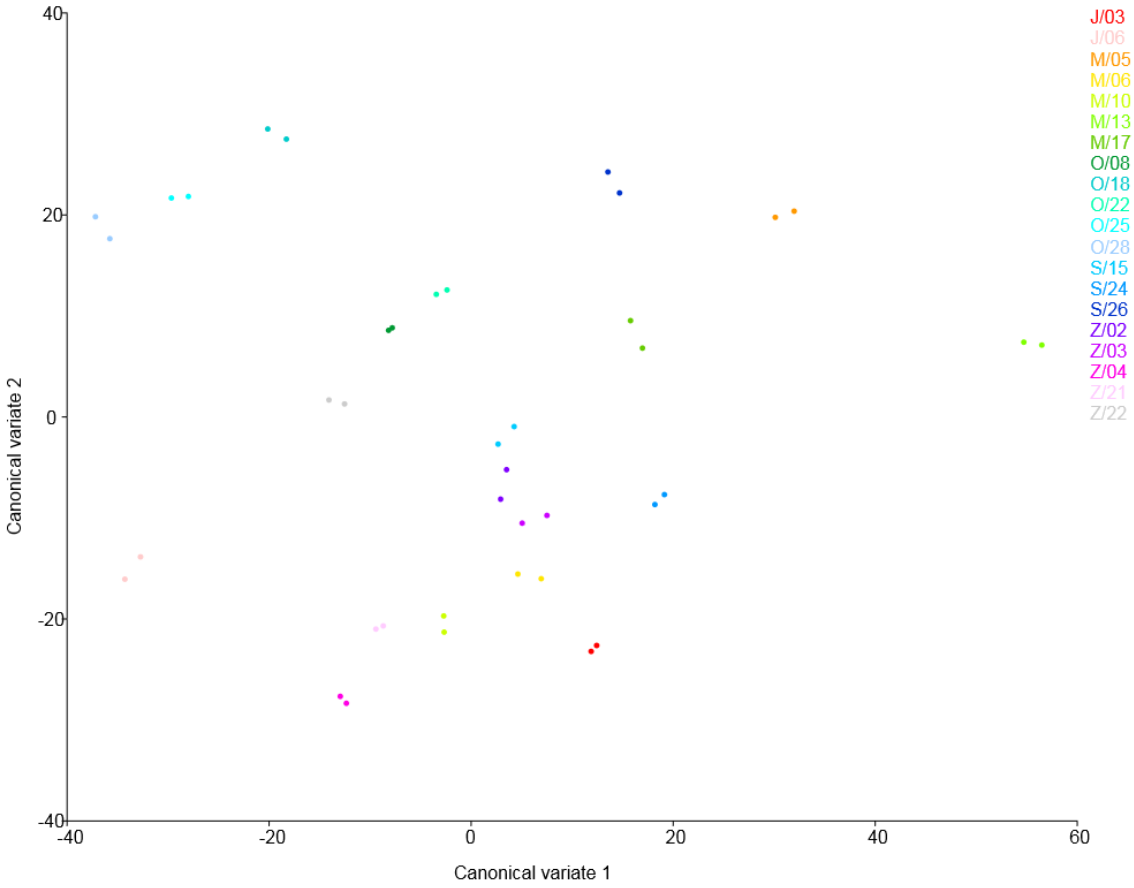


Figure 3.13: Canonical Variate test for adanal shield shape of 20 random males, repeated twice (J= Jaguar, M= Mozo, O= Australia, S= Sao Gabriel, Z= Juarez. Numbers in the key stands for the individual tick). Eigenvalues; CV1= 926.64, CV2= 575.61. % Variance; CV1= 44.63, CV2= 27.72. Cumulative %; CV1= 44.63, CV2= 72.36.

Results for Larvae

Scutum shape

Results from the CVA shows that *R.(B) microplus* groups with *R.(B) australis* and *R.(B) decoloratus*. There is a small amount of grouping seen with *R.(B) annulatus* and *R.(B) microplus* from Thailand (figure 3.14). All populations tended to favor the same shape, ranging from -1-1 in CV1 and -1-1 in CV2.

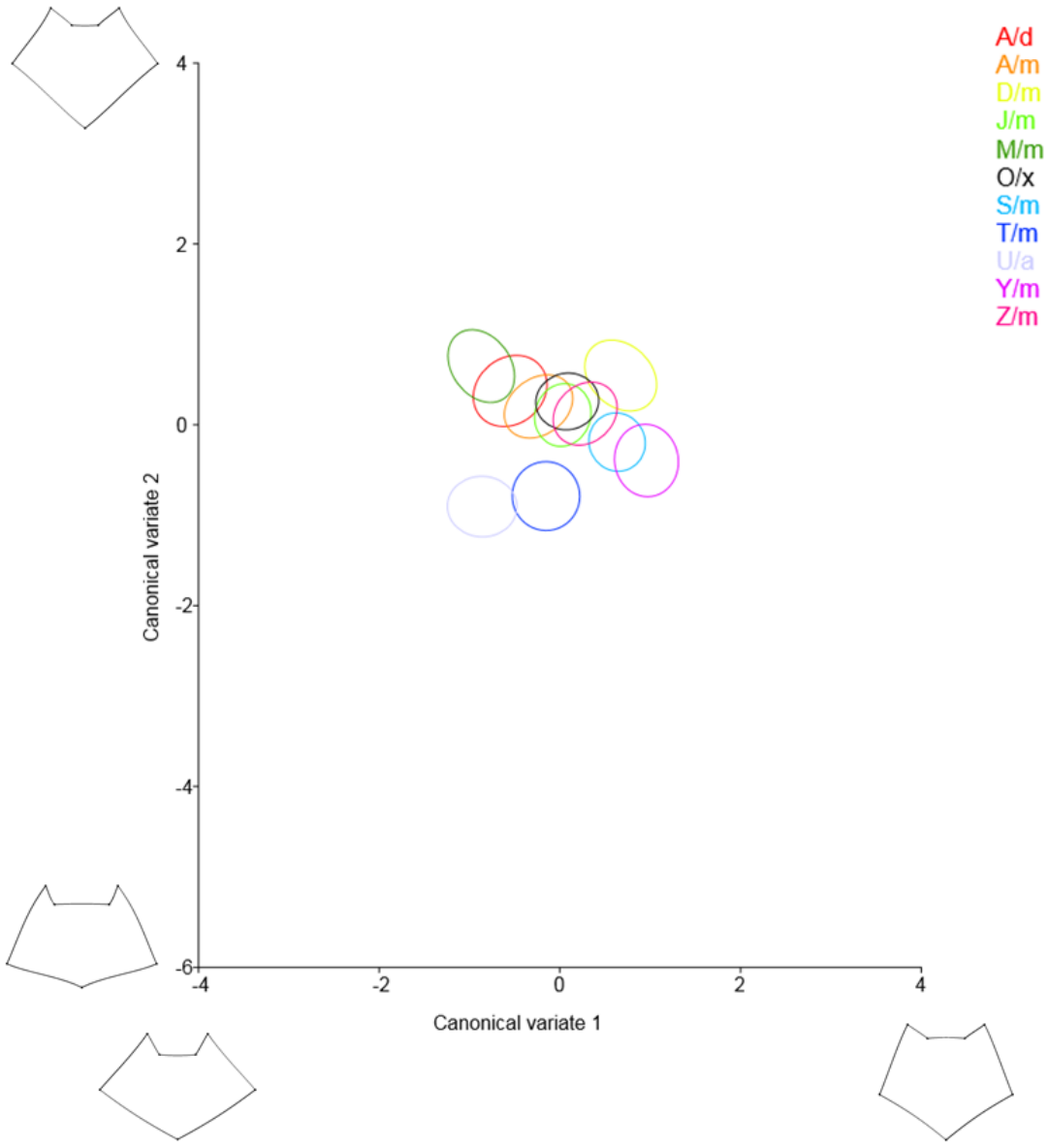


Figure 3.14: Canonical Variate test showing Confidence ellipses for larval scutum shape. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*. Eigenvalues; CV1= 0.34810943, CV2= 0.24357271. % Variance; CV1= 42.026, CV2= 20.406. Cumulative %; CV1= 42.026, CV2= 71.432.

The Mahalanobis distances allowed for further examination of the variation in scutum shape between populations. *R.(B) microplus* ticks from Mozo and Yucatan populations were the most different, closely followed by *R.(B) microplus* from Deutch and *R.(B) annulatus*. Ticks that were the most similar were from Australia and South Africa (table 3.35).

Table 3.35: Mahalanobis distances for larvae scutum shape among groups. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*

	A/d	A/m	D/m	J/m	M/m	O/x	S/m	T/m	U/a	Y/m
A/m	0.8115									
D/m	1.5239	0.9863								
J/m	1.7653	1.101	1.2631							
M/m	1.0998	0.9415	1.6326	1.2954						
O/x	0.8776	**0.5166**	0.8568	1.0678	1.1655					
S/m	1.3553	1.1045	1.0308	1.4882	1.8694	0.7704				
T/m	1.4718	1.0599	1.628	1.2418	1.6349	1.1039	1.1092			
U/a	1.5403	1.2871	2.1238	1.6345	1.589	1.5414	1.7471	0.7483		
Y/m	1.8581	1.3672	1.0359	1.4729	**2.1328**	1.1807	0.6678	1.2216	1.8977	
Z/m	1.483	1.0924	1.1528	0.842	1.3672	0.7787	0.9944	1.1769	1.7192	1.157

Results for Males

Conscutum shape

All populations of *R.(B) microplus* showed a degree of overlapping for shape change in both CV1 and CV2, grouping mainly towards the larger CV values. *R.(B) annulatus*, grouped away from the rest of the populations in CV1, favoring shape change towards -4, translating to a conscutum with an absent caudal appendage. *R.(B) decoloratus* also grouped away from the other populations in CV2, favoring shape change towards -2, thus indicating that *R.(B) decoloratus* males have a shorter, wider conscutum then the other species (figure 3.15). The largest amount of variation in shape can be observed in *R.(B) microplus* ticks from the Jaguar population in CV2 (figure 3.15). The rest of the populations, including

R.(B) decoloratus and *R.(B) annulatus* showed a small, but variable range of shape change, with *R.(B) annulatus* showing the least.

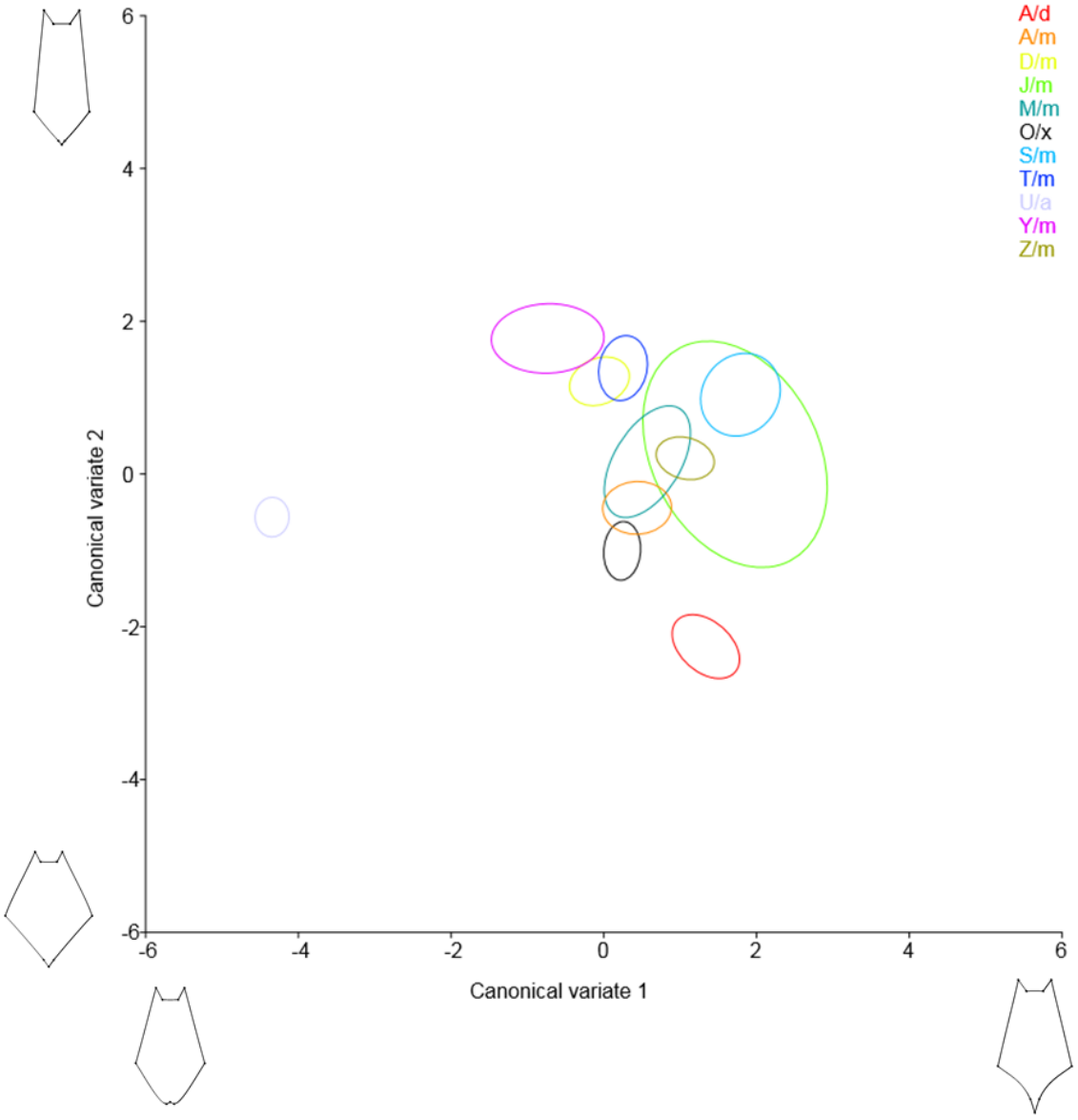


Figure 3.15: Canonical Variate test showing Confidence ellipses for male conscutum shape. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*. Eigenvalues; CV1= 2.98999232, CV2= 1.48942631. % Variance; CV1= 47.902, CV2= 23.862. Cumulative %; CV1= 47.902, CV2= 71.764.

The Mahalanobis distances allowed for further examination of the variation in conscutum shape between populations. *R.(B) microplus* from the Sao Gabriel population had the most different conscutum shape to *R.(B) annulatus.* The

populations that had the most similar conscutum shape were Mozo and Juarez (table 3.36).

Table 3.36: Mahalanobis distances for male conscutum shape among groups. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*

	A/d	A/m	D/m	J/m	M/m	O/x	S/m	T/m	U/a	Y/m
A/m	3.346									
D/m	3.8608	2.9442								
J/m	3.311	1.9913	2.9468							
M/m	3.1887	1.3707	2.0301	1.6978						
O/x	4.1962	1.9387	3.9038	3.411	2.506					
S/m	4.2271	2.4714	3.0484	1.3205	2.1301	3.7087				
T/m	4.2701	2.1755	1.7598	2.4199	1.6436	3.2915	2.6458			
U/a	6.1741	4.8965	4.9073	6.1713	5.0202	5.2481	**6.4489**	5.1046		
Y/m	4.6622	3.1269	1.0901	3.3188	2.311	4.0627	3.318	1.6018	4.4291	
Z/m	3.3845	1.1276	2.68	1.2914	**1.0774**	2.7521	1.8165	1.7644	5.5187	2.8975

Coxal shape

All populations, apart from *R.(B) decoloratus* shared a coxa shape favoring 0-2 in CV1 and -2-2 in CV2. *R.(B) decoloratus* clearly grouped away from rest of the populations in CV1, favoring a range of shape from -5- -6, meaning that male *R.(B) decoloratus* have wider, shorter coxa I, with a longer internal coxal spur then the other species (figure 3.16). The largest amount of variation in shape can be observed in *R.(B) microplus* ticks from the Jaguar population in both CV1 and CV2 (figure 3.16). The rest of the populations, including *R.(B) decoloratus* and *R.(B) annulatus* showed far smaller ranges in shape.

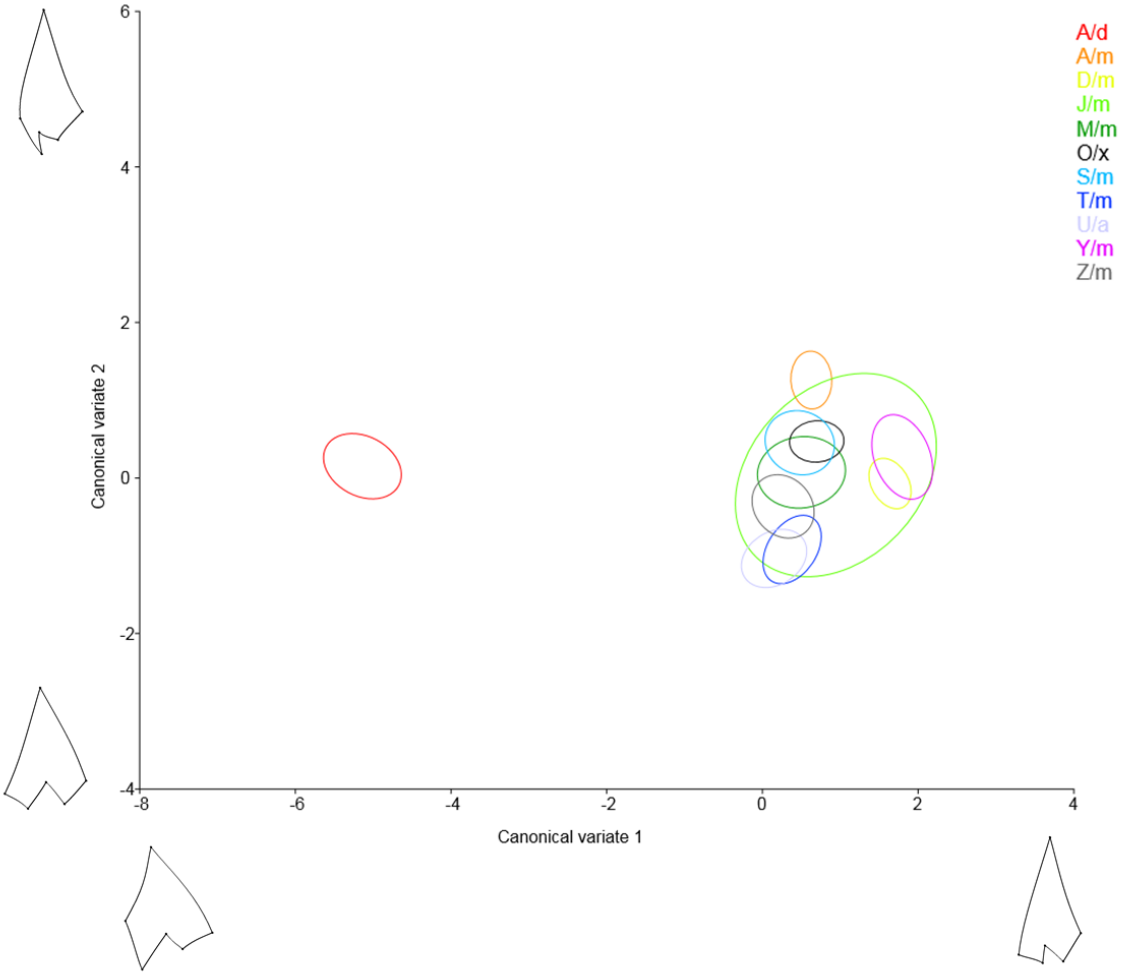


Figure 3.16: Canonical Variate test showing Confidence ellipses for male coxal shape. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*. Eigenvalues; CV1= 3.94136315, CV2= 0.48952585. % Variance; CV1= 76.209, CV2= 9.465. Cumulative %; CV1= 76.209, CV2= 85.674.

Based on the Mahalanobis distances, *R.(B) microplus* from Yucatan had the most different coxa shape to *R.(B) decoloratus.* The populations that had the most similar coxal shape were *R.(B) microplus* from Deutch and Jaguar populations (table 3.37).

Table 3.37: Mahalanobis distances for male coxal shape among groups. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*

	A/d	A/m	D/m	J/m	M/m	O/x	S/m	T/m	U/a	Y/m
A/m	5.9325									
D/m	6.799	1.9547								
J/m	6.1947	1.989	**1.0595**							
M/m	5.7775	2.3506	1.7018	1.408						
O/x	5.9266	1.7091	1.7658	1.9469	1.276					
S/m	5.6651	1.1172	1.5565	1.5947	1.8163	1.4264				
T/m	5.6777	2.4756	1.8085	2.0023	2.0483	2.0347	1.5749			
U/a	5.5701	2.5365	2.3775	2.3498	2.3627	2.0783	2.0799	1.8775		
Y/m	**7.0099**	2.1861	1.4963	2.1852	2.1176	1.6178	1.8455	2.1538	2.7428	
Z/m	5.4673	1.9173	1.4689	1.2435	1.5349	1.7766	1.117	1.2142	1.6904	2.291

Basis capituli shape

As with conscutum shape, the shape of the basis capituli varied more from *R.(B) annulatus* and *R.(B) decoloratus*. *R.(B) annulatus* differed most in CV2, favoring a wider base to *R.(B) microplus* and *R.(B) decoloratus*. *R.(B) decoloratus* favored CV1 values around 4, resulting in a wider then long basis capituli shape. The largest amount of variation in shape can be observed in *R.(B) microplus* ticks from Jaguar in CV1 (figure 3.17). The rest of the populations, including *R.(B) decoloratus* and *R.(B) annulatus* showed far smaller ranges in shape.

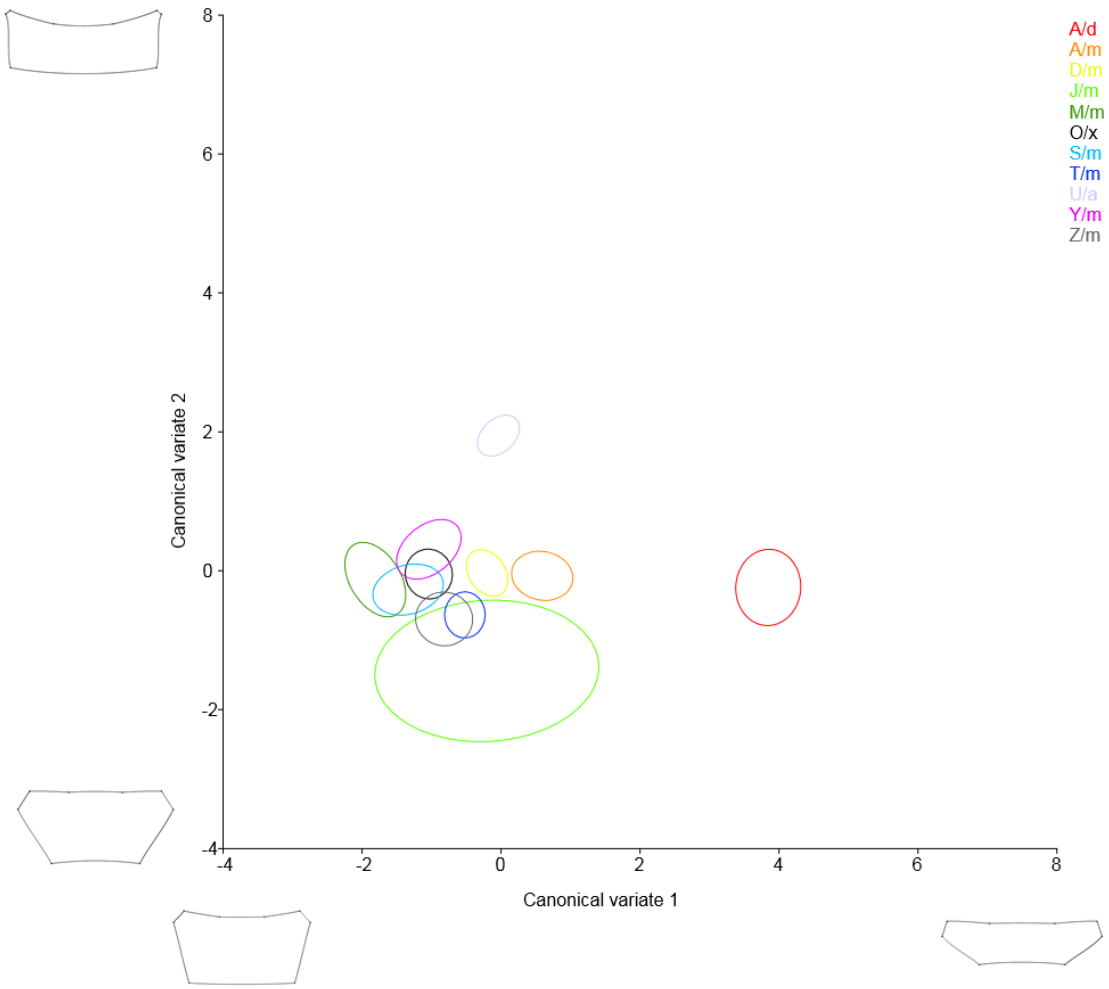


Figure 3.17: Canonical Variate test showing Confidence ellipses for male basis capituli shape. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*. Eigenvalues; CV1= 2.45102814, CV2= 0.62212544. % Variance; CV1= 64.035, CV2= 16.254. Cumulative %; CV1= 64.035, CV2= 80.289.

Mahalanobis distances showed that *R.(B) decoloratus* was highly different from all other populations, in particular it was most different from *R.(B) microplus* ticks from Mozo. The most similar basis capituli shape was found on ticks from the Australian and Yucatan populations (Table 3.38).

Table 3.38: Mahalanobis distances for male basis capituli shape among groups. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*

	A/d	A/m	D/m	J/m	M/m	O/x	S/m	T/m	U/a	Y/m
A/m	3.41									
D/m	4.3702	2.0541								
J/m	4.3335	1.9241	2.2223							
M/m	**5.729**	2.5825	2.7739	2.6325						
O/x	4.924	2.221	2.0148	2.026	1.4693					
S/m	5.229	2.0764	2.4142	1.932	0.9035	1.2042				
T/m	4.589	1.7126	1.4695	1.1483	2.3716	1.8382	1.7503			
U/a	4.4899	2.2506	2.5144	3.4326	2.9728	2.4461	2.6579	2.7758		
Y/m	4.9424	2.0319	1.5278	2.2655	1.4411	**0.8339**	1.2956	1.6753	2.0731	
Z/m	4.7269	1.9755	2.2056	1.6248	1.4743	1.1827	0.9209	1.7918	2.8921	1.4428

Adanal shield shape

Ticks from Mozo, Sao Gabriel, Juarez and Jaguar all had very similar adanal shield shape in both CV1 and 2. Ticks from Australian and *R.(B) microplus* from South Africa were found to have the same adanal shield shape in CV1 but slightly different in CV2. *R.(B) decoloratus* were found to have a completely different adanal shield shape in CV1 to the rest of the populations, favoring longer adanal shields with more pronounced spurs. The rest of the populations including *R.(B) annulatus*, were very similar in shape in CV1 but slightly different in CV2, ranging between 0-3 (figure 3.18). As with the other features, the population that had the greatest range of adanal shield shape was Jaguar in CV2.

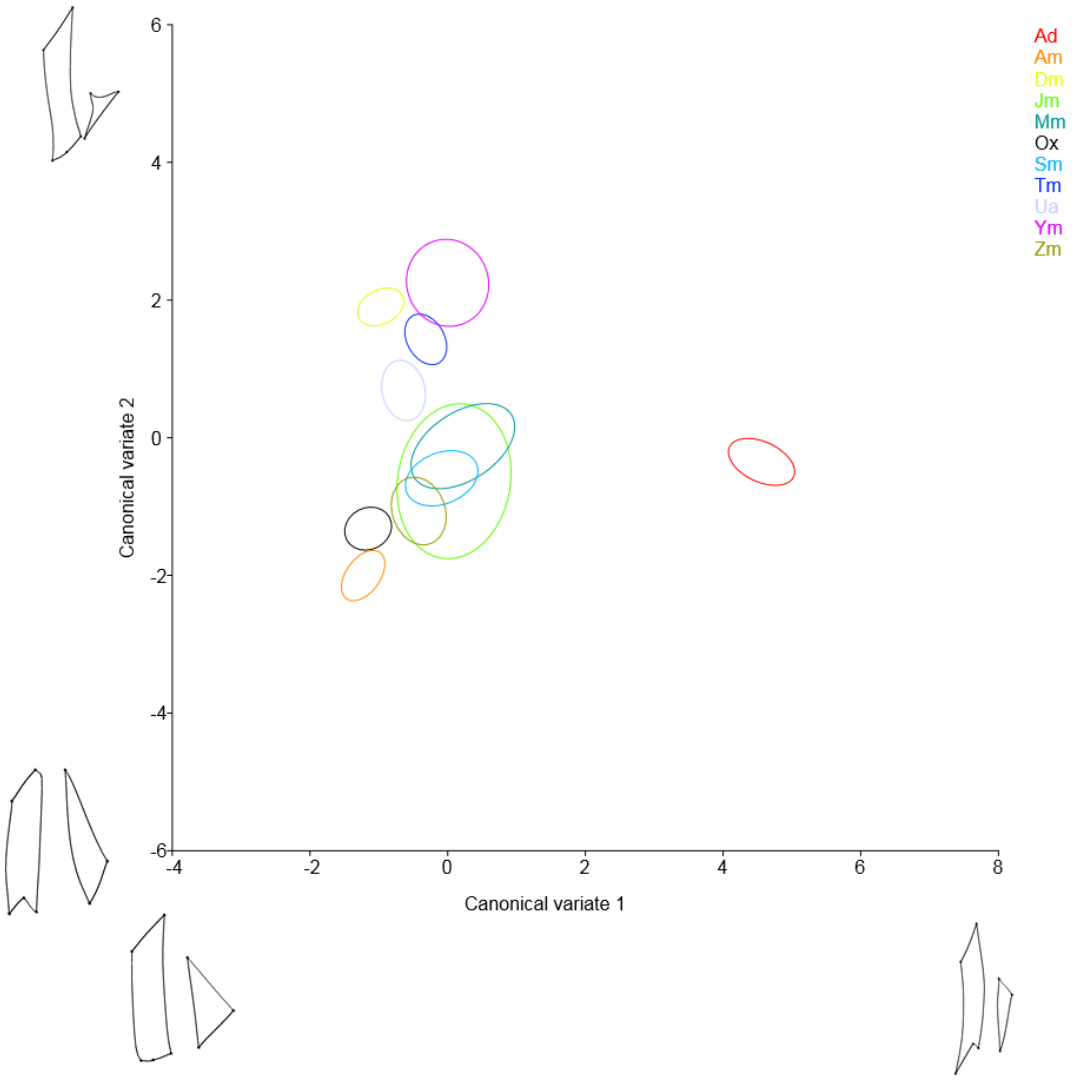


Figure 3.18: Canonical Variate test showing Confidence ellipses for male adanal shield shape. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*. Eigenvalues; CV1= 3.10078518, CV2= 1.88715615. % Variance; CV1= 46.994, CV2= 28.601. Cumulative %; CV1= 46.994, CV2= 75.595.

Populations that shared the most similar adanal shield shape were Mozo and Sao Gabriel. *R.(B) decoloratus* had adanal shields that were highly different to all other populations, however they were the most different from *R.(B) microplus* from Deutch (table 3.39).

Table 3.39: Mahalanobis distances for male adanal shield shape among groups. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*

	A/d	A/m	D/m	J/m	M/m	O/x	S/m	T/m	U/a	Y/m
A/m	6.0854									
D/m	**6.109**	4.1301								
J/m	4.9553	2.7885	3.6167							
M/m	4.6037	3.2411	3.245	2.3571						
O/x	5.8575	1.7583	3.6625	2.8458	2.2611					
S/m	4.818	2.3883	3.4026	2.1066	**1.5746**	1.9198				
T/m	5.2979	3.7814	2.2797	2.9415	2.2362	3.1402	2.2257			
U/a	5.6848	3.8905	3.3628	3.6019	2.2957	2.8478	2.7464	2.3581		
Y/m	5.5555	4.6736	2.8153	3.8984	3.6682	4.3637	3.2282	1.7365	3.4643	
Z/m	5.2074	2.0501	3.6913	2.6561	2.2923	2.0244	1.5806	2.8946	2.8108	3.7742

Results for Females

Scutum shape

Most populations were found to overlap, ranging -2-1 in both CV1 and CV2. Outlying populations, were *R.(B) annulatus* and Juarez, which favored a longer scutum (CV2) and *R.(B) annulatus* had longer anterolateral projections (CV1). Australia and Deutch varied most in CV1; favoring a wider scutum with longer anterolateral projections (figure 3.19).

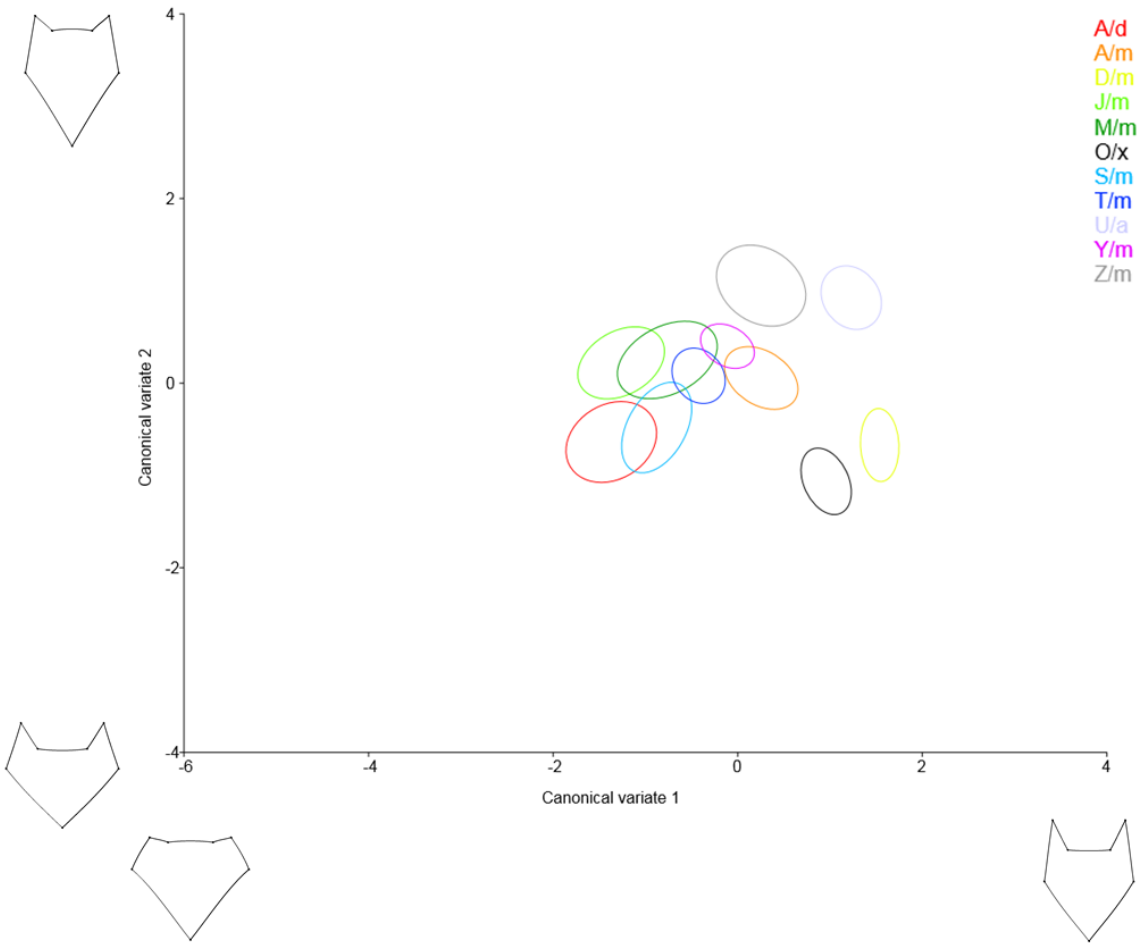


Figure 3.19: Canonical Variate test showing Confidence ellipses for female scutum shape. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*. Eigenvalues; CV1= 0.93364561, CV2= 0.42342295. % Variance; CV1= 54.045, CV2= 24.51. Cumulative %; CV1= 54.045, CV2= 78.555.

Scutum shape of *R.(B) decoloratus* varied the most from and *R.(B) annulatus.* The most similar scutum shapes were found in Jaguar and Mozo (table 3.38).

Table 3.40: Mahalanobis distances for female scutum shape among groups. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*

	A/d	A/m	D/m	J/m	M/m	O/x	S/m	T/m	U/a	Y/m
A/m	2.0099									
D/m	3.0128	1.9833								
J/m	1.1341	1.9684	2.9752							
M/m	1.2972	1.5677	2.5049	**0.5215**						
O/x	2.4834	1.776	1.0473	2.5881	2.2104					
S/m	0.7746	1.3665	2.5926	1.0841	1.018	2.0331				
T/m	1.2198	1.1249	2.1744	1.0027	0.613	1.8989	0.8592			
U/a	**3.0777**	1.6116	2.0754	2.8311	2.3997	2.2482	2.6352	2.0026		
Y/m	1.7253	1.5302	2.136	1.3424	0.9996	1.9267	1.5064	0.7853	1.672	
Z/m	2.6935	1.8377	2.2627	1.8539	1.4324	2.4036	2.2355	1.5918	2.0018	1.5017

Porose shape

Porose shape was very similar for most of the populations, with most grouping and overlapping in the middle range for both CV1 and CV2. However, three clear outlier groups can be observed (figure 3.25), *R.(B) annulatus* is divergent from the rest of the populations in CV1, favoring wider porose, that are angled down. The other two divergent groups were *R.(B) decoloratus* and Juarez, both divergent in CV1, *R.(B) decoloratus* more so (higher CV1 value) favoring narrower shaped porose areas (figure 3.20).

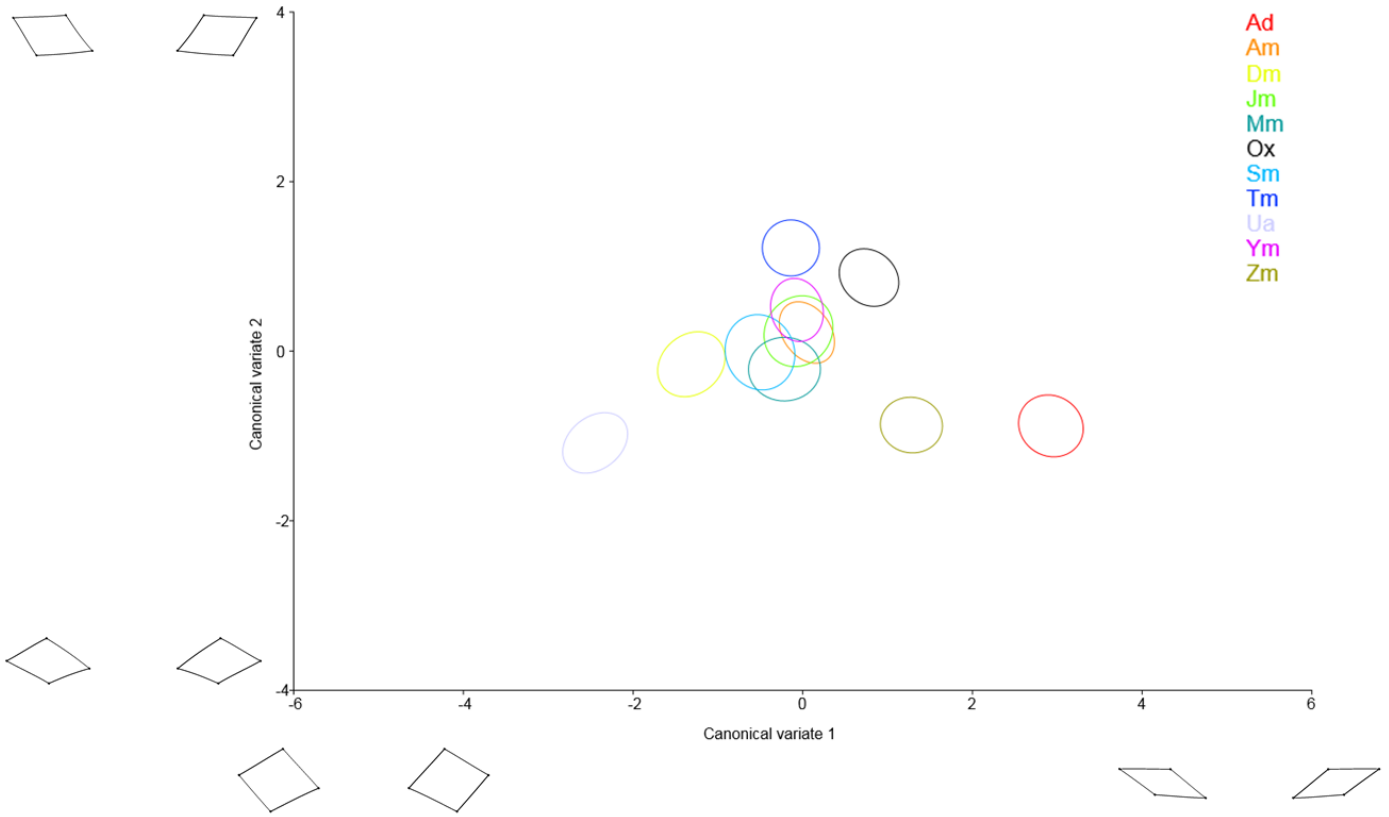


Figure 3.20: Canonical Variate test showing Confidence ellipses for female porose shape. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*. Eigenvalues; CV1= 1.76019546, CV2= 0.50792563. % Variance; CV1= 68.039, CV2= 19.633. Cumulative %; CV1= 68.039, CV2= 87.672.

R.(B) decoloratus had the most different porose area shape from the rest of the populations, the greatest difference was seen from *R.(B) annulatus* (table 3.41). Ticks from South Africa had the most similar porose area shape to ticks from Jaguar.

Table 3.41: Mahalanobis distances for female porose shape among groups. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*

	A/d	A/m	D/m	J/m	M/m	O/x	S/m	T/m	U/a	Y/m
A/m	3.1018									
D/m	**4.377**	1.5658								
J/m	3.2466	**0.6152**	1.8342							
M/m	3.2588	0.8053	1.5248	0.8858						
O/x	2.8403	1.0817	2.3819	1.4642	1.7222					
S/m	3.666	1.042	1.2689	1.3467	1.2038	1.6409				
T/m	3.7426	1.1548	1.8887	1.4249	1.5668	1.172	1.6633			
U/a	5.379	2.835	1.6095	2.8423	2.4612	3.8073	2.3893	3.2734		
Y/m	3.3916	0.9228	1.4876	1.443	1.2109	1.1837	1.2891	0.8443	2.9281	
Z/m	2.0135	1.9454	2.7263	2.382	2.0773	1.9586	2.2587	2.658	3.8655	1.9945

Coxal shape

Coxal shape for the females appeared to be very similar for all populations, except *R.(B) annulatus*, which differed in CV1, favoring a coxal shape absent of spurs and concave on the medial aspect (figure 3.21). The range of coxal shape varied between the different populations; however, Juarez showed the greatest amount of variation in shape in CV1.

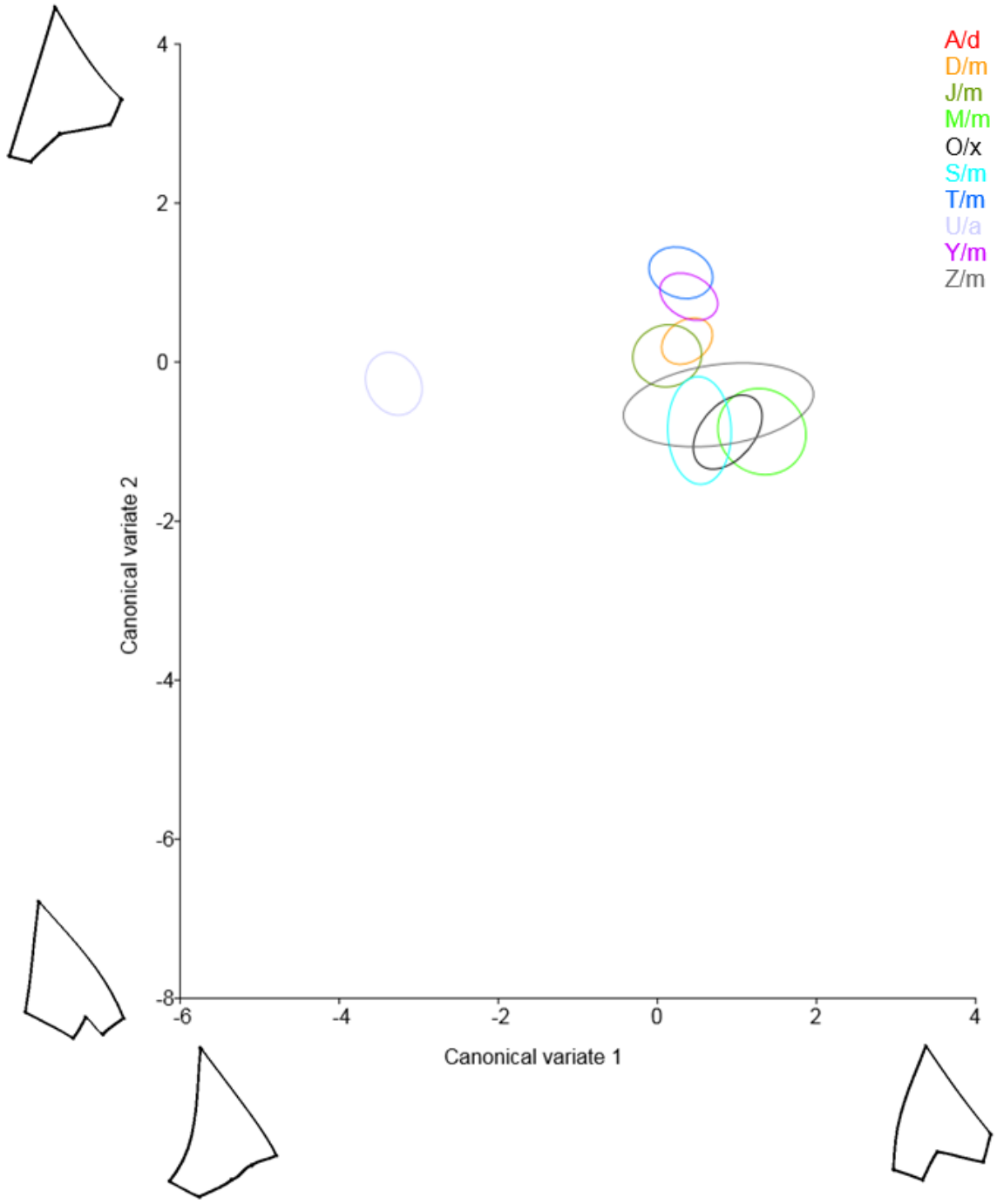


Figure 3.21: Canonical Variate test showing Confidence ellipses for female coxal shape. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*. Eigenvalues; CV1= 1.83887515, CV2= 0.70037619. % Variance; CV1= 53.191, CV2= 20.259. Cumulative %; CV1= 53.191, CV2= 73.449.

R.(B) decoloratus had the most different coxa shape, the greatest difference was observed from ticks from Thailand (table 3.42). The most similar coxa shape was seen between ticks from Thailand and Yucatan.

Table 3.42: Mahalanobis distances for female coxal shape among groups. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*

	A/d	D/m	J/m	M/m	O/x	S/m	T/m	U/a	Y/m
D/m	6.9056								
J/m	7.2556	1.5809							
M/m	6.5377	1.951	2.1516						
O/x	6.6445	2.4185	2.2892	1.3237					
S/m	5.9893	1.2261	1.9628	1.6148	2.0069				
T/m	**7.4328**	1.5559	1.9275	2.4851	2.4523	2.2833			
U/a	7.3375	3.8501	3.7108	4.7169	4.3932	4.0003	3.9129		
Y/m	7.2018	1.2862	1.9774	2.2012	2.1886	2.0224	**0.9002**	3.9155	
Z/m	5.9338	1.9845	1.8217	2.3535	2.5296	1.6975	2.3201	4.4453	2.3532

Basis capituli shape

The shape of the basis capituli was quite variable among populations (figure 3.22). Ticks from Yucatan and Deutch tended to share similar variations in shape. Ticks from Juarez, Jaguar, Mozo, Sao Gabriel and *R.(B) annulatus*, also shared similar basis capituli shape. The two populations that showed the most different basis capituli shape were *R.(B) decoloratus* and Thailand in CV1. *R.(B) decoloratus* tended to have a shorter basis capituli, with a more concave anterior border (lower CV1 values).

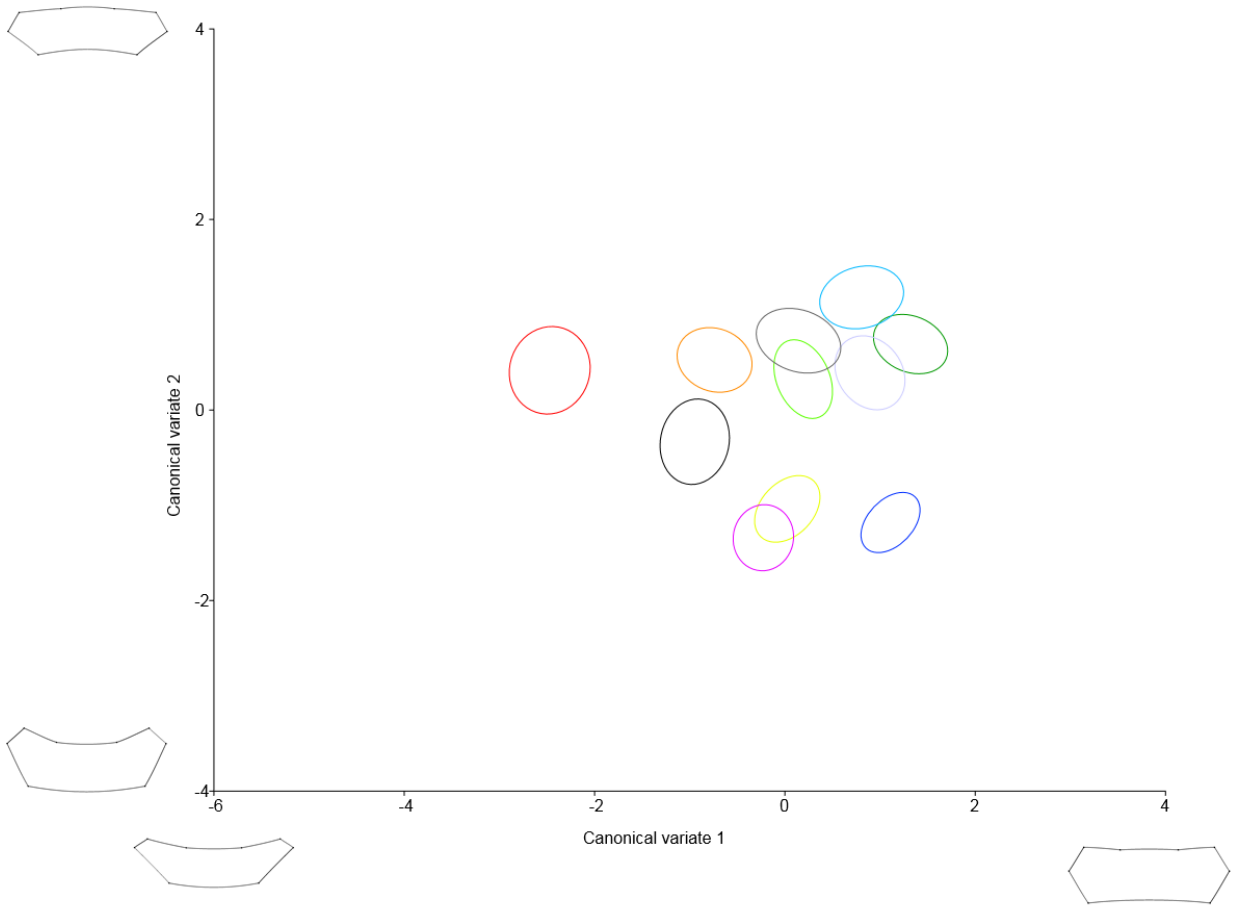


Figure 3.22: Canonical Variate test showing Confidence ellipses for female basis capituli shape. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*. Eigenvalues; CV1= 1.09497455, CV2= 0.70030563. % Variance; CV1= 43.483, CV2= 27.81. Cumulative %; CV1= 43.483, CV2= 71.292.

R.(B) decoloratus basis capituli shape was most different from Mozo, however they were also highly different from Sao Gabriel, Thailand, *R.(B) annulatus* and Yucatan populations (table 3.43). Ticks with the most similar basis capituli shape were from Jaguar and *R.(B) annulatus*.

Table 3.43: Mahalanobis distances for female basis capituli shape among groups. T/m= Thailand *R.(B) microplus*, A/m= South African *R.(B) microplus*, D/m= Deutch *R.(B) microplus*, Y/m=Yucatan *R.(B) microplus* J/m= Jaguar *R.(B) microplus*, M/m= Mozo *R.(B) microplus*, O/x= Australia *R.(B) australis*, S/m= Sao Gabriel *R.(B) microplus*, Z/m= Juarez *R.(B) microplus*, A/d= South African *R.(B) decoloratus*, U/a= North America *R.(B) annulatus*

	A/d	A/m	D/m	J/m	M/m	O/x	S/m	T/m	U/a	Y/m
A/m	2.1554									
D/m	2.9769	2.0288								
J/m	2.8079	1.0903	1.516							
M/m	**4.0266**	2.9047	2.7966	2.0649						
O/x	2.4294	2.0565	2.3488	2.138	2.6366					
S/m	3.4441	2.1778	2.5129	1.4303	1.1825	2.6555				
T/m	4.0129	2.6057	1.439	1.87	2.6492	2.9351	2.7087			
U/a	3.4285	1.7915	1.8356	**0.8711**	1.5351	2.4807	1.1249	1.6918		
Y/m	3.2136	2.7899	1.7907	2.4193	2.6226	1.5106	2.8653	2.3995	2.5205	
Z/m	2.7903	1.5088	2.0836	1.0683	1.5803	1.8766	0.9819	2.4556	1.0359	2.2761

Discussion

The first study within which a score matrix was implemented, *R.(B) annulatus* and *R.(B) decoloratus* were unambiguously differentiated from each other as well as from *R.(B) microplus* and *R.(B) australis* for females. This finding contradicted the reports that state due to the amount of variation seen in the morphology and the lack of points for comparison, differentiation between these Boophilid ticks is often inconsistent (Uilenberg, 1962, Lempereur *et al*., 2010, Barker and Walker, 2014). Males were mostly assigned unambiguously, with the exception of three ticks from the Yucatan population which were assigned to ‘mixed’, due to their lack of caudal appendage, a feature found on *R.(B) microplus* and not on *R.(B) annulatus* males. The combination of features would imply one of three things: 1) the emergence of a new variant, 2) hybridization, 3) a distinct species. *Rhipicephalus (Boophilus) annulatus* and *R.(B) microplus* are known to form hybrids (Osburn and Knipling, 1982) the morphology of which has not been previously described, but can be

reasonably deduced to contain a mixture of features from both species. However, the potential for hybridization between the Yucatan and *R.(B) annulatus* populations may be possible, it is highly unlikely to have occurred in the populations used in this study, as both sets of specimens have come from lab colonies originating from different locations (table 2.1).

In the case of *R.(B) australis* and *R.(B) microplus*, clear differentiation for both the males and females was inconsistent. Among females, the Australian population was assigned almost evenly to *R.(B) australis* and *R.(B) microplus*, with 8 individuals showing a mixture of features. This finding would support published claims that both species may be present in Australia (Estrada-Peña *et al*., 2012). The majority of the ticks in the rest of the regions were assigned to *R.(B) microplus*, which is to be expected as *R.(B) australis* is currently proposed to be in Australia, New Caledonia, Sumatra and Java (Indonesia), Cambodia, Philippines, Tahiti, Papua New Guinea and elsewhere in Asia (Barker and Walker, 2014). However, only females from Mozo were assigned exclusively to *R.(B) microplus.* The remaining regions had a number of ticks with mixed features and 6 ticks in South Africa, as well as 4 from Juarez were assigned to *R.(B) australis*. With regard to the males an entirely different pattern emerges. Most male ticks from all geographical locations were assigned to either *R.(B) australis* or had a mixture of both species' features, with a surprisingly small number scoring as *R.(B) microplus*. In males, the ventral spur of palp article i proved to be present not only in proposed *R.(B) australis*, but in individuals from most populations.

The observations suggest the majority of females to be *R.(B) microplus* and the majority of males to be *R.(B) australis*. This is clearly nonsensical. Hybridization of *R.(B) australis* and *R.(B) microplus*, could be potentially occurring in regions such as Thailand, where there is the potential presence of both populations (Barker and Walker, 2014). However, this should not be occurring in the regions where *R.(B) australis* is not reported. This therefore questions the validity of *R.(B) australis* as a morphologically distinct species on the basis of the previously published criteria. According to the criteria outlined by Estrada-Peña *et al*. (2012), the ticks sampled from Thailand would be classified as hybrids of *R.(B) australis* and *R.(B) microplus*.

The morphometric analysis showed a high degree of among-population variation for most of the traits measured and for almost every trait there were differences observed among different populations. This once again confirms the inconsistencies previously observed in the Boophilid ticks (Uilenberg, 1962, Lempereur *et al*., 2010, Barker and Walker, 2014). There was also more variation observed between populations that were supposed to be *R.(B) microplus* than there were between *R.(B) microplus* and the rest of the species covered.

For the larvae, the Fisher Pairwise comparison showed Australian ticks trended towards a smaller body length, idiosoma length and scutum width. The findings for a smaller body length and narrower scutum width were consistent with those proposed in the re-description of *R.(B) australis* (Estrada-Peña *et al*., 2012). For the rest of the morphological features, there was no obvious trend between the different populations or species. Different features were found to be suitable to differentiate between populations, in PC1 the strongest feature was scutum length and hypostome length. In PC2 the strongest feature was idiosoma length and in PC3 it was body length and hypostome length. The PCA didn't yield conclusive evidence that *R.(B) australis* should be considered as a separate species from *R.(B) microplus*, and there was no obvious differentiation of any of the species at all on a morphometric basis, even for *R.(B) decoloratus* and *R.(B) annulatus*.

For the males, the Fisher Pairwise comparison showed that for the majority of the features tested, Australian males were significantly different, trending toward the smaller means. This result was also supported by the score plot from the PCA, where clear grouping of the Australian ticks can be observed when males are plotted based on species (figure 3.8). As with the larvae, no patterns were seen in the other species, with *R.(B) decoloratus* and *R.(B) annulatus* grouping with *R.(B) microplus*. Features that were found to be strongest for differentiating between populations differed between PC1 and PC2. In PC1 both palpal lengths is proposed to be the strongest feature for differentiation and in PC2 the length of the ventral basis capituli.

The females yielded a mixed result. There was no real trend in the size of Australian ticks observed from the Fisher Pairwise comparison. However, *R.(B) decoloratus* tended to be smaller for most of the morphological features tested and *R.(B) annulatus* tended to be larger. This observation was not consistent with the

results from the PCA, as grouping of Australian ticks can be seen (figure 3.10). For differentiating between populations, both palpal lengths, width of the basis capituli and the length of the dorsal basis capituli were the strongest. In PC2 the length of the ventral basis capituli was the strongest feature for differentiating populations.

The geometric morphometric analysis also did not clearly and consistently enable the differentiation of any of the sample populations of ticks in this study. Each feature differed among samples in different sets of pairwise relationships. It's success in previous studies (Pretorius and Clarke 2000; Pretorius and Clarke 2001; Clarke and Pretorius 2005) indicated its potential as an approach, however based on the findings here, the lack of consistent morphological difference previously observed for the Boophilid ticks is once again confirmed (Barker and Walker, 2014, Uilenburg, 1962, Lempereur *et al*., 2010). With this three-way approach to understanding the morphological differences of the Boophilid ticks covered in this work we can draw the following conclusions. *R.(B) australis* and *R.(B) microplus* larvae and males are distinguishable from one another with regard to morphometrics, and the differences observed here are supportive of the previous findings of Estrada-Peña *et al*. (2012). The ventral spur on palp article i on males, which was reported by Estrada-Peña *et al*. (2012) is present, but inconsistantly, and is not limited to the Australian population. Females are indistinguishable from one another based on setae patterns, linear measurements and shape. This inconsistency in the resolution of the taxonomic status of *R.(B) microplus* confirms the claims of Barker and Walker (2014), however we have found no evidence that the geographical location impacts upon the ability to differentiate between *R.(B) microplus* and *R.(B) australis*.

Conclusion

The most consistent grouping from other populations was observed by *R.(B) decoloratus* across all three analyses, despite reports of difficulty in distinguishing between the Boophilid ticks (Uilenburg, 1962, Lempereur *et al*., 2010). We confirm that there is a vast amount of variation in the morphology of the Boophilid ticks covered in this work, and this appears to impact on the consistency and success of differentiation of the other three species *R.(B) microplus, R.(B) australis* and *R.(B) annulatus*. *Rhipicephalus (Boophilus) annulatus*, although

unambiguously differentiated from *R.(B) microplus* and *R.(B) australis* in the first analysis, was found to share similar morphology in the other two analyses. This confirms the findings of Barker and Walker (2014), who stated that *R.(B) microplus, R.(B) australis* and *R.(B) annulatus* were inseparable in their hands. We confirm that *R.(B) microplus* and *R.(B) australis* are indistinguishable based on the morphological criteria outlined by Estrada-Peña *et al*. (2012), a finding also consistent with that of Barker and Walker (2014). Based on our findings on morphology alone *R.(B) microplus* and *R.(B) australis* appear to be the same species, although the Australian population tends to be smaller, and *R.(B) annulatus* appear to be very closely related to *R.(B) microplus*, differing most prominently in males based on the presence/absence of the caudal appendage. *Rhipicephalus (Boophilus) decoloratus* was the most consistently distinct species out of the four, however we confirm that the morphology of the Boophilid ticks is both highly variable and limited for consistent differentiation.

CHAPTER 4

Phylogenetic analysis of Rhipicephaline ticks using cytochrome oxidase subunit I (*COX1*) gene sequence

Introduction

The genus *Rhipicephalus* has undergone re-organisation in the last 25 years. Up until 13 years ago, the genus *Rhipicephalus* was considered monophyletic, with a common evolutionary ancestor not shared with any other groups, despite a number of previous studies suggesting insufficient support (Klompen *et al*., 1997). With the development of molecular techniques, it was confirmed that some species of the *Rhipicephalus* genus are more closely related to species of the *Boophilus* genus then other members of *Rhipicephalus* (Murrell and Barker, 2003b). Evidence to support the synonymizing of *Boophilus* with *Rhipicephalus* has come mainly in the form of molecular and morphological; however, evidence is also observed when considering host preferences and biogeography. Previous studies using 12S rDNA, 16S rDNA and *ITS2* genes have shown weak evidence supporting the paraphyly of *Rhipicephalus* and *Boophilus* (Murrell *et al*., 1999, Mangold *et al*., 1998, Beati and Keirans, 2001, Murrell *et al*., 2001a). However the combination of 12S rDNA and *COX1* has shown strong support for a clade between *R. evertsi* and *R. pravus* with three *Boophilid* species; *R.(B) microplus*, *R.(B) annulatus* and *R.(B) decoloratus* with 93% bootstrap support, separate from the rest of *Rhipicephalus* (Murrell and Barker, 2003b, Murrell *et al*., 2000). As it stands *Boophilus* is considered a sub-genus of *Rhipicephalus*.

The resolution of the taxonomic status of *R.(B) microplus* with regard to the validity of *R.(B) australis*, has not been straight forward. The use of *ITS2* indicated that the Australian ticks could not be separated from other populations of *R.(B) microplus* (Barker, 1998). This result was contradicted by a later study that used 12S rDNA which proposed a separation of the Australian ticks from ticks from South Africa and Mexico (Campbell and Barker, 1999). Other studies have used a combination of 16S rDNA, 12S rDNA and microsatellites which yielded no conclusive results (Labruna *et al*., 2009). More recently however, *COX1* has been proposed to be a suitable mitochondrial gene with the ability to clarify complex region groupings that have not been resolved clearly by using other mitochondrial

genes (Burger *et al.,* 2014). Although *COX1* has a similar range of application to that od *ITS2,* there is evidence to suggest that it is evolving slightly faster then *ITS2* and so may be developing polymorphisms that can be used to differentiate populations of the same species (Cruickshank, 2002). However most of the work up until now has been done using ticks from a limited number of locations. In this study, we aim to build upon results obtained by previous work (Burger *et al.,* 2014) by using a larger number of ticks from more geographical locations, most of which have not been previously included in a taxonomic study. In doing so we aim to confirm previous findings that *COX1* can be used to resolve complex relationships within the *R.(B) microplus* clade and to determine whether specimens clustered according to region. The second aim of this study was to determine whether *R.(B) australis* should be considered as a separate species from *R.(B) microplus*.

Materials and methods

Tick Samples

220 samples were used in total, comprising 180 putative *R. (B) microplus* from 9 locations table 2.1), 20 *R. (B) decoloratus* from South Africa and 20 *R. (B) annulatus* from Texas. One cell line (CTVM2), derived from *R.(B) microplus*, was used as a positive control throughout the optimization process. The tick cell line was provided by the Tick Cell Biobank at The Pirbright Institute. The passage level tested, species and instar of origin, geographic origin and acaricide resistance status of the parent tick for CTVM2 are listed in table 4.1.

Table 4.1: Tick cell lines used in the optimisation of *COX1* primers.

Cell line	Passage level tested	Instar of origin	Year initiated	Geographical origin (strain)	Resistance status	Reference
BME/CTVM2	140	Embryo	1983	Costa Rica (Paquera)	Susceptible	(Bell-Sakyi, 2004)

Primer design

Primers were designed using CLC Genomics Workbench 7. All available published sequences for *R.(B) microplus*, *R.(B) australis*, *R.(B) annulatus* and

R.(B) decoloratus for *COX1* were searched and downloaded into CLC Genomics from the National Center for Biotechnology Information (NCBI) website (appendix table A1). Sequences were then aligned and primers were designed manually using general guidelines (appendix table A2) (Green *et al*., 2012). The primers that were designed were then submitted to NCBI BLAST to ensure their specificity for the target gene.

When published sequences for the *COX1* primers (Murrell *et al*., 2000, Simon *et al*., 1994, Kambhampati and Smith, 1995) (appendix table A1) were aligned in CLC Genomics with sequences for *COX1* it was found that there were many polymorphisms in the primer binding sites, both among species and within the *R.(B) microplus* group. As a result, it was decided that the primers would be re-designed manually using the technique described above. The primers are shown in table 4.2.

Table 4.2: Sequence length and Oligo name of manually designed *COX1* primers

Oligo Name	Sequence (5'-3')
COI-F1	CAGGAAGATTAATTGGTAATGATC (24)
COI-R1b	TAATAGCCCCTGCTAAAACAGG (22)

PCR Optimization

The first stage in end-point polymerase chain reaction (PCR) optimization required the testing of the newly designed *COX1* primers on all three species (*R.(B) microplus* cell line CTVM2 as positive control, *R.(B) annulatus* and *R.(B) decoloratus* both extracted from own samples) with two different master mixes: master mix 1 (MM1) ddH2O 15µl, Buffer 10x 2.5 µl, MgCl2 1.25 µl, dNTPs (10 mM) 0.5 µl, forward primer 0.25 µl, reverse primer 0.25 µl, HotStar Taq 0.25 µl and master mix 2 (MM2) ddH2O 10 µl, Buffer 10x 2.5 µl, MgCl2 1.25 µl, dNTPs (10 mM) 0.5 µl, forward primer 0.25 µl, reverse primer 0.25 µl, HotStart Taq 0.25 µl, Qmix 5 µl. Three different annealing temperatures were used; 55ºC, 57ºC and 59ºC. Master mix recipes were made up using reagents from the Qiagen HotStar Taq plus DNA polymerase kit.

A 1.5% agarose gel was run with 5 µl of PCR product from each reaction at 140 volts for 40 minutes (figure 4.1). It was found that the *COX1* primer set worked well on *R.(B) microplus* with both master mixes and at all annealing temperatures; the best conditions for *R.(B) annulatus* and *R.(B) decoloratus* was 55ºC and master mix 1. From this it was decided that the *COX1* primer set would be used on the main DNA plates.

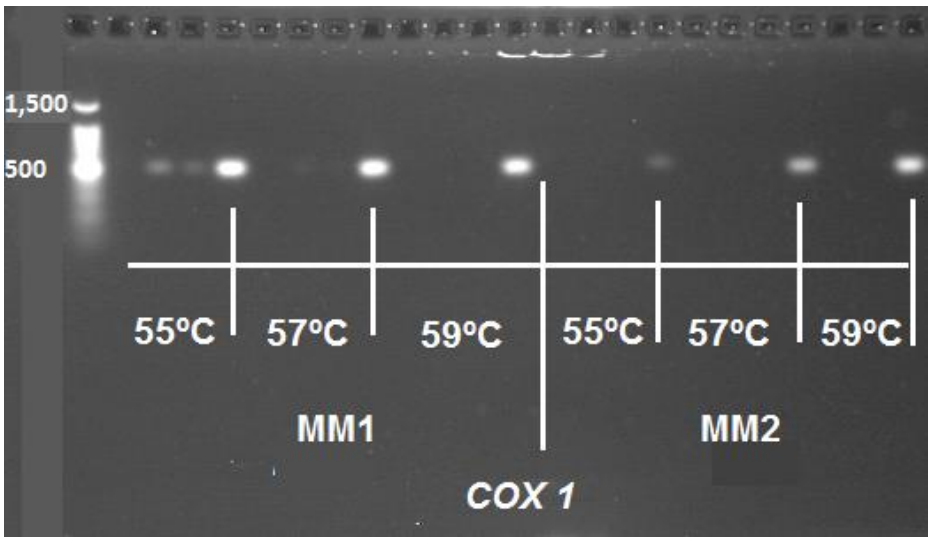


Figure 4.1: Gel photo showing the results obtained from first round of PCR optimization for *COX1*. 'MM 1' = master mix 1, 'MM2' = master mix 2.

The *COX1* primer set was tested on *R.(B) microplus* samples taken from a DNA extraction that had low A260/280 ratios (see chapter 2), as the *R.(B) microplus* used in the first optimization step were from cell line CTVM2, that had a high concentration and quality of DNA. No bands were observed for the two lower quality DNA *R.(B) microplus* samples with the *COX1* primer set (figure 4.2).

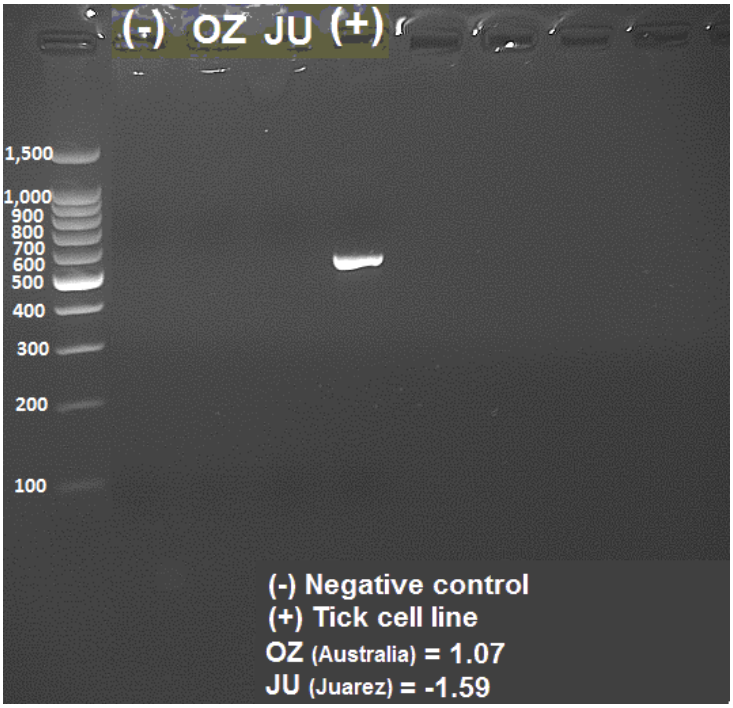


Figure 4.2: Gel photo showing the results obtained from second round of PCR optimization for *COX1.*

Subsequently, the *COX1* primer set was tested using a Thermo scientific Phusion Green Hot Start II High-Fidelity DNA Polymerase kit master mix and protocol. The kit did not require added $MgCl_2$, was designed to overcome PCR inhibition problems caused by ethanol, and required a shorter PCR cycle time, as well as already having dye in the master mix which would require no added dye to run a gel. The master mix was set up using the Thermo kit recipe (table 4.3). The PCR protocol was set up on the thermocycler; 30 seconds at 98ºC, 10 seconds at 98ºC, 30 seconds at 55ºC, 30 seconds at 72ºC, steps 2-4 repeated 40 times, 10 minutes at 72ºC.

Table 4.3: Master mix recipe for 20 µl used for third round of PCR optimization for *COX1* primers using the Thermo scientific Phusion Green Hot Start II High-Fidelity DNA Polymerase kit.

Thermo scientific Phusion Green Hot Start II High-Fidelity DNA Polymerase kit master mix- 20 µl
ddH_2O 8.4 µl 5x Phusion green HF buffer 4 µl dNTPs (10mM) 0.4 µl Forward primer 1 µl Reverse primer 1 µl Phusion hot start II DNA Polymerase (2 U/µl) 0.2 µl +5 µl DNA

The samples used in this PCR were as before with the addition of *R.(B) annulatus* and *R.(B) decoloratus*.

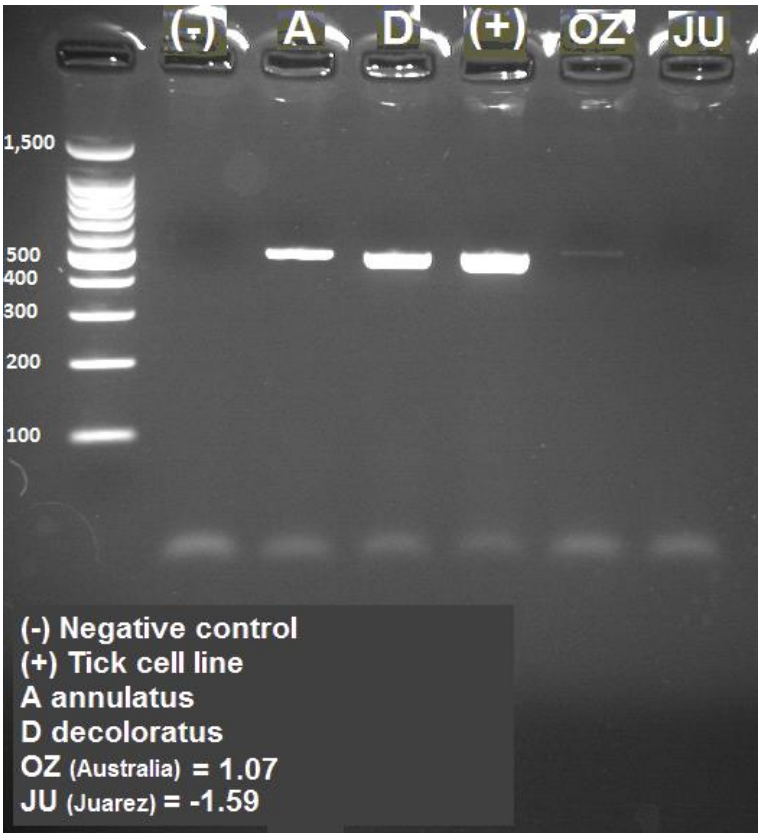


Figure 4.3: Gel photo showing the results obtained from third round of PCR optimization for *COX1* primers.

Strong bands were seen for *R.(B) annulatus* and *R.(B) decoloratus*, as well as *R.(B) microplus* (cell line CTVM2) (figure 4.3). A faint band was also seen for the

Australian sample (OZ) which had a low ratio of 1.07, however no band was seen for the *R.(B) microplus* sample from Juarez which had the lowest ratio of -1.59. It was decided to use the Phusion kit for the main DNA plates as it showed to successfully amplify even low quality DNA with the *COX1* primer set; however, the master mix was increased to 50 µl (table 4.4).

Table 4.4: Master mix recipe for 50 µl for main DNA plates (*COX1* primers) using the Thermo scientific Phusion Green Hot Start II High-Fidelity DNA Polymerase kit.

Thermo scientific Phusion Green Hot Start II High-Fidelity DNA Polymerase kit master mix- 50 µl
ddH_2O 23.5 µl 5x Phusion green buffer HF 10 µl dNTPs (10mM) 1 µl Forward primer 2.5 µl Reverse primer 2.5 µl Phusion hot start II DNA Polymerase (2 U/µl) 0.5 µl +10 µl DNA

This master mix, along with the annealing temperature of 55ºC was then used on the 3 main DNA plates.

Sequencing and Analysis

PCR products were then cleaned using the AMPure XP kit before being used in the sequencing reaction. Sequences were obtained using the Applied Biosystems kit and protocol on an Applied Biosystems 3130XL Genetic Analyzer. All analysis used CLC Genomics Workbench 7.5.2 and all phylogenetic trees were created using MEGA 6.06 software using maximum likelihood tree setting. Phylogeny was tested using the Bootstrap method, with 500 Bootstrap replication. Any branches with a value of less than 50% were considered unresolved and the branch was collapsed. Goodness of fit was not tested for with a molecular clock, instead BEAST 2.4.3 (Drummond and Rambaut, 2007) was used to do a relaxed clock Bayesian analysis which allowed the estimation of topology as well as divergence. This analysis was conducted based on settings in (Gou *et al*., 2013). BEAUti 2.4.3 was used to generate the XML document needed for running in BEAST 2.4.3. Settings for partitions and tip dates were as default based on (Gou *et al*., 2013); site model was set to generalized time reversible (GTR); Gamma Category Count

was set at “4 with estimate substitution rate and shape checked”; frequency set to “estimated”. The clock model was set to “relaxed clock log normal”, with the rest of the settings as default. For priors, the default Yule model was used. Most recent common ancestor (MRCA) was calibrated using fossil records discussed below. A normal distribution was used, mean (M) and Sigma (S) were set according to the approximate date of the fossil samples; *Ixodes*: M= 37.5 S= 1.25, *Amblyomma*: M= 27.5 S= 6.25 and *Hyalomma:* M= 42.5 S= 3.75. These values specified a distribution based on the date range of the fossil samples, with a central 95% probability range covering *Ixodes* 35-40 million years, *Amblyomma* 15-40 million years and *Hyalomma* 35-50 million years. Two separate Markov chain Monte Carlo (MCMC) runs were performed, one of 10 million generations and the second of 15 million generations, with sampling every 1000 generations. These independent runs were then combined, removing 10% burn-in, using LogCombiner 2.4.3. MCMC samples were checked using Tracer 1.6 (Drummond and Rambaut, 2007). Effective sample size (ESS) values of all parameters were checked to be >200, which is considered a sufficient level of sampling. Sampled posterior trees were summarized using TreeAnnotator 2.4.3 and a maximum clade credibility tree was created. The topology created in BEAST was visualized using FigTree 1.4.3.

To calibrate the clock model, three fossils were used which covered three different genera. The first genus used was *Ixodes*, based on *Ixodes succineus* found in amber (35–40 Mya) (Weidner, 1964). The most recent common ancestors of the *Ixodes* clade used were *Ixodes pavlovskyi*, *Ixodes persulcatus*, *Ixodes bakeri*, *Ixodes cornuatus*, *Ixodes ricinus*, and *Ixodes hirsti*. The second genus covered was *Hyalomma*, based on a un-speciated (ticks identified to genus level) specimen found in amber (35–50 Mya) (De La Fuente, 2003). The most recent common ancestors of the *Hyalomma* clade used were *Hyalomma truncatum*, *Hyalomma marginatum*, *Hyalomma rufipes*, *Hyalomma lusitanicum*, *Hyalomma dromedarii*, *Hyalomma asiaticum asiaticum* and *Hyalomma asiaticum*. The third genus was *Amblyomma*, based on a un-speciated specimen found in amber (15–40 Mya) (Poinar, 1992). The most recent common ancestors of the *Amblyomma* clade used were *Amblyomma triguttatum*, *Amblyomma pattoni*, and *Amblyomma variegatum*.

Results

182 out of 220 extracted samples successfully amplified yielding a PCR product of around 500 base pairs (bp). Forward and reverse sequences were aligned a maximum likelihood trees were created using sequences obtained from this study along with published sequences. Un-edited sequences (ranging in length from 301 bp to 1539 bp) and sequences that had all been trimmed to be the same length (294 bp) were created to compare the difference in tree output (figure 4.4).

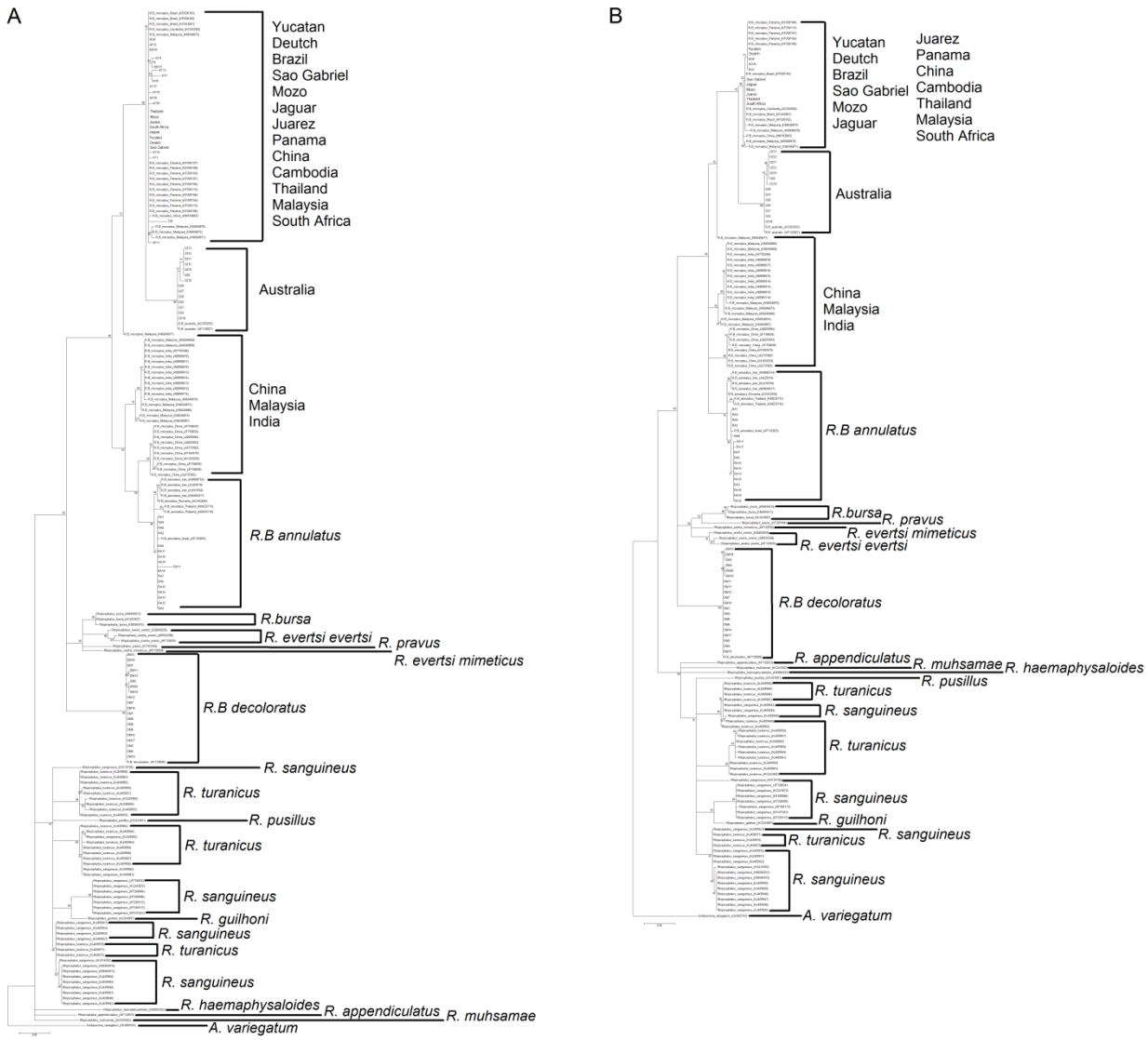


Figure 4.4: Maximum Likelihood tree inferred from *COX1* sequences. Comparison of trees created from non-trimmed sequences (A) and sequences trimmed to be the same length, with partial sequences omitted (B). Trees are rooted using *Amblyomma variegatum*. Phylogeny was tested using the Bootstrap method, with 500 Bootstrap replication. Any branches with a value of less than 50% were considered unresolved and the branch was collapsed. Bootstrap values visible on figure 4.5-4.9.

It was found that the grouping of *R.(B) microplus*, *R.(B) annulatus* and *R.(B) decoloratus* trees did not differ materially between the trimmed and untrimmed alignments, although the tree for the trimmed sequences had fewer out-groups with low bootstrap support. Some differences were observed between trimmed and untrimmed sequences in the broader *Rhipicephalus* clade (figure 4.4). It was decided based on this that the trimmed tree B would be used for inferring phylogeny.

Any groups with less than 50% bootstrap support were collapsed. Within the *R.(B) microplus* clade a number of groups can be observed (figure 4.5 A-J). Group A (red) included ticks from Panama (published), Mexico (Yucatan isolate), USA (Deutch isolate), and three individual ticks from Brazil (Sao Gabriel isolate) with 61% bootstrap support. Group B (dark green) consists of ticks from South America (Brazil (published), Sao Gabriel, Jaguar, Juarez and Mozo), Thailand, Cambodia (published) and two from Malaysia (published) with a low bootstrap support of 52%. Group C (blue) consisted of a Chinese and two Malaysian isolates (both from published sequences), with a 95% bootstrap support. Based on the low bootstrap support for group B, it would be reasonable to consider collapsing this group into group C. These ticks are not that divergent from one another and appear to be experiencing a similar amount of genetic change.

Group D (dark brown) indicates the group comprising published *R.(B) australis* sequences and the Australian samples from this study with 100% bootstrap support. The Australian ticks appear to be divergent from the other populations of *R.(B) microplus*, showing evidence of undergoing a large amount of genetic change. Within group D there appear to be two additional group of the Australian ticks with 62% and 80% bootstrap support.

Malaysian isolate KM246877 (pale purple E) was found to be unresolved with bootstrap support of 52%. There appears to be a clear grouping of the published Malaysian and Indian ticks (92% bootstrap support) distinct from the published Chinese ticks (99% bootstrap support). Within this group there is a main group of Malaysian and Indian (purple F) ticks with bootstrap support of 59%, there are also an additional two groups of Malaysian ticks (pale blue G and pale green H) with 85% and 93% bootstrap support respectively. Within the Chinese group (yellow I

and brown J) there is a divergent group with 62% bootstrap support (yellow I). Groups F-H are divergent from the rest of *R.(B) microplus* (A-C).

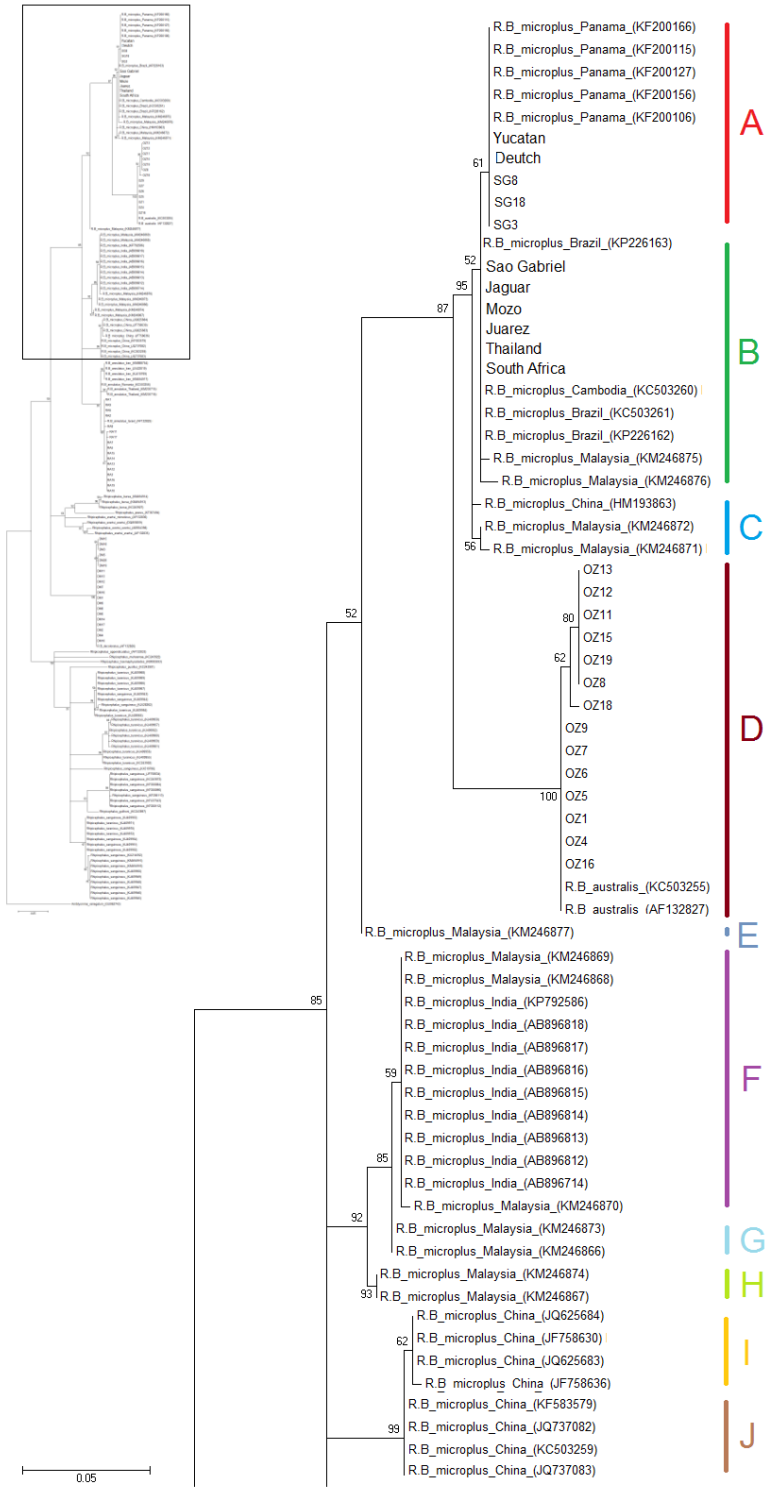


Figure 4.5: Maximum Likelihood tree inferred from *COX1* sequences. Tree shown here was created using the highly stringent sequences (figure 5.4 B). Percentage bootstrap support is indicated on each node. Any nodes with <50% support have been collapsed. Specimens sequenced in this study are indicated by the absence of accession number. Data shown here is for the entire *R.(B) microplus* sequences, groups are indicated by colours and letters. Conclusion of species identification is shown in black to the right. Position on the main tree is shown by the black box to the left. Individual sequences have been amalgamated into a region label where majority of samples fall on the tree (e.g. Sao Gabriel), anomalies have retained their original unique label (e.g. SG8). Tree is rooted using *Amblyomma variegatum*.

R.(B) annulatus was found to be closely associated with members of the *R.(B) microplus* clade, branching with 93% bootstrap support (figure 4.6). It can also be seen that *R.(B) annulatus* is undergoing a similar amount of genetic change as *R.(B) microplus* (I-J). Within *R.(B) annulatus* further groups can be observed based on geographical location. Published Iranian sequences group with 86% bootstrap support from the published Romanian sequences. Similarly, *R.(B) annulatus* from Thailand grouped with 94% bootstrap support. *R.(B) annulatus* from America was found to split into two different groups; the first group with 80% bootstrap support included a sequence from Israel; the second grouping with 61% bootstrap support. The amount of genetic change was similar throughout *R.(B) annulatus* clades.

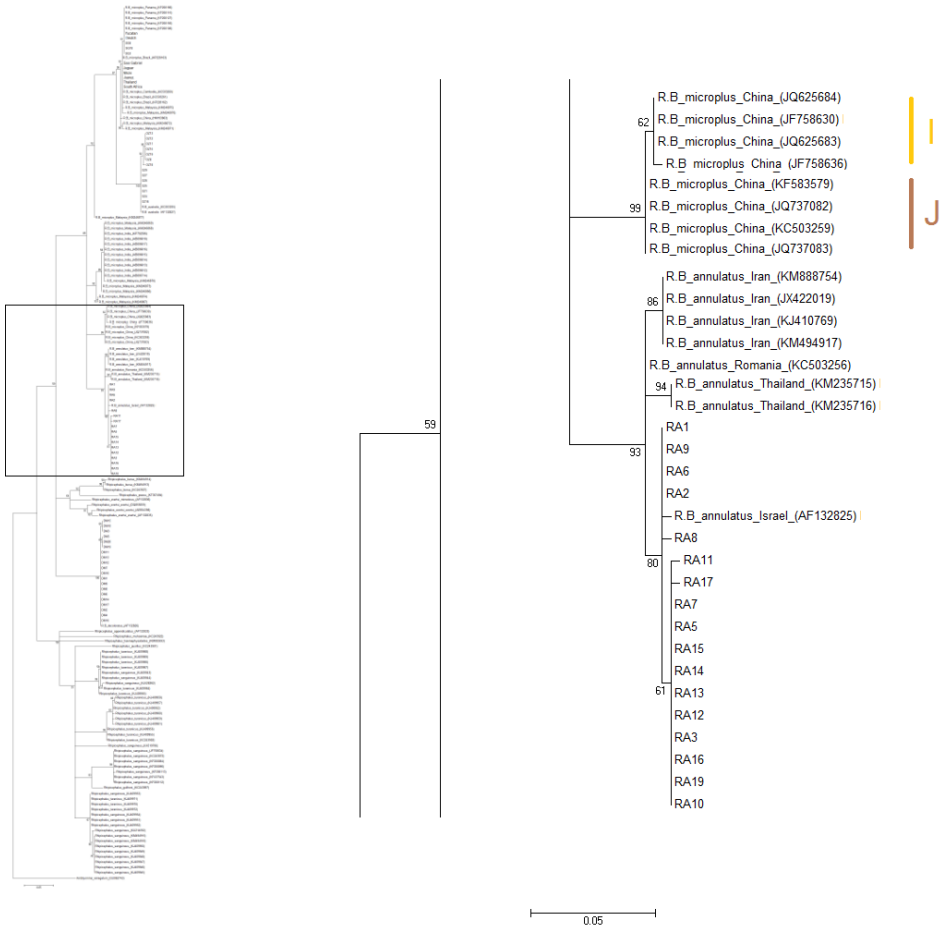


Figure 4.6: Maximum Likelihood tree inferred from COX1 sequences. Tree shown here was created using the highly stringent sequences (figure 5.4 B). Percentage bootstrap support is indicated on each node. Any nodes with <50% support have been collapsed. Specimens sequenced in this study are indicated by the absence of accession number. Data shown here is for the Chinese R.(B) microplus sequences in relation to R.(B) annulatus. Position on the main tree is shown by the black box to the left. Tree is rooted using Amblyomma variegatum.

R.(B) decoloratus is divergent from both *R.(B) annulatus* and *R.(B) microplus* with 100% bootstrap support and therefore a different species (figure 4.7). Within *R.(B) decoloratus* two small groups diverge with 55% and 69% bootstrap support, however these two groups are undergoing a very slow rate of divergence. *R.(B) decoloratus* with *R. bursa, R. pravus* and *R. evertsi* appears to be paraphyletic with repect to the res tof the *Boophilid* clade.

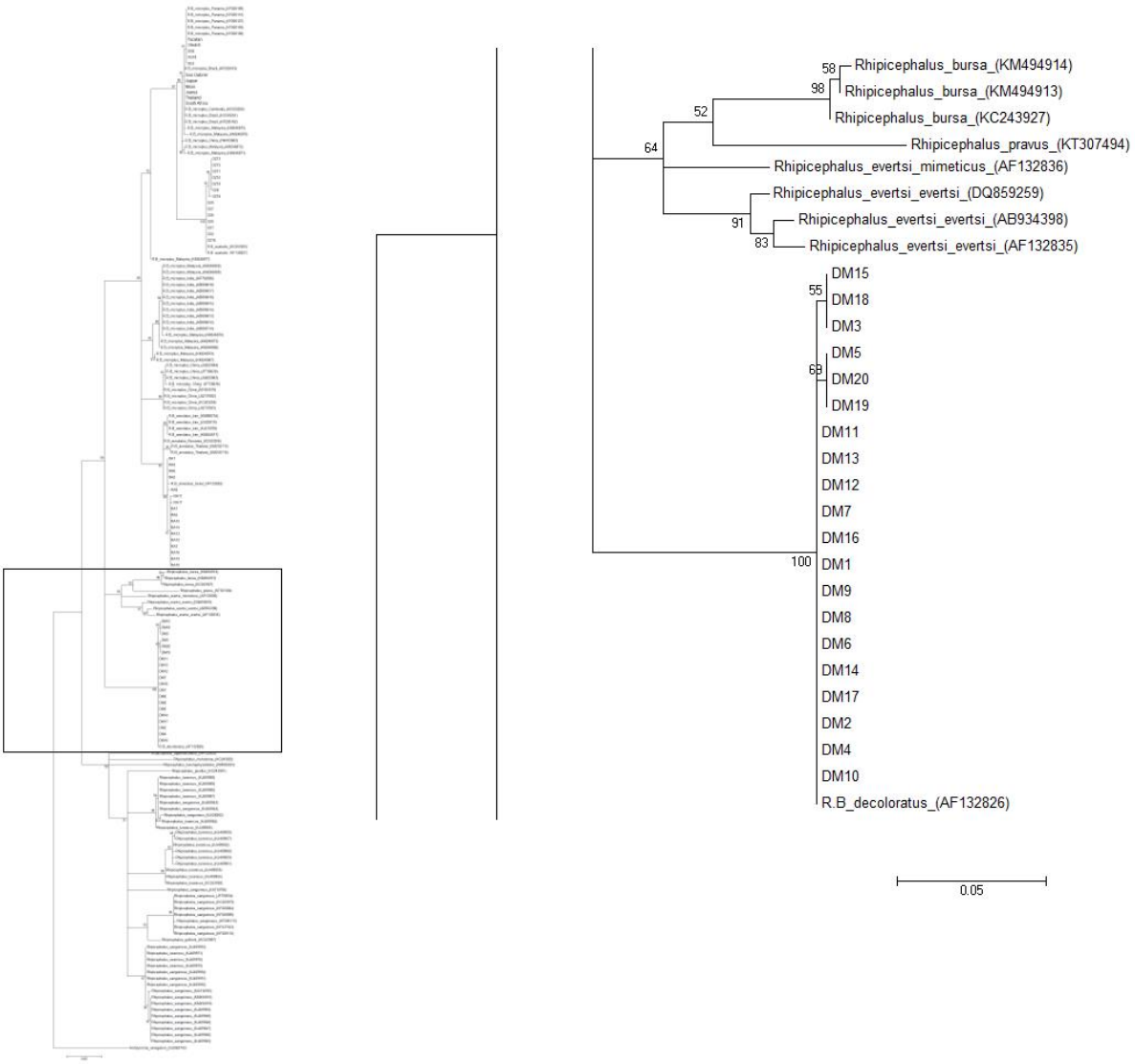


Figure 4.7: Maximum Likelihood tree inferred from *COX1* sequences. Tree shown here was created using the highly stringent sequences (figure 5.4 B). Percentage bootstrap support is indicated on each node. Any nodes with <50% support have been collapsed. Specimens sequenced in this study are indicated by the absence of accession number. Data shown here is for *R.(B) decoloratus* sequences, including some other *Rhipicephalus* species. Position on the main tree is shown by the black box to the left. Tree is rooted using *Amblyomma variegatum*.

The rest of the *Rhipicephalus* genus appears to be supportive of the currently accepted theories of organisation, with exception of *R. sanguineus* and *R. turanicus,* which appear to share a complex relationship (figure 4.8).

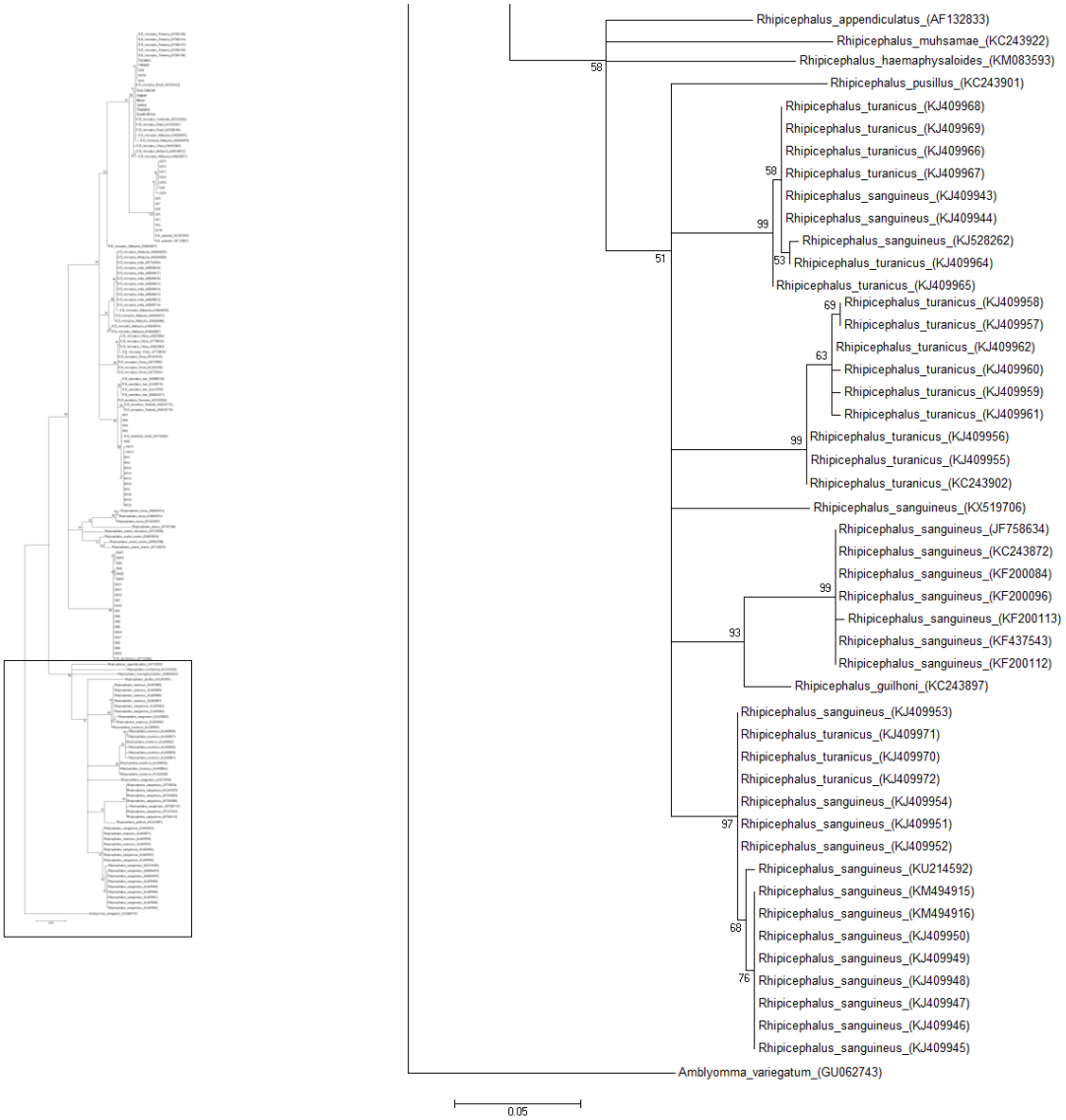


Figure 4.8: Maximum Likelihood tree inferred from *COX1* sequences. Tree shown here was created using the highly stringent sequences (figure 5.4 B). Percentage bootstrap support is indicated on each node. Any nodes with <50% support have been collapsed. Specimens sequenced in this study are indicated by the absence of accession number. Data shown here is for the rest of the *Rhipicephalus* sequences. Position on the main tree is shown by the black box to the left. Tree is rooted using *Amblyomma variegatum*.

When a maximum likelihood tree is created using published sequences for *ITS2* (Burger *et al*., 2014) it can be seen that *R.(B) australis* diverges from *R.(B) microplus* with 86% bootstrap support (figure 4.9). Similarly, *R.(B) annulatus* is also divergent from *R.(B) microplus* with 62% bootstrap support. Divergence of *R.(B) decoloratus* from *R.(B) annulatus*, *R.(B) microplus* and *R.(B) australis* is also

supported, with 76% bootstrap support. In the case of *ITS2*, *R.(B) decoloratus* does not group with *R. bursa* and *R. evertsi*.

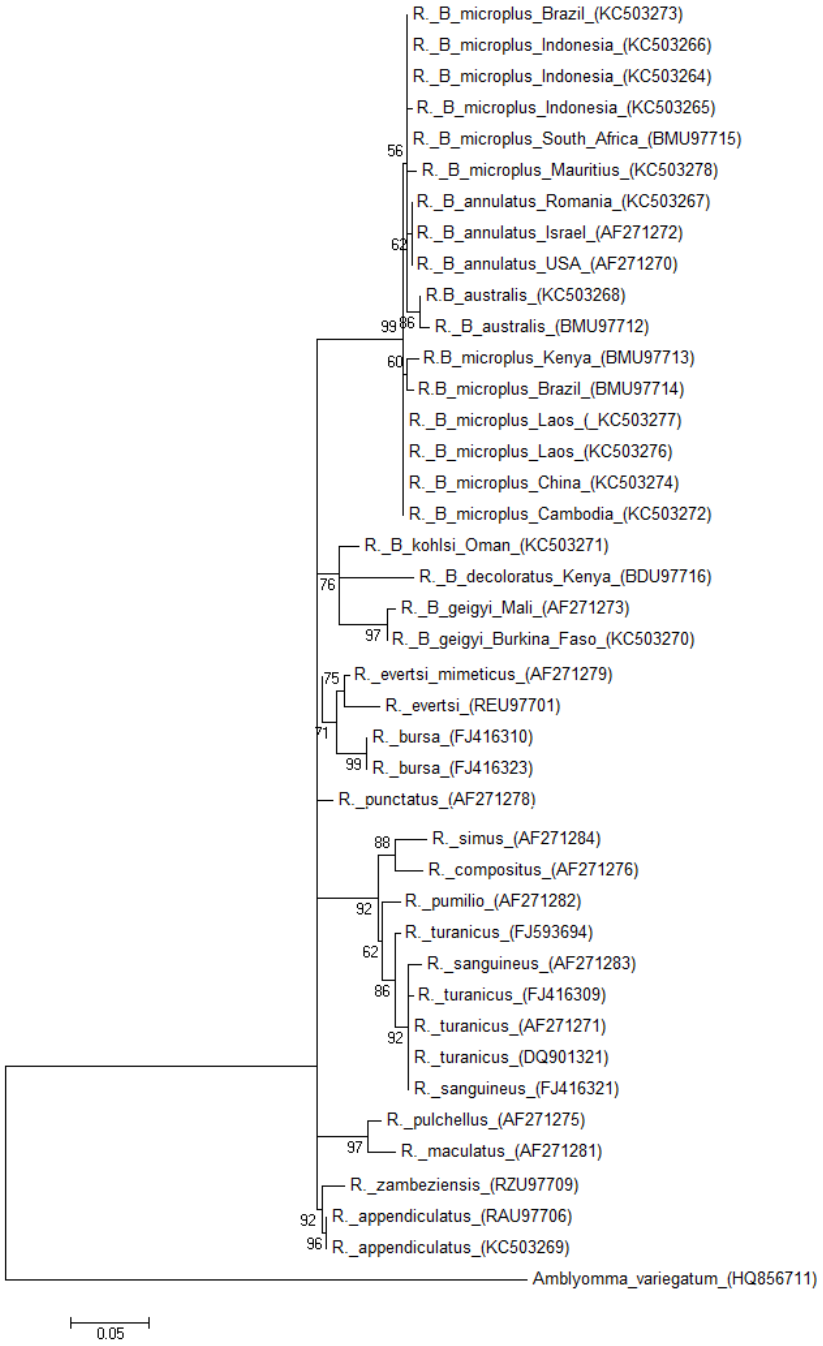
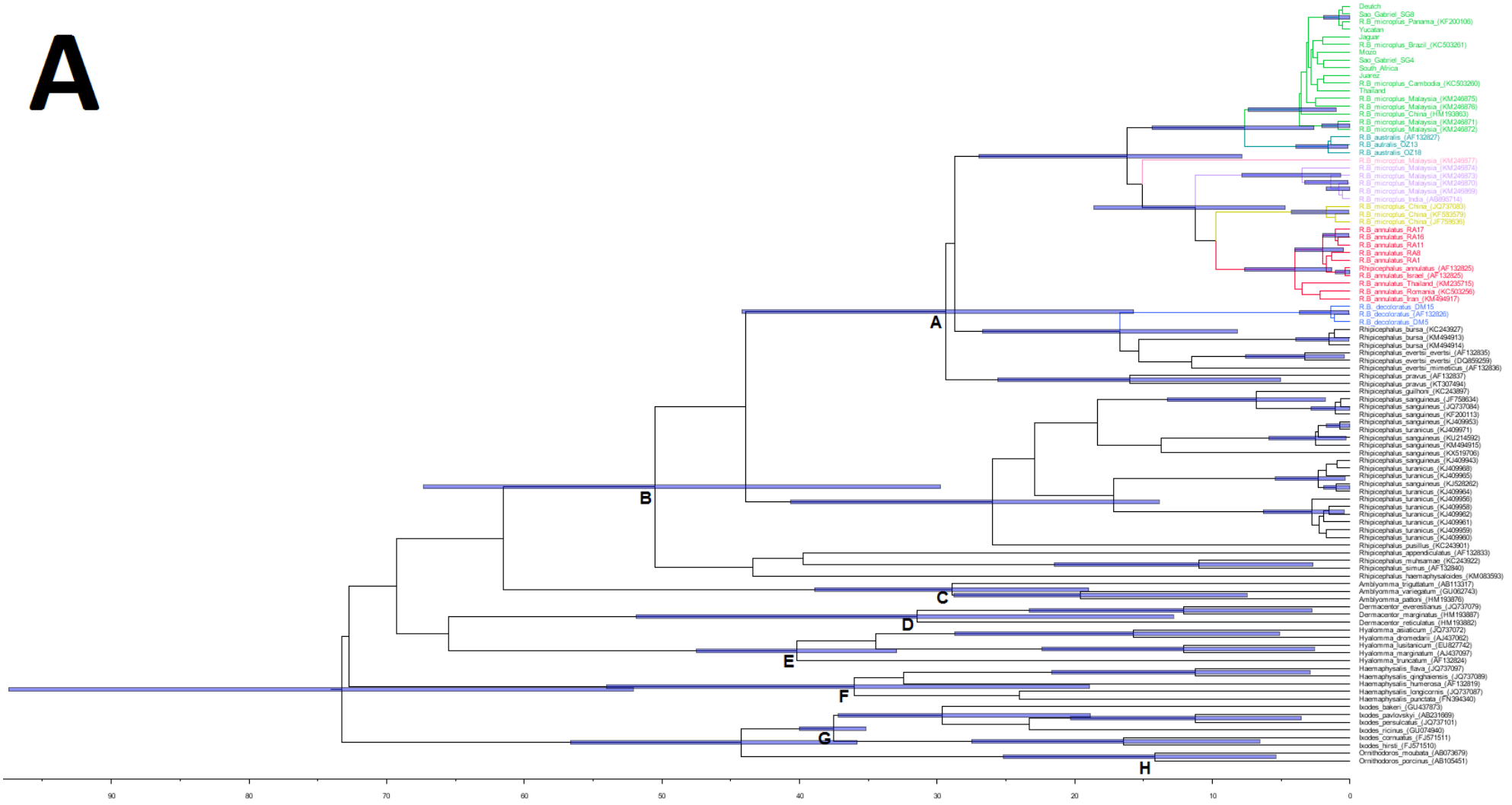


Figure 4.9: Maximum Likelihood tree inferred from *ITS2* sequences. Percentage bootstrap support is indicated on each node. Any nodes with <50% support have been collapsed. Specimens shown here were reproduced from Burger *et al*., 2014. Tree is rooted using *Amblyomma variegatum*.

Results from the relaxed-clock Bayesian analysis showed support for the general groupings discussed above (figure 4.10). Groups A-C as seen in figure 4.5, is depicted as the green clade in 4.10, with identical sequences being collapsed.

Members of this clade appear to have evolved from the same ancestor as *R.(B) australis* at around 7.65 million years (Mya) (95% highest possible density (HPD) interval 2.62-14.39 Mya). The green *R.(B) microplus* clade diverged approximately 3.69 Mya (95% HPD 1.02-7.38 Mya). Based on this analysis, *R.(B) australis* diverged later at approximately 1.59 Mya (95% HPD 0.15-3.92 Mya). Groups F-H (figure 4.6) is colored purple in figure 4.10. This group, made up of published Malaysian and Indian *R.(B) microplus* sequences, diverged 3.49 Mya (95% HPD 0.69-7.86 Mya). Groups I and J (figure 4.5) made up of published Chinese sequences, colored yellow (figure 4.10) appears to have diverged 1.72 Mya (95% HPD 0.12-4.25 Mya). This group shared a common ancestor with *R.(B) annulatus* (red, figure 4.10) 9.75 Mya, diverging around 3.98 Mya (95% HPD 1.31-7.69 Mya). Both the Chinese (yellow), *R.(B) annulatus* (red) and Malaysian/Indian (purple) clades share a common ancestor 11.22 Mya (95% HPD 4.72-18.62 Mya). *R.(B) decoloratus* (blue) is highly divergent from the rest of the *Boophilid* ticks covered in this work, sharing a common ancestor 28.73 Mya but diverging 1.39 Mya (95% HPD 0.07-3.67 Mya). 16.69 Mya (95% HPD 8.2-26.72 Mya) *R.(B) decoloratus* shared a common ancestor with *R. bursa* and *R. evertsi*. The genus *Rhipicephalus* appears to have formed 50.53 Mya (95% HPD 29.74-67.33 Mya). Along with members of *Boophilus*, three species of *Rhipicephalus*; *R. bursa*, *R. evertsi* and *R. pravus* appear to have diverged from the rest of *Rhipicephalus* at around 29.38 Mya (95% HPD 15.72-44.2 Mya) (A figure 4.10).

Figure 4.10: A: Chronogram created from the molecular dating analysis conducted in BEAST 2.4.3 and edited in FigTree 1.4.3. Shaded bars represent the 95% highest posterior density interval for divergence estimates. Clades/groups (figure 4.5) are indicated by colour; Green= group A-C, Blue= group D (*R.(B) australis*), Pink= Malaysian KM246877, Purple= groups F-H, Yellow= groups I + J, Red= *R.(B) annulatus*, Blue= *R.(B) decoloratus*. A-H indicates the times at which genera formed: A: *Boophilus* (including some *Rhipicephalus*) 29.38 Mya (95% HPD 15.72-44.2 Mya) B: *Rhipicephalus* 50.53 Mya (95% HPD 29.74-67.33 Mya) C: *Amblyomma* 28.93 Mya (95% HPD 18.98-38.92 Mya) D: *Dermacentor* 31.45 Mya (95% HPD 12.84-51.87 Mya) E: *Hyalomma* 40.2 Mya (32.93-47.49 Mya) F: *Haemaphysalis* 36.03 Mya (18.92-54.03 Mya) G: *Ixodes* 37.55 Mya (35.18-40.03 Mya) H: *Ornithodoros* 14.18 Mya (5.4-25.18 Mya). B: Close up image of group A.


A
Deutch
Sao_Gabriel_SG8
R.B_microplus_Panama_(KF200108)
Yucatan
Jaguar
R.B_microplus_Brazil_(KC503261)
Mozo
Sao_Gabriel_SG4
South_Africa
Juarez
R.B_microplus_Cambodia_(KC503260)
Thailand
R.B_microplus_Malaysia_(KM246875)
R.B_microplus_Malaysia_(KM246876)
R.B_microplus_China_(HM193863)
R.B_microplus_Malaysia_(KM246871)
R.B_microplus_Malaysia_(KM246872)
R.B_australis_(AF132827)
R.B_australis_OZ13
R.B_australis_OZ18
R.B_microplus_Malaysia_(KM246877)
R.B_microplus_Malaysia_(KM246874)
R.B_microplus_Malaysia_(KM246873)
R.B_microplus_Malaysia_(KM246870)
R.B_microplus_Malaysia_(KM246869)
R.B_microplus_India_(AB868714)
R.B_microplus_China_(JQ737083)
R.B_microplus_China_(KF583579)
R.B_microplus_China_(JF758936)
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R.B_annulatus_RA16
R.B_annulatus_RA11
R.B_annulatus_RA8
R.B_annulatus_RA1
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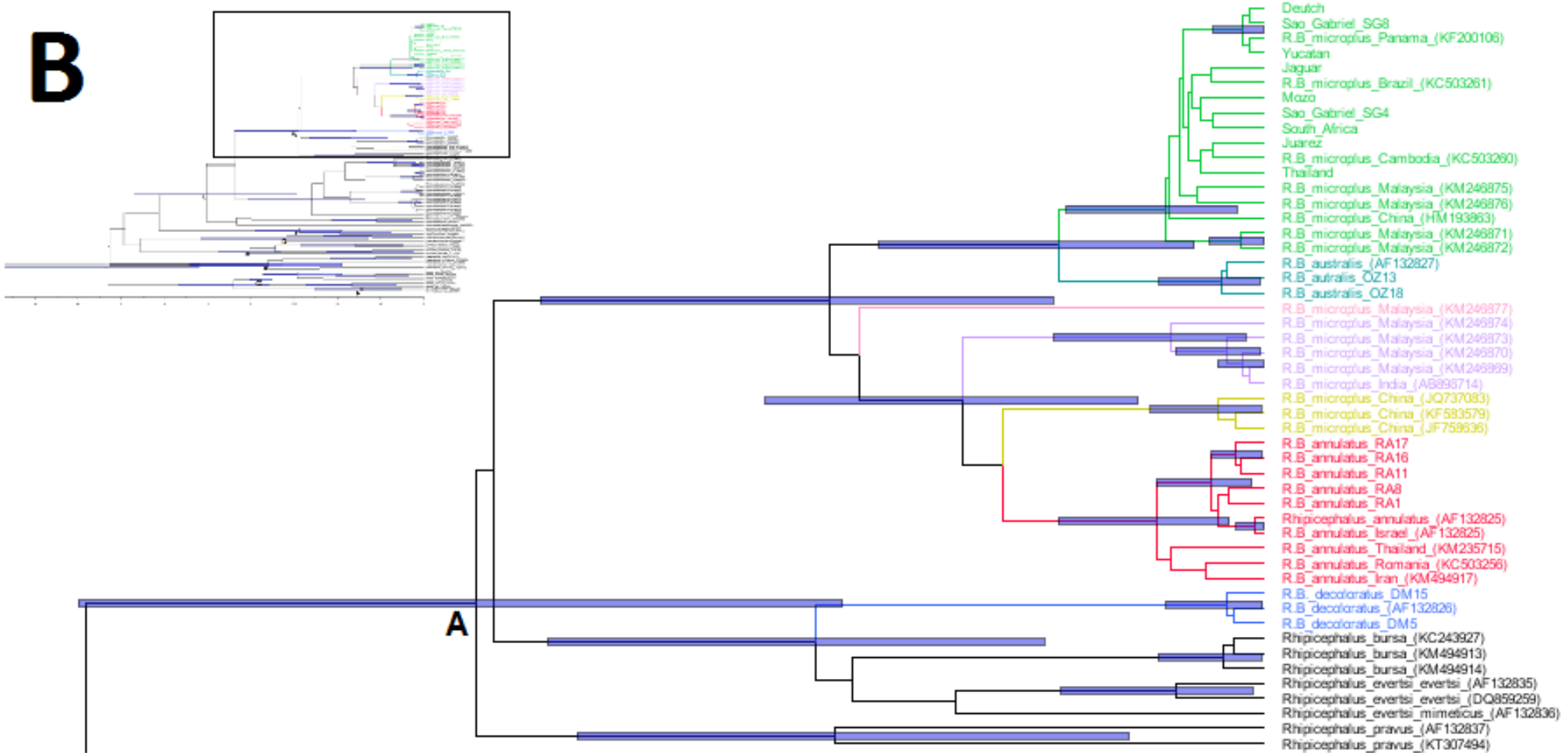
R.B_annulatus_Thailand_(KM235715)
R.B_annulatus_Romania_(KC503256)
R.B_annulatus_Iran_(KM494917)
R.B_decoloratus_DM15
R.B_decoloratus_(AF132826)
R.B_decoloratus_DM5
Rhipicephalus_bursa_(KC243927)
Rhipicephalus_bursa_(KM494913)
Rhipicephalus_bursa_(KM494914)
Rhipicephalus_evertsi_evertsi_(AF132835)
Rhipicephalus_evertsi_evertsi_(DQ859259)
Rhipicephalus_evertsi_mimeticus_(AF132836)
Rhipicephalus_pravus_(AF132837)
Rhipicephalus_pravus_(KT307494)
Rhipicephalus_guilhoni_(KC243897)
Rhipicephalus_sanguineus_(JF759834)
Rhipicephalus_sanguineus_(JQ737084)
Rhipicephalus_sanguineus_(KF200113)
Rhipicephalus_sanguineus_(KJ409953)
Rhipicephalus_turanicus_(KJ409971)
Rhipicephalus_sanguineus_(KU214592)
Rhipicephalus_sanguineus_(KM494915)
Rhipicephalus_sanguineus_(KX519706)
Rhipicephalus_sanguineus_(KJ409943)
Rhipicephalus_turanicus_(KJ409968)
Rhipicephalus_turanicus_(KJ409965)
Rhipicephalus_sanguineus_(KU528262)
Rhipicephalus_turanicus_(KJ409964)
Rhipicephalus_turanicus_(KJ409956)
Rhipicephalus_turanicus_(KJ409958)
Rhipicephalus_turanicus_(KJ409962)
Rhipicephalus_turanicus_(KJ409961)
Rhipicephalus_turanicus_(KJ409959)
Rhipicephalus_turanicus_(KJ409960)
Rhipicephalus_pusillus_(KC243901)
Rhipicephalus_appendiculatus_(AF132833)
Rhipicephalus_muhsamae_(KC243922)
Rhipicephalus_sirnus_(AF132840)
Rhipicephalus_haemaphysaloides_(KM083593)
Amblyomma_triguttatum_(AB113317)
Amblyomma_variegatum_(GU062743)
Amblyomma_pattoni_(HM193876)
Dermacentor_everestianus_(JQ737079)
Dermacentor_marginatus_(HM193887)
Dermacentor_reticulatus_(HM193882)
Hyalomma_asiaticum_(JQ737072)
Hyalomma_dromedarii_(AJ437062)
Hyalomma_lusitanicum_(EU827742)
Hyalomma_marginatum_(AJ437097)
Hyalomma_truncatum_(AF132824)
Haemaphysalis_flava_(JQ737097)
Haemaphysalis_qinghaiensis_(JQ737089)
Haemaphysalis_humerosa_(AF132819)
Haemaphysalis_longicornis_(JQ737087)
Haemaphysalis_punctata_(FN394340)
Ixodes_baikeri_(GU437873)
Ixodes_pavlovskyi_(AB231669)
Ixodes_persulcatus_(JQ737101)
Ixodes_ricinus_(GU074940)
Ixodes_cornuatus_(FJ571511)
Ixodes_hirsti_(FJ571510)
Ornithodoros_moubata_(AB073679)
Ornithodoros_porcinus_(AB105451)
A
B
C
D
E
F
G
H
90
80
70
60
50
40
30
20
10
0


B
A
Deutch
Sao_Gabriel_SG8
R.B_microplus_Panama_(KF200106)
Yucatan
Jaguar
R.B_microplus_Brazil_(KC503261)
Mozo
Sao_Gabriel_SG4
South_Africa
Juarez
R.B_microplus_Cambodia_(KC503260)
Thailand
R.B_microplus_Malaysia_(KM246875)
R.B_microplus_Malaysia_(KM246876)
R.B_microplus_China_(HM193963)
R.B_microplus_Malaysia_(KM246871)
R.B_microplus_Malaysia_(KM246872)
R.B_australis_(AF132827)
R.B_autralis_OZ13
R.B_australis_OZ18
R.B_microplus_Malaysia_(KM246877)
R.B_microplus_Malaysia_(KM246874)
R.B_microplus_Malaysia_(KM246873)
R.B_microplus_Malaysia_(KM246870)
R.B_microplus_Malaysia_(KM246869)
R.B_microplus_India_(AB893714)
R.B_microplus_China_(JQ737083)
R.B_microplus_China_(KF583579)
R.B_microplus_China_(JF759636)
R.B_annulatus_RA17
R.B_annulatus_RA16
R.B_annulatus_RA11
R.B_annulatus_RA8
R.B_annulatus_RA1
Rhipicephalus_annulatus_(AF132825)
R.B_annulatus_Israel_(AF132825)
R.B_annulatus_Thailand_(KM235715)
R.B_annulatus_Romania_(KC503256)
R.B_annulatus_Iran_(KM494917)
R.B._decoloratus_DM15
R.B_decoloratus_(AF132826)
R.B_decoloratus_DM5
Rhipicephalus_bursa_(KC243927)
Rhipicephalus_bursa_(KM494913)
Rhipicephalus_bursa_(KM494914)
Rhipicephalus_evertsi_evertsi_(AF132835)
Rhipicephalus_evertsi_evertsi_(DQ859259)
Rhipicephalus_evertsi_mimeticus_(AF132836)
Rhipicephalus_pravus_(AF132837)
Rhipicephalus_pravus_(KT307494)

Discussion

This phylogenetic analysis of *COX1* sequences is consistent with the reinstatement of *R.(B) australis* as previously proposed (Estrada-Peña *et al*., 2012). There is also evidence to suggest that *R.(B) microplus* is a clade composed of five groups including *R.(B) australis* and *R.(B) annulatus*. The first group (A-E, figure 4.5) has a world-wide distribution, including *R.(B) microplus* from South America, North America, South Africa, Thailand and published sequences from Cambodia, Malaysia and one from China. The second group (D, figure 4.5) consists of *R.(B) australis*. The third group (F-H, figure 4.5) consists of published Indian and Malaysian samples. The fourth group (I+J, figure 4.5) is composed exclusively of Chinese samples. *R.(B) annulatus* shares a common ancestor with the Chinese group (I+J, figure 4.5), diverging approximately 9.75 Mya (figure 4.10) indicating that *R.(B) annulatus* is probably monophyletic with *R.(B) microplus*. These groupings support the observations made previously (Burger *et al.,* 2014), where it was proposed that *R.(B) microplus* is a complex made up of *R.(B) australis, R.(B) annulatus* and two *R.(B) microplus* clades; A: Brazil, China and Cambodia; B: China. The later findings of Low *et al* (2015) proposed an additional clade, named clade C, made up of Malaysian and Indian specimens are also supported. With the addition of the sequences generated in this study, it is possible to expand on this proposed complex, increasing the size of *R.(B) microplus* clade A, with the addition of multiple locations.

R.(B) decoloratus shares a common ancestor with *R.(B) microplus* and *R.(B) annulatus*, diverging approximately 28.73 Mya, however it appears to be monophyletic with to *R. bursa, R. evertsi* and *R. pravus* (figure 4.10). This supports the findings from previous studies that proposed the synonymizing of *Boophilus* with *Rhipicephalus* (Klompen *et al*., 1997, Murrell and Barker, 2003b, Murrell *et al*., 2001a).

The maximum likelihood tree for the nuclear gene *ITS2* broadly supports the findings for *COX1* (figure 4.9) (Burger *et al*., 2014). However, due to the lack of *ITS2* published sequences covering the geography used in this study, it is hard to confirm completely the relationships discussed. In general, *ITS2* shows evidence that *R.(B) australis* and *R.(B) annulatus* share a common ancestor with *R.(B) microplus*, and are therefore are probably monophyletic with *R.(B) microplus*.

The question then arises as to what the relationship is of these groups within the *R.(B) microplus* clade, and what they should be called. The first suggestion is that the *R.(B) microplus* is made up of sub-species. However, first the term subspecies needs to be defined (Mayr and Ashlock, 1969). One interpretation is that subspecies should be less divergent then species. In the case of the chronogram created here (figure 4.10), based on the dates given, the groups discussed, including *R.(B) australis* and *R.(B) annulatus*, are diverging too long ago. Another interpretation of subspecies is a group that can successfully interbreed with another group, producing fertile offspring, but do not in practice due to geographical isolation. If this definition is accepted, then further work in the form of a mating study, where several crosses spanning the group would be needed.

It should also be noted that within species divergence times appear very high because of the lack of recent calibration points. The evolutionary rates that can be inferred in this analysis tend to be time scale dependent and therefore may be biased. There are a number of reasons that this bias may arise, purifying selection, ancestral polymorphisms, calibration errors and sequence errors, to name but a few (Ho *et al*., 2015). In the case of this work, a mixture of sources was used for the acquisition of tick specimens. Selected strains from research stations, ticks direct from the field as well as ticks taken from the field and maintained in a laboratory environment which have not undergone selection pressures, were all used in this analysis and could influence the diversity observed.

Conclusion

Overall, this study supports the reinstatement of *R.(B) australis*. *R.(B) microplus* is potentially made up of 5 groups; one group that's made up of specimens from around the world, *R.(B) australis*, Malaysian/Indian group, Chinese group and *R.(B) annulatus*. The taxonomic status of these groups requires further investigation with two potential outcomes. Either *R.(B) microplus* is a complex clade made up of several different sub-species, or all 5 groups are in fact separate species and the validity of the name *R.(B) microplus* as well as which group it belongs to will need re-addressing. There is an assumption that because the type specimen *R.(B) microplus* comes from South America then all sequences from

South America represent that species. However, if the latter is true, then the type specimen for *R.(B) microplus* which is currently accepted as being discovered by Canestrini in 1887 in Chaco australe, Paraguay, may not be the type specimen.

A number of taxonomic relationships can be observed from this analysis, firstly *R.(B) australis* (pale blue, figure 4.10) is monophyletic with the top *R.(B) microplus* group (green, figure 4.10). *R.(B) annulatus* can be seen to be monophyletic with the Chinese group of *R.(B) microplus* (yellow, figure 4.10). *R.(B) decoloratus* (blue, figure 4.10) is paraphyletic to the rest of *Boophilus* covered in this analysis, and monophyletic with *R. bursa* and *R. evertsi*. This observation supports previous studies that called for *Boophilus* to be synonymized with *Rhipicephalus*.

CHAPTER 5

Variation in genomic nucleotide sequence of the *Bm86* gene in *R.(B) microplus* ticks from multiple geographical locations

Introduction

Bm86 is a concealed glycoprotein antigen that is expressed in the midgut of the tick and, is the basis of the only vaccine against ticks that has been available commercially. Tick GARD^{PLUS} was one version of the vaccine, developed and available for use in Australia from the 1990's but was discontinued; Gavac was developed in South America and is still currently available (Freeman *et al*., 2010). The vaccine has been shown to variably reduce the number of ticks engorging and the reproductive rate of those that do engorge for ticks of the species, *R.(B) microplus*, *R.(B) decoloratus*, *R.(B) annulatus*, *R. appendiculatus*, *Hyalomma anatolicum anatolicum* and *H. dromedarii* (Odongo *et al*., 2007, Pipano *et al*., 2003, de Vos *et al*., 2001). *Bm86* was first identified as a potentially protective antigen in the late 1980's (Rodríguez *et al*., 1994, Willadsen *et al*., 1989, Johnston *et al*., 1986, Opdebeeck *et al*., 1988, Kemp *et al*., 1989, Willadsen *et al*., 1988), Using immunogold labeling, it was found that *Bm86* was located mainly on the microvilli of digest cells within the ticks midgut (Gough and Kemp, 1993).

Since the introduction of several commercial vaccines based on the antigen, variability in the responsiveness of *R.(B) microplus* in different regions have been observed (García-García *et al*., 1999, Willadsen *et al*., 1995, García-García *et al*., 2000). Studies have set out to investigate why the efficacy is so variable. One proposed theory is the presence of a divergence in the amino acid sequence between recombinant *Bm86* vaccine and native *Bm86* expressed in ticks from different geographical locations (Freeman *et al*., 2010). It has also been proposed that there is an inverse correlation between efficacy of the vaccine and variation in the *Bm86* sequence (García-García *et al*., 1999).

A number of *Bm86* homologues were identified and analyzed from a range of ticks representing the soft ticks (Argasidae) and the hard ticks (Ixodidae) (Nijhof *et al*., 2010). Results from this study indicated that *Hyalomminae* were embedded within the *Rhipicephalinae*, which was previously proposed in a review on the

systematics of ticks, published the year before (Nava *et al*., 2009). A clear difference between *Bm86* homologues of Prostriate (*Ixodes*) and Metastriate (*Hyalomma, Amblyomma, Decoloratus, Haemaphysalis* and *Rhipicephalus*) was also observed. A clear grouping based on species of *R.(B) microplus*, *R.(B) decoloratus* and *R.(B) annulatus* with 99%, 100% and 96% Bootstrap support respectively was observed. There was also some divergence within the *R.(B) microplus* group, with the Mexican (FJ456928) (Canales *et al*., 2009) and Brazilian (EU352677) (Andreotti *et al*., 2008) *Bm86* sequences grouping with 96% Bootstrap support.

Bm86 has been sequenced from a number of different locations and compared to the original Yeerongpilly strain. Polymorphism among geographically separate *R.(B) microplus* has been reported to be associated with the reduced effectiveness of the vaccines (García-García *et al*., 1999). Reports questioning the efficacy of the *Bm86* derived from Australian *R.(B) microplus* against *R.(B) microplus* strains in South America has supported the questioning of the validity of the taxonomic status of *R.(B) microplus* in Australia (Labruna *et al*., 2009). In Argentina, the original vaccine, based on *Bm86* was cloned in Australia from Yeerongpilly strain ticks and had a reported very low protective efficacy (10-15%) against the Argentinean Strain A of *R.(B.) microplus*. The Argentinean strain A *Bm86 w*as subsequently cloned and expressed in *Pichia pastoris* and named *Bm95*. Testing of this vaccine was alleged to yield a great improvement in efficacy of the vaccine on herds with low protection from Bm86 (García-García *et al*., 2000).

Differences in the *Bm86* sequences have been reported between *R.(B) microplus* from the USA, Australia and Brazil (Freeman *et al*., 2010) further supporting the theory of geographic variation. Another study aimed to determine the level of *Bm86* polymorphisms within Thailand in order to ascertain a relationship between sequence variation and vaccine effectiveness on Thai *R.(B) microplus* (Kaewmongkol *et al*., 2015). This study found that sequences grouped based on their regional location within the country. All sequences were found to be distinct from those reported in South America for *R.(B) microplus* and only one group of Thai ticks grouped closely with the original Australian Yeerongpilly strain (Kaewmongkol, *et al*., 2015). This study was the first to demonstrate regional

variation in the *Bm86* gene coherently within a country and demonstrates the need for similar investigation in other locations.

It has been claimed (de la Fuente *et al*., 2000; Ali *et al*., 2016; García-García *et al*., 2000; García-García *et al*., 1999) as well as discussed in informal discussions and conferences, that the variation in vaccine efficacy that has been observed was partly or fully attributable simply to the fact that distinct species are being considered, without reference to whether sequence variation conformed differences among the proposed species. This concept was further driven by the proposal for reinstatement of the species *R.(B) australis* (Estrada-Peña *et al*., 2012).

At present, the relationship between *Bm86* sequence and function is unclear. Only transcript sequences and expressed sequence-tag (EST) are available in the public domain, as of yet there is nothing available on the *Bm86* gene genomic sequence. Because all the work undertaken so far on the gene has made use of cDNA synthesized from RNA and the existing sequences in public database show indications of multiple splice variants in this gene, we decided to examine genomic DNA sequence in a limited region of the gene, to determine whether there was variation in the gene that clustered according to the proposed new classification.

In this study, we attempted to amplify the *Bm86* gene from genomic DNA extracted from three *Boophilid* tick species; *R.(B) microplus* (multiple locations), *R.(B) annulatus* (USA) and *R.(B) decoloratus* (South Africa). The locations covered for *R.(B)* microplus were; Australia, South Africa, South America (Jaguar, Mozo, Sao Gabriel and Juarez), North America (Mexico, Texas) and Thailand (table 2.1).

Proposed structure of *Bm86*

Bm86 is an epidermal-growth factor (EGF)-like membrane glycoprotein found on the surface of cells in the midgut of ticks (Gough and Kemp 1993). Its function and structure is not fully understood, however there are some theories regarding the architecture of this gene. A basic glycoprotein comprises a polypeptide chain made up of the intracellular domain, the hydrophobic amino acid domain (inserted

in the lipid bilayer of the cell membrane) and an extracellular domain, attached to a carbohydrate (Montreuil, 1980) (figure 5.1).

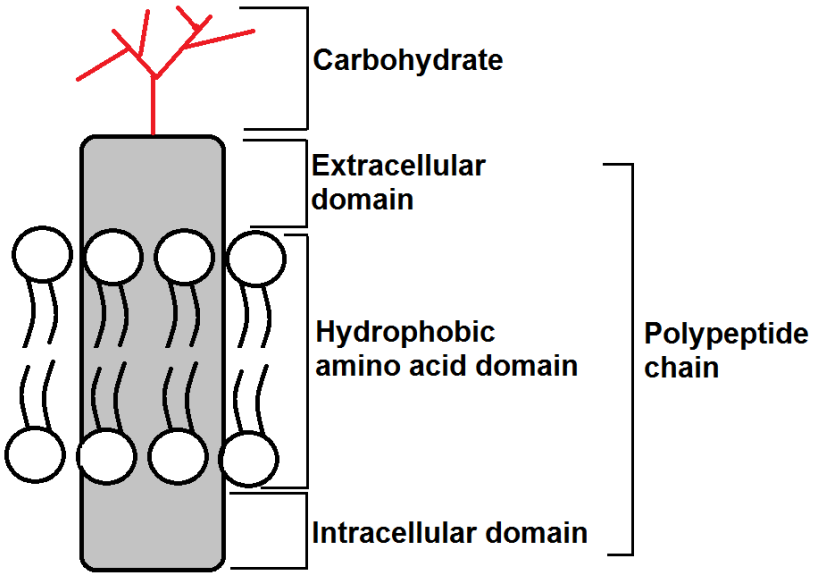


Figure 5.1: The structure of a basic glycoprotein. (Source: Authors own drawing)

The research group that first pioneered studies behind the discovery of *Bm86* first suggested its structure. Initially it was found that the amino acid sequence shares similarities with previously characterized proteins; a putative protective antigen from *Plasmodium falciparum* (homology was short but significant) and an epidermal growth factor precursor molecule, with which it shared an extended and highly significant homology (Willadsen *et al*., 1989). Another feature that was observed was a high proportion of cysteine residues, which is characteristic of extracellular proteins, in particular cell surface proteins (Willadsen *et al*., 1989). The amino acid configuration of *Bm86* was predicted in more detail in the same year. Based on the cDNA sequence, it was thought that *Bm86* contains 650 amino acids, made up of a 19 amino acid signal sequence, a 23 amino acid hydrophobic region (adjacent to the carboxyl terminus) and a repeated pattern of 6 cysteine residues. The latter feature alluded to the potential presence of several epidermal growth factor-like domains, and further confirmed *Bm86* similarity with EGF-like proteins (Rand *et al*., 1989). It wasn't until 1993 did the location of *Bm86* get confirmed. By using immunogold labeling, it was found that *Bm86* was located on the microvilli of digest cells (Gough and Kemp, 1993). With the location confirmed, it has been possible to narrow the theories on what the function of

Bm86 is, however further work was required on its structure. The nature of the anchorage of *Bm86* to the digestive cells has been discovered to be via a glycosyl-phosphatidyl inositol (GPI) linkage to the extracellular surface of the cell (Richardson *et al*., 1993). This study also further confirmed the presence of 66 cysteines, indicating the presence of multiple epidermal growth-like domains (Richardson *et al*., 1993).

The proposed structure of *Bm86* is that it is made up of a signal peptide region followed by two EGF-like domains, a partial EGF-like domain, six EGF-like domains and then a GPI anchor. Potential O-linked carbohydrate additions are suggested to be located close to the GPI anchor and potential N-linked carbohydrate additions are proposed at EGF-like domain two and three, partial EGF-like domain and in between EGF-like domain six and seven (Nijhof *et al*., 2010) (figure 5.2).

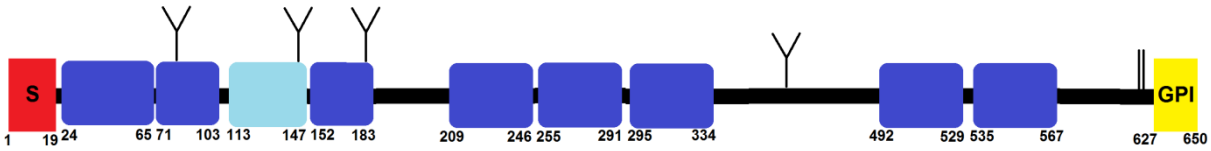


Figure 5.2: Proposed structure for *Bm86.* Red box 'S': signal peptide, dark blue boxes: epidermal growth factor (EGF)-like domains, light blue box: partial EGF-like domain, yellow box 'GPI': glycosyl-phosphatidylinositol (GPI) anchor, vertical line: potential O-linked carbohydrate additions, 'Y': potential N-linked carbohydrate additions. The numbers corresponds to the amino acid positions of the start and end of each protein domain. Reproduced from (Nijhof *et al*., 2010).

Materials & Methods

There are a number of published *Bm86* sequences in the public domain (appendix table A3). In this part of the study both partial and complete sequences were imported from the National Center for Biotechnology Information (NCBI) GenBank, and aligned using CLC Genomics Workbench 7.5.2. All phylogenetic trees were created using MEGA 6.06 software using Construct/test neighbor-joining tree setting. Phylogeny was tested using the Bootstrap method (with a 500 Bootstrap replication). Any branches with a value of less than 50% were considered unresolved and the branch was collapsed.

Tick samples

220 samples were used in total, comprising 180 putative *R.(B) microplus* from 9 locations table 2.1), 20 *R. (B) decoloratus* from South Africa and 20 *R.(B) annulatus* from Texas. Two cell lines (BME/CTVM 2 and BME/CTVM 5), derived from *R.(B) microplus*, was used as a positive control throughout the primer optimization process. The tick cell lines were provided by the Tick Cell Biobank at The Pirbright Institute. The instar of origin, geographical origin and acaricide resistance status of the parent tick for all cell lines used are listed in table 5.1.

Table 5.1: Tick cell line used in this study, including information on tick cell lines; BME/CTVM 6, 23 and 30 covered in the discussion.

Cell line	Instar of origin	Year initiated	Geographical origin (strain)	Resistance status	Reference
BME/ CTVM 2	Embryo	1983	Costa Rica (Paquera)	Susceptible	(Bell-Sakyi, 2004)
BME/ CTVM 5	Embryo	1983	Colombia (Paso Ancho)	Resistant to organophosphates, organochlorines and Amitraz	(Bell-Sakyi, 2004)
BME/ CTVM 6	Embryo	1983	Colombia (Paso Ancho)	Resistant to organophosphates, organochlorines and Amitraz	(Bell-Sakyi, 2004)
BME/ CTVM 23	Embryo	2005	Mozambique	Unknown	(Alberdi *et al*., 2012a)
BME/ CTVM 30	Embryo	2005	Mozambique	Unknown	(Alberdi *et al*., 2012a; Alberdi *et al*., 2012b)

Primer design

No known genomic sequences are available and the previous work undertaken on this gene were all done using cDNA (appendix table A3). All available published sequences were used to create an alignment, and were found to be variable in

length. In particular identification of spliced exons in the alternate transcript in the Mozambique strain EU191620 (Canales *et al*., 2008) was observed. By using M29321 (Rand, 1989) as a reference sequence a section from 1-585bp was selected as a target region for amplification. This segment was searched using BLAST against the whole genome shotgun (wgs) contigs (overlapping reads with no gaps), restricted to the *Rhipicephalus* genus (taxid 34630) (figure 5.1).

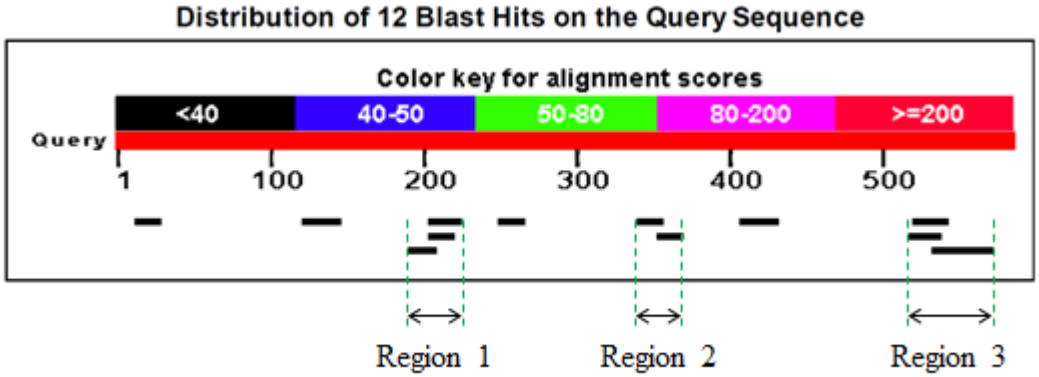


Figure 5.3: BLAST results for *Bm86* isolate GenBank accession number: M29321 (Rand *et al*., 1989) against all available ESTs. Three within exon regions identified labelled as region 1-3. The positions of region 1: 192-226, 2: 340-370 and 3: 517-571.

Three regions where sequences can be seen to overlap were identified to lie within exons (figure 5.3). It is not known how long the exons/introns are or where their boundaries lay, therefore only the sequences within the three regions identified were used to design the primers as they were most likely to produce *Bm86* gene specific primers, without amplifying unwanted DNA from other genes. To attempt to check that this was unlikely to occur, the designed primers (table 5.2) were checked using primer-Blast for specificity. It is also likely that an intron or introns of unknown length or lengths might exist between the identified regions so more than one set of manually designed primers, in the form of degenerate primers, would be required to increase the probability of successful PCR amplification of *Bm86* genomic sequence.

Table 5.2: Manually designed *Bm86* primer sequences and regions based on figure 5.3

Oligo Name	Sequence (5'-3')	Region of location (figure 4.1)
Bm86-F1	ATGTACTTCAATGCTGCTG (19)	Within region 1
Bm86-R1a	CGTCCGATGCTTCGCAG (17)	Around region 2
Bm86-F2a	GACGATCTAACGCTACAATGC (21)	Around region 2
Bm86-F2b	GACACTCTAACGCTACAATGC (21)	Around region 2
Bm86-R2	AGACACGTGGTAGGGACAC (19)	Between region 2 and 3 (closer to region 3)

F2a and F2b are similar but not identical primers designed to be used together as degenerate primers allowing variations in the target gene to be amplified. The degenerate primers are designed to cover the variations expected from all four species *R.(B) microplus*, *R.(B) australis*, *R.(B) annulatus* and *R.(B) decoloratus.*

PCR optimisation using Qiagen HotStar Taq plus

The newly designed *Bm86* primers were tested on all three species *(R.(B) microplus* cell line CTVM2, *R.(B) annulatus* and *R.(B) decoloratus* all extracted from own samples) using the Qiagen HotStar Taq plus DNA polymerase kit. Each 25 µl reaction volume consisted of 2.5 µl of 10X buffer, 0.5 µl of 10 mM dNTPs, 0.25 µl each of 10 mM forward and reverse primers and 0.25 µl of HotStar Taq. Optimization of PCR was also tested with additional Q solution added to each reaction as recommended by manufacturer's instructions for potentially high GC-rich content.

Both reaction mixes (with and without Q solution) were tested at annealing temperature of 55ºC, 57ºC and 59ºC.

PCR Optimisation using Phusion

The master mix was set up using the Thermo kit recipe (table 5.3). The PCR protocol was set up on the thermocycler; 30 seconds at 98ºC, 10 seconds at 98ºC, 30 seconds at 55ºC, 30 seconds at 72ºC, steps 2-4 repeated 40 times, 10 minutes at 72ºC.

Table 5.3: Master mix recipe for 50 µl for main DNA plates using the Thermo scientific Phusion Green Hot Start II High-Fidelity DNA Polymerase kit.

Thermo scientific Phusion Green Hot Start II High-Fidelity DNA Polymerase kit master mix- 50 µl
ddH_2O 23.5 µl 5x Phusion green buffer HF 10 µl dNTPs (10mM) 1 µl Forward primer 2.5 µl Reverse primer 2.5 µl Phusion hot start II DNA Polymerase (2 U/µl) 0.5 µl +10 µl DNA

Both *Bm86* primer sets were then tested again on *R.(B) microplus* (cell line BME/CTVM 2), testing the two different 5X Phusion Green buffers; HF and GC, supplied with the kit at different annealing temperatures (52ºC, 57ºC and 59ºC).

Additional published primers were tested using both the Phusion and the Qiagen kits (table 5.4).

Table 5.4: Sequence length and Oligo name of published Bm86 primers, including information on which annealing temperature was chosen for the PCR.

Oligo Name	**Sequence (5'-3')**	**Annealing temperature (ºC)**	**Reference**
Bm86-F3	ATGCGTGGCATCGCTTTGTT (20)	53ºC	(Freeman *et al*., 2010)
Bm86-R5	TTTCAGCAGCATTGAAGTAC (20)		
Bm86-IntFor	ATCGACAAAGCTGCTATTGTCC (22)	58ºC	(Freeman *et al*., 2010)
Bm86-IntRev	TTTCTCTGCTATGAGTCTTGCC (22)		

Agarose Electrophoresis

For all PCR, a 1.5% agarose gel was run with 5 µl of PCR product from each reaction at 140 volts for 40 minutes, with additional 6X Type I loading dye added where necessary.

Results

Currently published sequence information

An initial construct/test neighbor-joining tree using a Bootstrap analysis was inferred using all 100 published *Bm86* sequences (appendix table A3). These sequences were first aligned in CLC Genomics Workbench 7.5.2 and were found to vary in length from 258-2225 bp. The alignment was then exported from CLC Genomics Workbench 7.5.2 as a fasta alignment before being imported into MEGA 6.06 where the construct/test neighbor-joining tree using a Bootstrap analysis was created. Trees were then rooted using *R. appendiculatus* and checked for sequences from the same location that shared the same amount of genetic change within the same group on the tree. These sequences did not differ in SNPs and so were removed from the alignment, leaving one example from each region for each group on the tree. The construct/test neighbor-joining tree was then repeated creating a more coherent tree (figure 5.4).

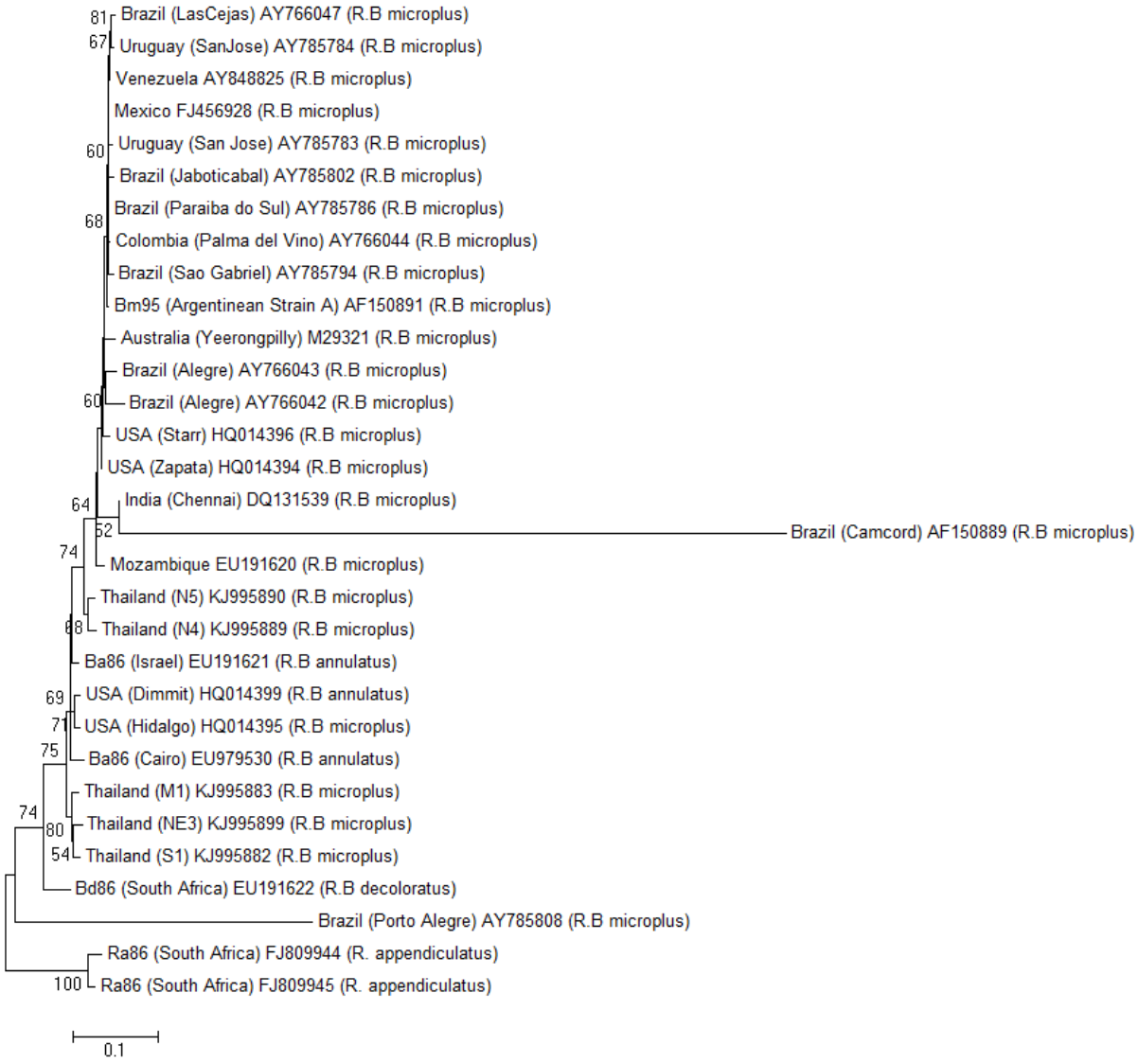


Figure 5.4: Construct/test neighbor-joining tree inferred from unedited published *Bm86* sequences (appendix table A3). Specimens from the same location that demonstrated the same amount of genetic change within the same group has been removed. Percentage bootstrap support is indicated on each node. Only nodes with >50% support show value of support. Strain/isolate is indicated by the first set of brackets where necessary. The tree is rooted using *R. appendiculatus*.

There is no clear differentiation of populations of *R.(B) microplus* based on the *Bm86* sequences used for this tree. There mostly appears to be a mix of geographical location, with the exception of the Thai sequences which tended to group with 80% Bootstrap support (figure 5.4). The South American, Australian and USA sequences tended to share groups and demonstrated a similar amount of genetic change. *R.(B) microplus* and *R.(B) annulatus* didn't appear to be distinguishable from one another based on *Bm86* sequences. *R.(B) decoloratus* was divergent from both *R.(B) microplus* and *R.(B) annulatus* with 74% Bootstrap support. The Brazilian Camcord (AF150889) (de la Fuente *et al*., 2000) and Porto

Alegre (AY785808) (Sossai *et al*., 2005) strains showed a very different amount of genetic change compared to the rest of the tree. When checking the alignment in CLC Genomics Workbench 7.5.2, this difference can be accounted for by how incomplete, and poorly aligned these sequences were compared to the rest.

A second construct/test neighbor-joining tree using a Bootstrap analysis was constructed using the same sequences, but in this instance sequences were edited from position 278 to 1077, resulting in a final sequence length of 794-797 bp. This length represented the shortest, most complete sequence that aligned with the reference sequence M29321 (Rand, 1989). Five partial sequences: India (Chennai) DQ131539 (Anbarasi *et al*., unpublished), Brazil (Camcord) AF150889 (de la Fuente *et al*., 2000), Brazil (Alegre) AY785811 (Sossai *et al*., 2005), Brazil AY785808 (Sossai *et al*., 2005) and Brazil AY785809 (Sossai *et al*., 2005) were excluded. As with the first tree, sequences from the same location that shared the same amount of genetic change within the same group on the tree were removed. The resulting tree has more sequences then the first (figure 5.4), this is because the edited tree appeared to show more genetic diversity then the un-edited.

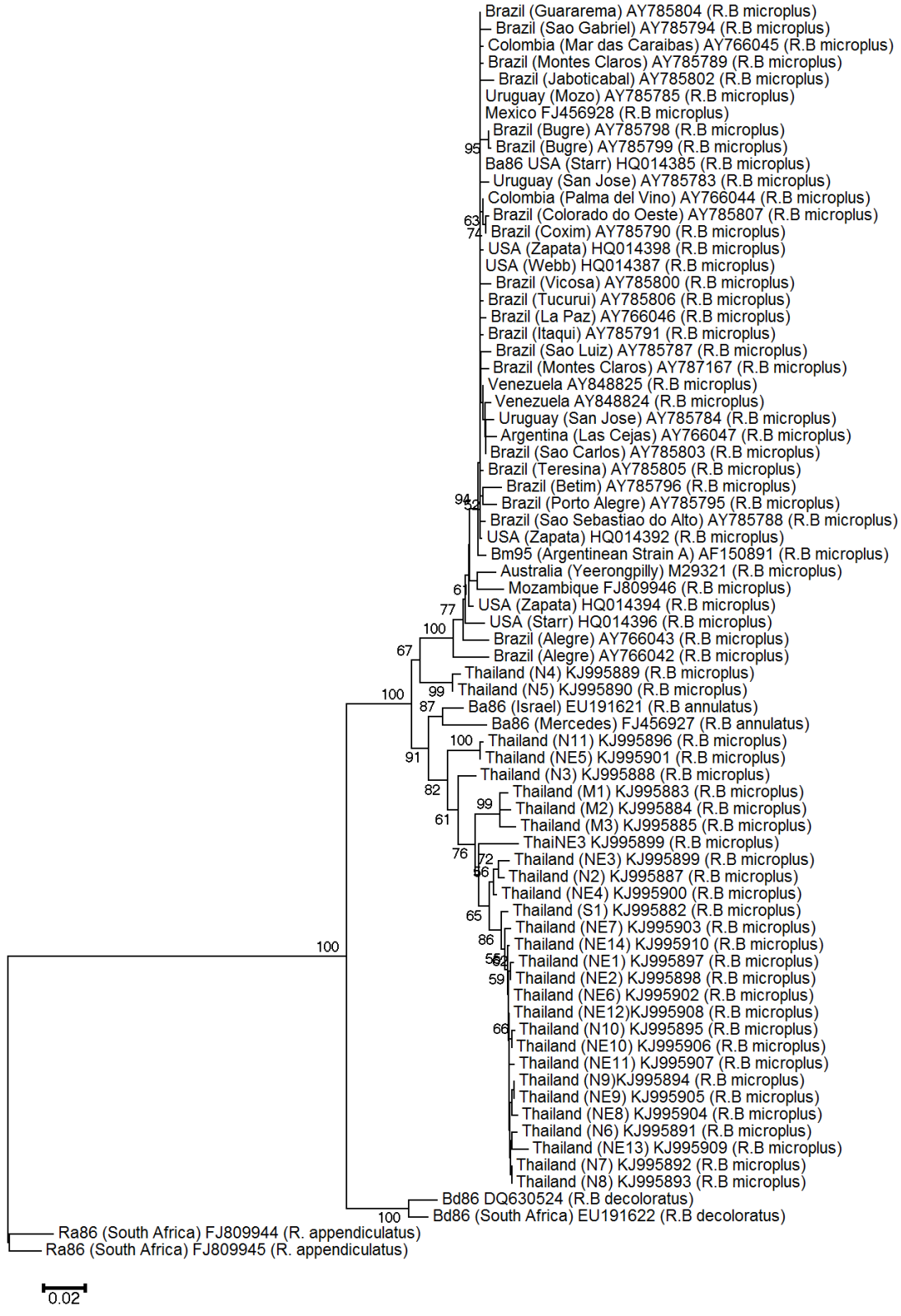


Figure 5.5: Construct/test neighbor-joining tree inferred from edited published *Bm86* sequences (appendix table A3). Specimens from the same location that demonstrated the same amount of genetic change within the same group has been removed. Percentage bootstrap support is indicated on each node. Only nodes with >50% support show value of support. Strain/isolate is indicated by the first set of brackets where necessary. The tree is rooted using *R. appendiculatus*.

As with the first tree, no clear grouping of *R.(B) microplus* can be seen, with the exception of the ticks from Thailand. In this tree, more Thai sequences were included as a greater amount of variation amongst the population became more obvious with the trimmed sequences. Multiple groups can be seen in this tree for the Thai samples, ranging in Bootstrap support from 55-100%. *R.(B) annulatus* grouped with 87% Bootstrap support and appears to share a common ancestor with the Thai samples. *R.(B) decoloratus* grouped away from both *R.(B) microplus* and *R.(B) annulatus* with 100% Bootstrap support. The Australian strain (M29321) diverged with Mozambique (FJ809946) with 61% Bootstrap support (figure 5.6). Variations can be seen in the Brazilian samples, with a number of groups' observable (figure 5.6). Both Bugre strains from Brazil (AY785798 and AY785799) were found to diverge with 95% Bootstrap support. The Colombian strain Palma del vino (AY766044) diverged with the Brazilian strains Colorado do oeste (AY785807) and Coxim (AY785790) with 63% Bootsrap support. Brazilian strains Colorado do oeste (AY785807) and Coxim (AY785790) further diverged from Colombian strain Palma del vino (AY766044) with 74% Bootstrap support. A final grouping of Brazilian strains Betim (AY785796) and Porto Alegre (AY785795) grouped with 52% Bootstrap support and with such a low percentage can be considered unresolved.

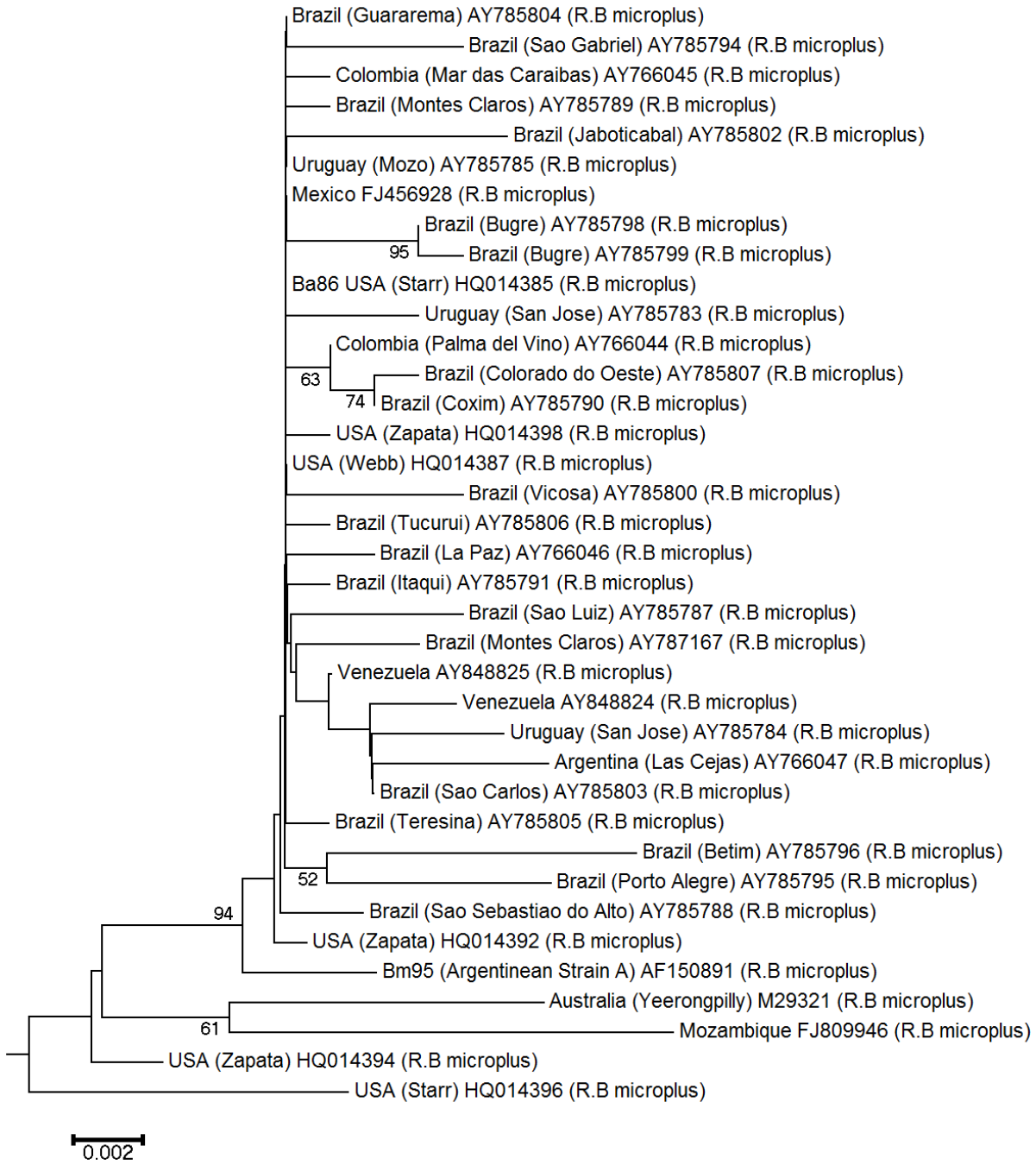


Figure 5.6: Close up of Construct/test neighbor-joining tree inferred from edited published Bm86 sequences for South America, USA and Australia (figure 5.5). Specimens from the same location that demonstrated the same amount of genetic change within the same group has been removed. Percentage bootstrap support is indicated on each node. Only nodes with >50% support show value of support. Strain/isolate is indicated by the first set of brackets where necessary. The tree is rooted using *R. appendiculatus* but not shown in this close up.

A number of insertions and deletions were also observed in these trimmed sequences. An insertion of 3bp was found in both *R. appendiculatus* sequences starting at position 424 (figure 5.7). This insertion was different for both

sequences; AGC for FJ809944 (Nijhof *et al*., 2009) and AAG for FJ809945 (Nijhof *et al*., 2009).

Figure 5.7: Position of insertions for both *R. appendiculatus* sequences, indicated by the red box.

A section of 6 bp deletion at position 311, was observed in four *R.(B) annulatus* sequences; EU979530 (Shahein *et al*., unpublished), HQ014401 (Freeman *et al*., 2010), HQ014400 (Freeman *et al*., 2010) and HQ014399 (Freeman *et al*., 2010) (figure 5.8). This 6 bp deletion was also observed in the USA *R.(B) microplus* sample HQ014395 (Freeman *et al*., 2010).

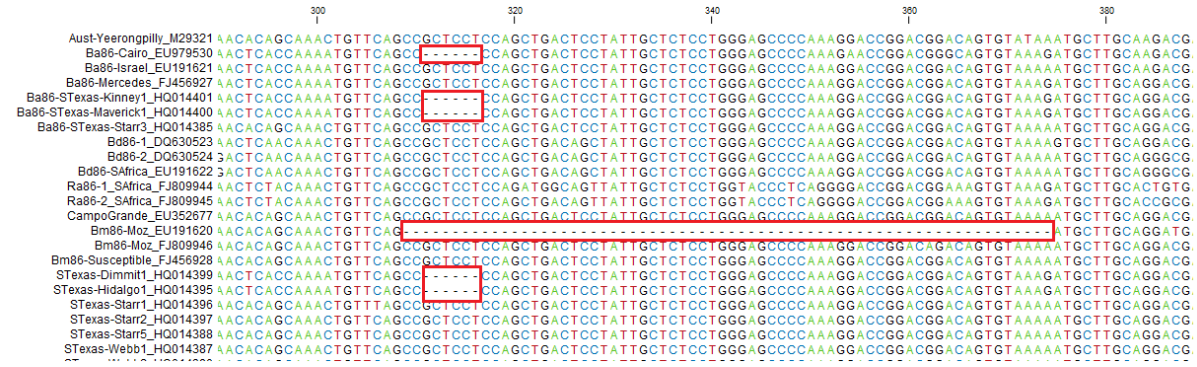


Figure 5.8: Position of deletions for six *R.(B) microplus* sequences, indicated by the red box.

The aligned sequences showed a large amount of variation, with approximately 350 different SNPs. There was evidence of alternative splicing in the Mozambique strain (EU191620) from 554-619. Insertion/deletions (indels) were also observed for a number of *R.(B) annulatus* (EU979530, HQ014401 and HQ014400) and North American *R.(B) microplus* (HQ014399 and HQ014395) from 556-561 (figure 5.8). There was also a large deletion between 310 and 375 observed in one of the Mozambique sequences EU191620 (Canales *et al*., 2008).

PCR Optimization using Qiagen HotStar Taq plus

The first stage in end-point polymerase chain reaction (PCR) optimization required the testing of the newly designed *Bm86* primers on all three species (*R.(B) microplus* cell line BME/CTVM2, *R.(B) annulatus* and *R.(B) decoloratus* both extracted from own samples)

The *Bm86* set of primers that was found to work best was *Bm86* primer set 1 (Bm86-F1 + Bm86-R1a) which produced a strong single band for *R.(B) microplus* at all three annealing temperature when master mix 1 was used (figure 5.9). *Bm86* primer set 2 (Bm86-F2a/ Bm86-F2b + Bm86-R2) was found to produce a strong band for *R.(B) microplus* at 57ºC when master mix 2 was used. When master mix 1 was used, multiple bands were present. From this it was decided that the *Bm86* primer set 1 would be used on the main DNA plates.

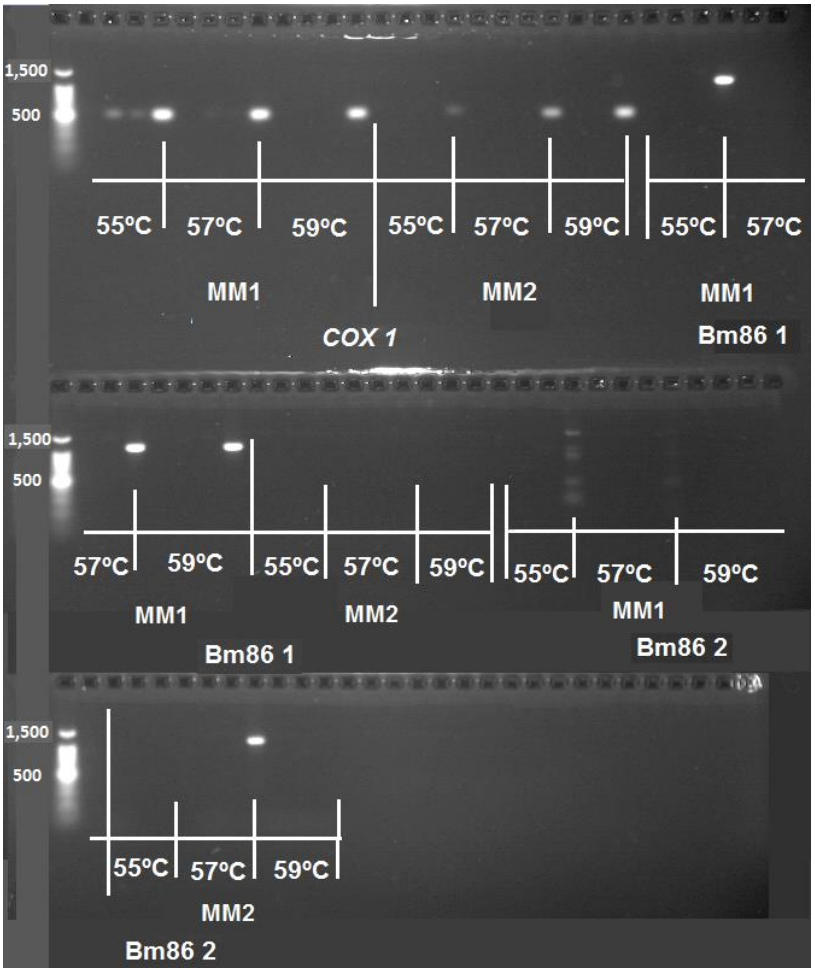


Figure 5.9: Gel photo showing the results obtained from first round of PCR optimization for both Bm86 and *COX1*. 'MM 1' = master mix 1, 'MM2' = master mix 2, 'Bm86 1' = Primer set F1 +R1a and 'Bm86 2' = Primer set F2a/F2b + R2.

The *Bm86* primer set (Bm86-F1 + Bm86-R1a) was repeated using master mix 1 with an annealing temperature of 52ºC in an attempt to obtain bands for *R.(B) annulatus* and *R.(B) decoloratus* (figure 5.10).

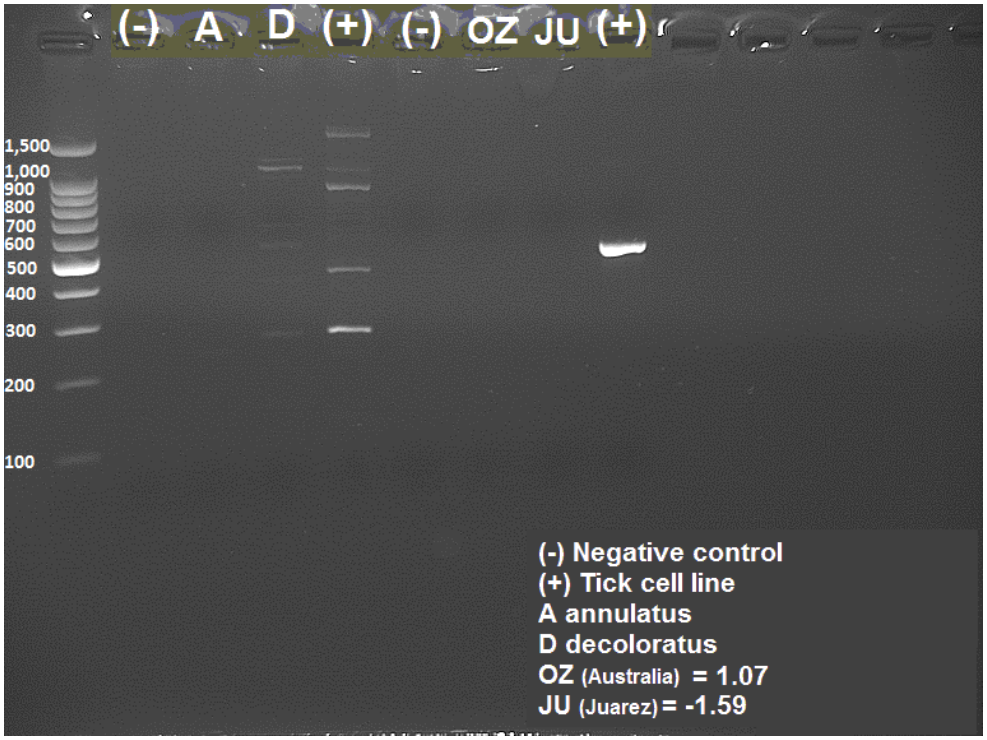


Figure 5.10: Gel photo showing the results obtained from second round of PCR optimization for both *Bm86* (primer set F1 +R1a) and *COX1.*

For this *Bm86* primer set, it was found that the lower annealing temperature was too low and resulted in multiple bands for *R.(B) decoloratus* and *R.(B) microplus* (figure 5.10).

PCR Optimisation using Phusion Green Hot Start II

It was then decided to use a Thermo scientific Phusion Green Hot Start II High-Fidelity DNA Polymerase kit master mix and protocol. The kit did not require added $MgCl_2$, was designed to overcome PCR inhibition problems caused by ethanol and required a shorter PCR cycle time, as well as already having dye in the master mix which would require no additional dye to run a gel.

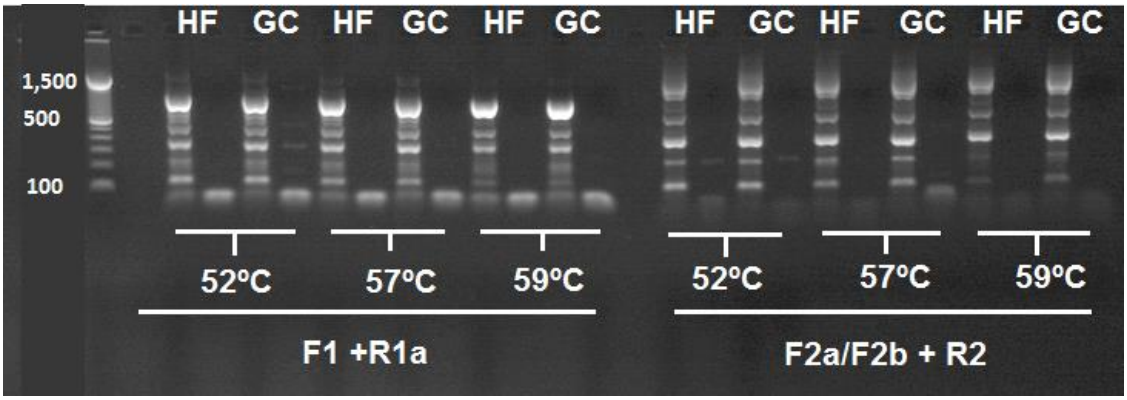


Figure 5.11: Gel photo showing the results obtained from third round of PCR optimization for both *Bm86* primer sets; F1 +R1a and F2a/F2b +R2. ('HF' = 5X Phusion Green buffer HF and 'GC' = 5X Phusion Green buffer GC). Results are set in pairs where the first well contains *R. (B) microplus* (cell line CTVM 2) with tested buffer and second well contains negative control.

Both *Bm86* primer sets yielded multiple bands when the Phusion kit was used, regardless of buffer and annealing temperature used (figure 5.11). The Qiagen kit was re-tested with *R.(B) microplus* (cell line CTVM2), *R.(B) microplus* (cell line BME/CTVM 5) and two extracted samples; OZ (Australia) and Mex (Mexico). Results from that PCR (figure 5.12) showed that the Qiagen kit, when used with an annealing temperature of 55 ºC, yielded strong single bands at the correct length for the two *R.(B) microplus* cell lines; however, no bands were seen for the Australian or Mexican samples.

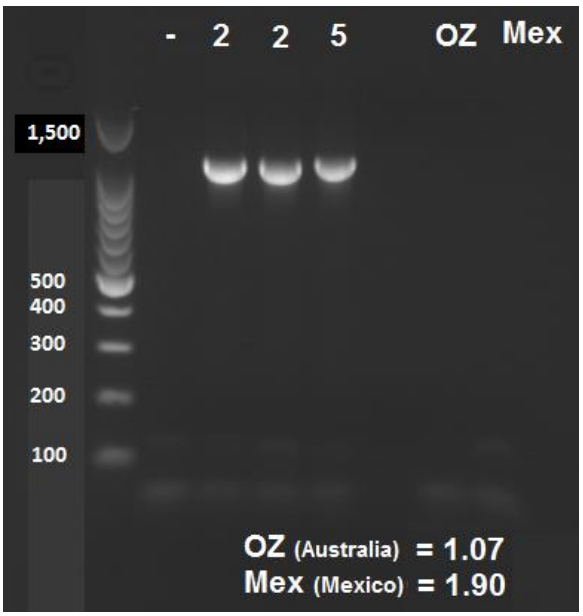


Figure 5.12: Gel photo showing the results obtained from fourth round of PCR optimization for *Bm86* primer set F1 +R1a ('-' = negative control, '2' = contains *R. (B) microplus* cell line CTVM 2 of which there are two and '5' = contains *R. (B) microplus* cell line CTVM 5).

The final step in optimizing the *Bm86* protocol was to attempt the PCR using two primer sets used in published work (table 5.4) using both the Qiagen and Phusion protocols described previously.

The Phusion kit once again produced multiple bands; however, no bands were observed when the Qiagen kit was used (figure 5.13).

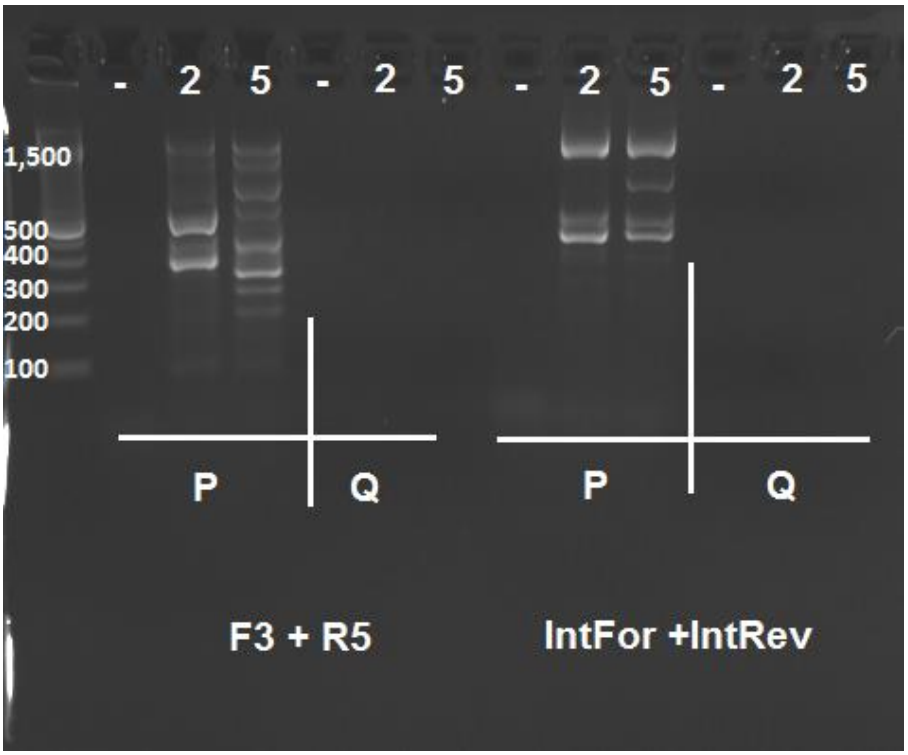


Figure 5.13: Gel photo showing the results obtained from fifth round of PCR optimization for Bm86 primer sets F3 +R5 and IntFor + IntRev. '-' = negative control, '2' = contains *R. (B) microplus* cell line CTVM 2 , '5' = contains *R. (B) microplus* cell line CTVM 5, 'P' = Phusion protocol and master mix used and 'Q' = Qiagen protocol and master mix used.

Primer set Bm86-F1 + Bm86-R1a proved to be the most consistent at producing bands when used with the Qiagen kit at an annealing temperature of 55ºC, however when the primer set was used on the main DNA plates it failed with almost all the extracted DNA. It was concluded that the method by which the ticks were preserved resulted in some degradation in the genomic DNA, which is supported by the low-quality scores observed from the NanoDrop readings (chapter 2).

Discussion

Our attempts to obtain *Bm86* sequence data from gDNA extracted from ethanol preserved ticks collected for this study were not successful. This was likely a result of inadequate DNA quality. However, an analysis of published cDNA sequences revealed a large number of polymorphisms which suggests that the primer sets designed in this study were not suitable.

For the published sequence data, grouping of *Bm86* from *R.(B) microplus* according to geographical location was only evident for Thailand (figure 5.5). There was also a large amount of sequence variation between the Thai samples, as reported previously (Kaewmongkol *et al*., 2015). *R.(B) decoloratus* and *R. appendiculatus* demonstrated distinct groups from *R.(B) microplus* and *R.(B) annulatus*. *R.(B) annulatus* also formed a distinct group with 87% Bootstrap support, but was also found to group closely with the Thai samples. Again, this finding was previously reported (Kaewmongkol *et al*., 2015).

The amount of variation observed in the rest of the *R.(B) microplus* specimens indicates that the gene is highly polymorphic, and the Australian isolate is divergent from the rest of the samples (>50% Bootstrap support). Whether this divergence is enough to support the findings of García-García *et al*., 1999; 2000 within which it was proposed that a vaccine derived from Australian ticks lacked efficacy on some South American tick populations is not yet convincing. The fact that there is evidence from previous studies indicating a similar efficacy of the Australian derived *Bm86* vaccine on other species including *R.(B) decoloratus*, *H. anatolicum anatolicum* and *H. dromedarii* (de Vos *et al*., 2001), also disaudes the concept that efficacy is linked to sequence variation based upon species.

Previous work demonstrating the phylogenetic relationships between species and region grossly shows concensus with the trees created in this study (Nijhof *et al*., 2010). These studies indicated that *Bm86* grouped mainly based on species (*R.(B) microplus, R.(B) annulatus, R.(B) decoloratus* and *R. appendiculatus*) but regional variation was not obvious (Nijhof *et al*., 2010). Some regional variation was observed with Mexican and Brazilian samples grouping away from Mozambique and Australian samples with 96% Bootstrap support. This was again similar to what was found in this study (figure 5.6), however the work done here

included many more sequences, acting to shed light on the extent of variation in the *Bm86* gene. The Indian Chennai isolate was previously found to have 97% homology with the reference Australian strain (Anbarasi *et al*., 2014), however when aligned with all the sequences, it was found to only be partial and was omitted. Polymorphisms have also been documented in South American strain of *R.(B) microplus* (Sossai *et al*., 2005). In this study, it was found that Alegre and Betim strains were the most divergent from both the *Bm86* reference and *Bm95*. In this study, Alegre and Betim strains diverged with only 52% Bootstrap support but with such a low level of support were considered unresolved. It is clear there is still a large amount of information on the *Bm86* gene still lacking in annotation. Although in this study we have attempted to scratch the surface, it is clear that there is a need for further investigation into the variation of *Bm86* both regionally within countries and between geographically separate locations.

There are a number of different primers that have been used in the *Bm86* literature (appendix table A3); however, these have all been used on cDNA. In this study, these primers along with a set that were designed in-house were tested on genomic DNA extracted from tick samples and from established tick cell lines. With regard to the extracted tick DNA, all primer sets failed and it was concluded to be due to the quality of the DNA extracted, however without knowledge of the actual gene structure of *Bm86*, it is difficult to design primers suitable for this study. The primers were also used on six *R.(B) microplus* tick cell lines from the Tick Cell Biobank at The Pirbright Institute, in another project within the group. It was found that for two of the cell lines (BME/CTVM23 and BME/CTVM30) (table 5.1) sequencing revealed further nucleotide variation, despite only one band observed on the gel (figure 5.14). This could have been caused by the fact that the cell lines are composed of multiple individuals, and as previous studies have indicated (Kaewmongkol *et al*., 2015), there can be variation in the *Bm86* sequence from ticks of the same species from the same geographical location.

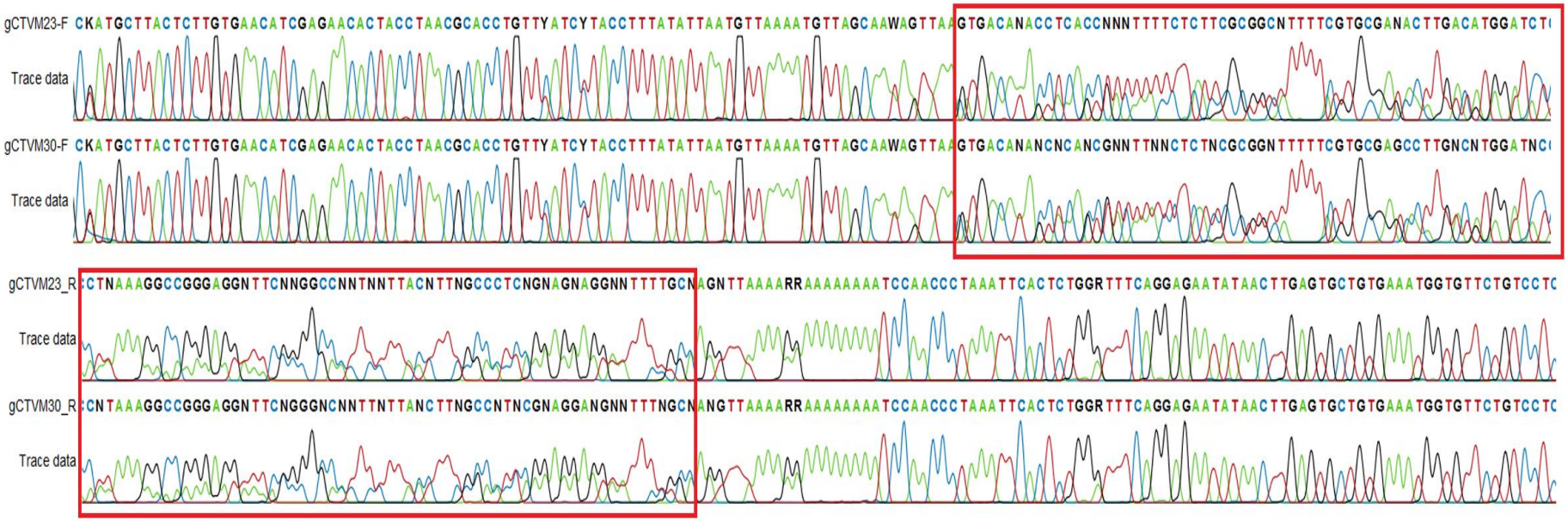


Figure 5.14: Evidence of multiple sequences taken from a section of the *Bm86* gene for tick cell lines CTVM23 and CTVM30 shown by red boxes from forward primer (top) and reverse primer (bottom).

The sequenced data from the cell lines was aligned with the published *Bm86* sequences (appendix table A3). As with the published data, a large number of SNPs and indels were observed in the tick cell line sequences (figure 5.15). Cell line BME/CTVM2 appeared to only align in partial segments. This could be due to the lack of information regarding the size and arrangement of the *Bm86* gene, particularly the number and size of the exons and introns present in the gene. This is important for designing primers, as with unknown intron/exon length it is impossible to know what size product will be amplified and if it will be specific enough to produce insight into the *Bm86* gene. It is also potentially a transcript variant that hasn't previously been isolated; however, this is just a speculation without further annotation available on the gene.

Figure 5.15: Example of the amount of variation observed in the cell lines CTVM2, CTVM5 and CTVM6. SNPs highlighted in blue.

Conclusion

The aim of this chapter was to describe variation in the *Bm86* gene, and determine whether the variation is in any way partitioned according to species or is independent of it. As expected, it mostly seems to be independent of the proposed species and regional classification, particularly with regard to the proposed *R.(B) australis* vs *R.(B) microplus*. There are researchers who are claiming that the Australian strain derived vaccine does not work on South American ticks because they are different species and have sequence differences. The work done in this study shows that the *Bm86* gene is highly polymorphic, is probably expressed as different isoforms, and previous studies on vaccine efficacy show results that cannot possibly be explained by simple polymorphisms in the cDNA sequences that have been published so far. While there are many alternative explanations for these results, they suggest a hypothesis that the apparent high degree of intronic variation, particularly seen in the BME/CTVM cell cultures, might be relatively more important with respect to the expression of the gene and likely vaccine efficacy than variation in the sequence. It is clear that the Bm86 story is a complicated one with evidence of indels, transcript variants and alternative splicing to name but a few problems, the overall issue with working on this gene is the lack of knowledge of the actual gene structure. Without knowing the size of the gene itself, l*et al*one the position/size of its introns and exons, the designing of optimal primers becomes impossible. For work to progress on this gene it needs to be understood in better depth so that all the issues discussed above can be resolved.

CHAPTER 6

Variation in genomic nucleotide sequence of the *βAOR* gene in *R.(B) microplus* ticks from multiple geographical locations

Introduction

R.(B) microplus is a tick that has major economic impact on the cattle industry, infecting an estimated 75% of cattle worldwide (Sales-Junior *et al*., 2005). Due to the combination of the one-host cycle and short generation interval of approximately two months under favourable environmental and climatic conditions, *R.(B) microplus* has become resistant to acaricides at a rapid rate. *Rhipicephalus (Boophilus) microplus* has become resistant to a large number of currently used acaricides across the world (table 1.5), with some strains having developed resistance to all commonly used acaricides (Reid, 1989). Acaricide resistance has evolved via several mechanisms, the most common of which is modification of the target site and metabolic resistance (Abbas *et al*., 2014, Guerrero *et al*., 2014, Guerrero *et al*., 2012).

G-protein-coupled receptors (GPCRs) are seven-transmembrane proteins that convert signals received in the extracellular domain into physiological responses in the cellular domain (Broeck, 2001). In terms of arthropods, the GPCR family is quite extensive with around 270 different types having been described in both *Drosophila melanogaster* and *Anopheles gambiae* genomic research (Brody and Cravchik, 2000, Hill *et al*., 2002). Regardless of the diversity of type and function, the structure of GPCRs is relatively uniform and heptahelical, with 7 trans-membrane domains (Broeck, 2001, Probst *et al*., 1992, Bockaert and Pin, 1999). The extracellular domain contains an N-terminus, followed by seven transmembrane (TM) α-helices (TM1-TM7) connected by three intracellular (IL1-IL3) and three extracellular loops (EL1-EL3), ending with a C-terminus in the intracellular domain (Yeagle and Albert, 2007). The tertiary structure folds in such a way to resemble a barrel, causing the seven transmembrane helices within the plasma membrane to form a cavity, which acts as a ligand-binding domain (Yeagle and Albert, 2007). Ligand binding at the extracellular side of the receptor leads to conformational changes in the cytoplasmic side (Warne *et al*., 2011). The

transmembrane domain (TM) 5 and 6 move out, further into the cytoplasm. Ligand binding has been proposed to create an active receptor conformation that interacts with the heterotrimeric G-protein (made up of alpha, beta and gamma subunits) to form a ternary complex of agonist, receptor, and G-protein (Broeck, 2001). This then triggers a series of events; GTP is exchanged for GDP on the G-protein alpha subunit (Strange, 1999); the G-protein alpha subunit GTP and G-protein beta subunit then dissociate from the receptor; either or both subunits can then go on to regulate the activity of effector proteins (e.g. enzymes) within the cell. Re-association of the subunits occurs when GDP hydrolyses back into GTP (Broeck, 2001).

Previous studies in insects have demonstrated that octopamine receptors are the target for formamidine-based acaricides, such as amitraz (Baxter and Barker, 1999b, Hollingworth and Lund, 1982). In insects, octopamine receptors can be classified into three main types; tyraminergic, alpha-adrenergic-like and beta-adrenergic-like (Evans and Maqueira, 2005). Corley *et al* (2012) identified 8 distinct GPCR from *R.(B) microplus* using a targeted, degenerate primer PCR approach and presented a phylogenetic analysis of the family of genes. They argued that the product of *βAOR* was the most likely target of amitraz. Subsequent work (Corley *et al*., 2013) showed that the entire coding sequence lay within a single exon of 1644 bp, and identified two SNPs, both non-synonymous, within the gene. One of these SNPs (A181T) was tightly associated with resistance to amitraz in an extended field trial. The second SNP (T185C) was from a population of unknown resistance status. Further diversity in this gene was demonstrated by Koh-Tan *et al*. (2016), using *in vitro* cell cultures of ticks. In that study, a 36 bp insertion was identified at position 190 in a cell culture from a resistant isolate of ticks from Latin America. The existance of a new, related gene was also inferred from consistent multiple primer binding sites and ambiguous sequence information.

The overall aim of this study was to quantify regional and taxonomic variation in the *βAOR* gene among *R.(B) microplus* ticks from multiple geographical locations (table 6.1). We also included the North American *R.(B) annulatus* and South African *R.(B) decoloratus* samples by way of comparison.

Materials and methods

Tick specimens and their acaricide resistance status

220 samples were used in total, comprising 180 putative *R.(B) microplus* from 9 locations table 6.1), 20 *R.(B) decoloratus* from South Africa and 20 *R.(B) annulatus* from Texas. All specimens had variable resistance status, in the case of the Australian, Thai and South African ticks, specimens were collected wild from the the field and so resistance status was unknown. The rest of the specimens were selected strains from research groups consisting of ticks direct from the field and maintained in a laboratory environment, as well as lab colony ticks that have been selected and maintained for their resistance status (table 6.1).

Table 6.1: Resistance status of ticks used. For the Australian, Thai and South African ticks the resistance status is unknown as these species were taken from wild populations.

Location/strain	Abbreviation used	Species	Resistance status
Australia	OZ	*R.(B) australis*	Unknown
Thailand	T	*R.(B) microplus*	Unknown
South Africa	AF	*R.(B) microplus*	Unknown
	DM	*R.(B) decoloratus*	Unknown
North America	RA	*R.(B) annulatus*	Susceptible
Central America/ Yucatan	AY	*R.(B) microplus*	**Strong resistance:** Pyrethroids and Ivermectin. **Weak resistance:** Amitraz and Organophosphates.
North America/ Deutch	AD	*R.(B) microplus*	Susceptible
South America/ Sao Gabriel	SG	*R.(B) microplus*	Pyrethroid, Organophosphates and Amitraz
South America/ Mozo	MO	*R.(B) microplus*	Susceptible
South America/ Juarez	JU	*R.(B) microplus*	**Strong resistance:** All compounds Fluazuron susceptible
South America/ Jaguar	JA	*R.(B) microplus*	**Strong resistance:** All compounds

Primers

Primers used in this study were those developed by (Koh-Tan *et al*., 2016) (table 6.2). These primers were designed to produce an amplicon of 183 bp.

Table 6.2: Sequence length and Oligo name of *βAOR* primers (Koh-Tan *et al*., 2016).

Oligo Name	Sequence (5'-3')
βAOR For	GAAATCTGACGGACGAGGAA (20)
βAOR Rev	GCGACACGATGAAGTAGTTG (20)

PCR

The first stage in end-point polymerase chain reaction (PCR) was carried out using the Qiagen HotStar Taq plus DNA polymerase kit. A standard master mix of ddH2O 15 µl, Buffer 10x 2.5 µl, MgCl2 1.25 µl, dNTPs (10 mM) 0.5 µl, forward primer 0.25 µl, reverse primer 0.25 µl, HotStar Taq 0.25 µl was used. PCR conditions were: 95 °C for 5 minutes; 35 cycles of 95 °C for 1 minute, annealing temperature 55°C for 1 minute and 72 °C for 1 minute; and final extension at 60 °C for 30 minutes. A 1.5% agarose gel was run with 5 µl of PCR product from each reaction at 140 volts for 40 minutes to check results after each DNA plate was run. PCR products were then cleaned using the AMPure XP kit before being used in the sequencing reaction.

Sequencing

Plates containing cleaned PCR product were set up for the sequencing reaction using Applied Biosystems kit and protocol. They were then analyzed using the Applied Biosystems 3130XL Genetic Analyzer. All sequences analysis was done using CLC Genomics Workbench 7.5.2 and all phylogenetic trees were created using MEGA 6.06 software using construct/test neighbor joining tree setting, including the published sequences (Corley *et al*., 2013, Koh-Tan *et al*., 2016).

Statistics and other analysis

All statistics were performed using Minitab 17 Statistical software. A chi-square test was run to determine the frequency of mutations in the susceptible and resistant populations. Membrane-spanning domains were predicted using TMHMM Server at the Center for Biological Sequence Analysis, Technical University of Denmark (http://www.cbs.dtu.dk/services/TMHMM/). The predicted 2-D structure of the *βAOR* protein and any deviation due to non-synonymous SNPs was created by TMRPres2D (Spyropoulos *et al.*, 2004).

Results

βAOR was successfully amplified from 85 samples, spanning all the species (*R.(B) microplus, R.(B) annulatus* and *R.(B) decoloratus*), across the geographical regions (table 6.1). The resulting amplicon was 183 bp, however these sequences were trimmed to 177 bp as the beginning and end of the sequences were poorly amplified. Eight distinct SNPs were identified in the samples that were sequenced in this study. Results showed no grouping of *βAOR* sequence according to location or species (figure 6.1). However, a number of different SNPs can be observed, both previously published and new. The majority of samples aligned with the genomic reference strain for *βAOR* (JN974909) (Corley *et al*., 2013) and so individual samples were collapsed into region (i.e. more than one 'SG' sample became Sao Gabriel). Most SNPs were observed in the resistant South American populations of Jaguar, Juarez and Sao Gabriel. Two individuals from Jaguar were found to share a SNP at position T185C with an Australian isolate from a population of unknown resistance status (Corley *et al*., 2013) (figure 6.1). Three ticks from Jaguar and Juarez were also found to share a SNP at position A181T with another Australian isolate (Corley *et al*., 2013).

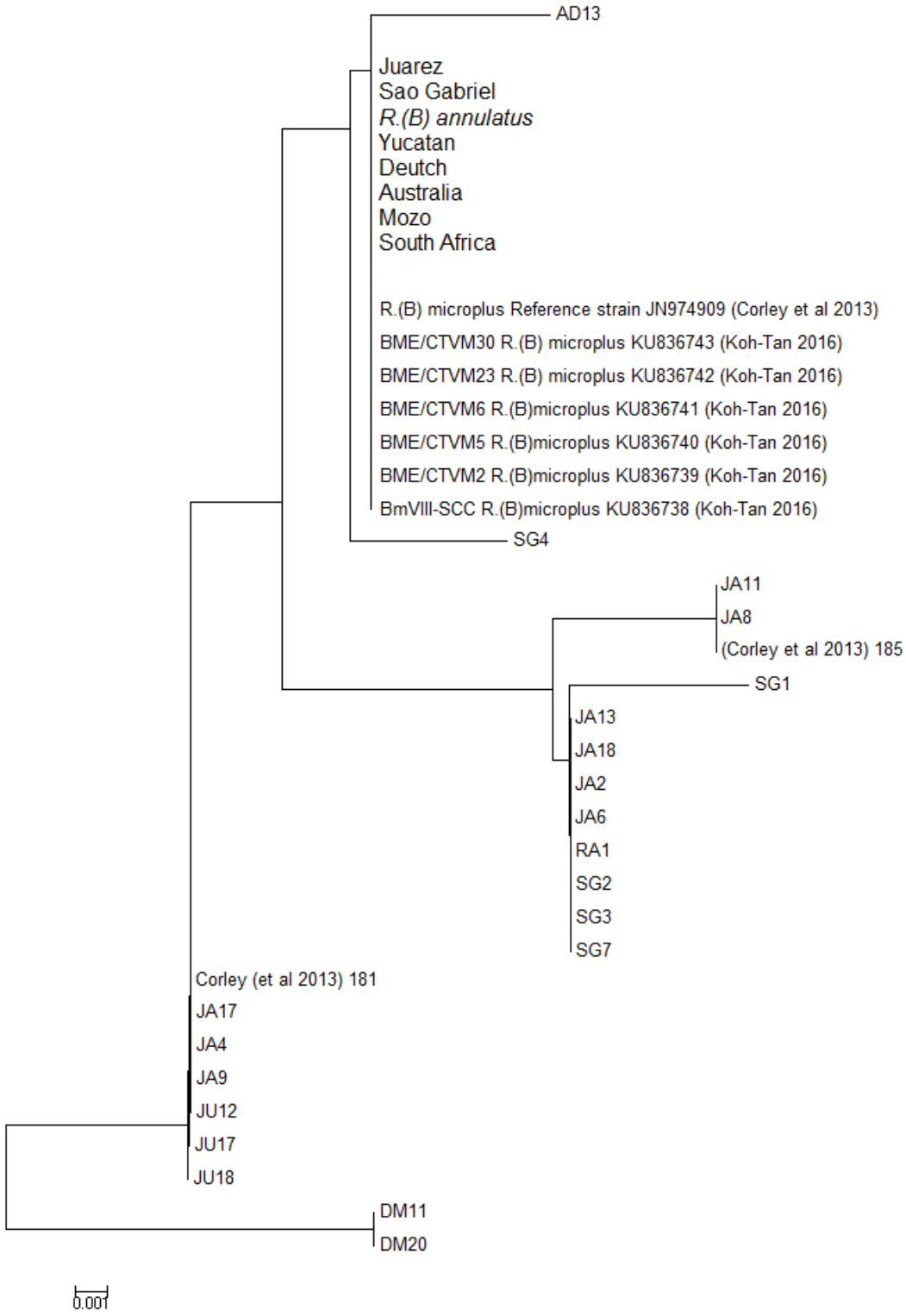


Figure 6.1: Construct/test neighbor joining phylogenetic tree inferred from *βAOR* sequences generated in this study and published sequences (Koh-Tan *et al*., 2016, Corley *et al*., 2013). Tree is unrooted. AD: North American (Deutch) *R.(B) microplus*, SG: South American (Sao Gabriel) *R.(B) microplus*, JA: South American (Jaguar) *R.(B) microplus*, RA: North American *R.(B) annulatus*, JU: South American (Juarez) *R.(B) microplus* and DM: South African *R.(B) decoloratus*.

In total eight different single nucleotide polymorphisms (SNPs) were found in the populations tested (table 6.3). A181T, T185C and A225G were also found in previous studies (Corley *et al*., 2013, Koh-Tan *et al*., 2016). A225G was previously found in *R. appendiculatus*, *R. evertsi* and *R. sanguineus* and this is the first time it has been reported in *R.(B) decoloratus*. T123C, C126T and A225G were found to be synonymous. Despite the 36 bp insertion in the tick cell line BME/CTVM6 (Koh-Tan *et al*., 2016) it was still found to group with the rest of the cell lines, as well as the majority of the sequences generated in this study including reference sequence (JN974909).

Table 6.3: Identified SNPs from *βAOR* sequences. Position from start codon, base pair substitution and consequence are given.

Position from coding sequence start codon	SNP	Amino Acid	Sample
123	T → C	Synonymous	DM11, DM20
126	C → T	Synonymous	DM11, DM20
181	A → T	I → F	JA17, JA4, JU17, JU18, JU12, JA9, DM11, DM20, JA11, JA8
185	T → C	I → T	SG1, SG4, JA11, JA8
225	A → G	Synonymous	DM11, DM20
263	A → C	Y → S	JA13, SG3, JA6, JA2, RA1, SG2, JA18, SG7, SG1
264	C → A	Y → S	JA13, SG3, JA6, JA2, RA1, SG2, JA18, SG7, SG1
265	T → A	F → I	AD13

The SNPs at positions A263C and C264A are both predicted to, cause a tyrosine to serine mutation (table 6.3). T265A caused a change from phenylalanine to isoleucine. These substitutions changed the characteristics of the amino acid in

the chain, from an aromatic compound to a hydroxylic compound in the case of serine and an aliphatic compound in the case of isoleucine.

The 2-dimentional structure of the *βAOR* protein was predicted for the reference protein and then compared with that of the protein with amino acid substitution (figure 6.2-6.4).

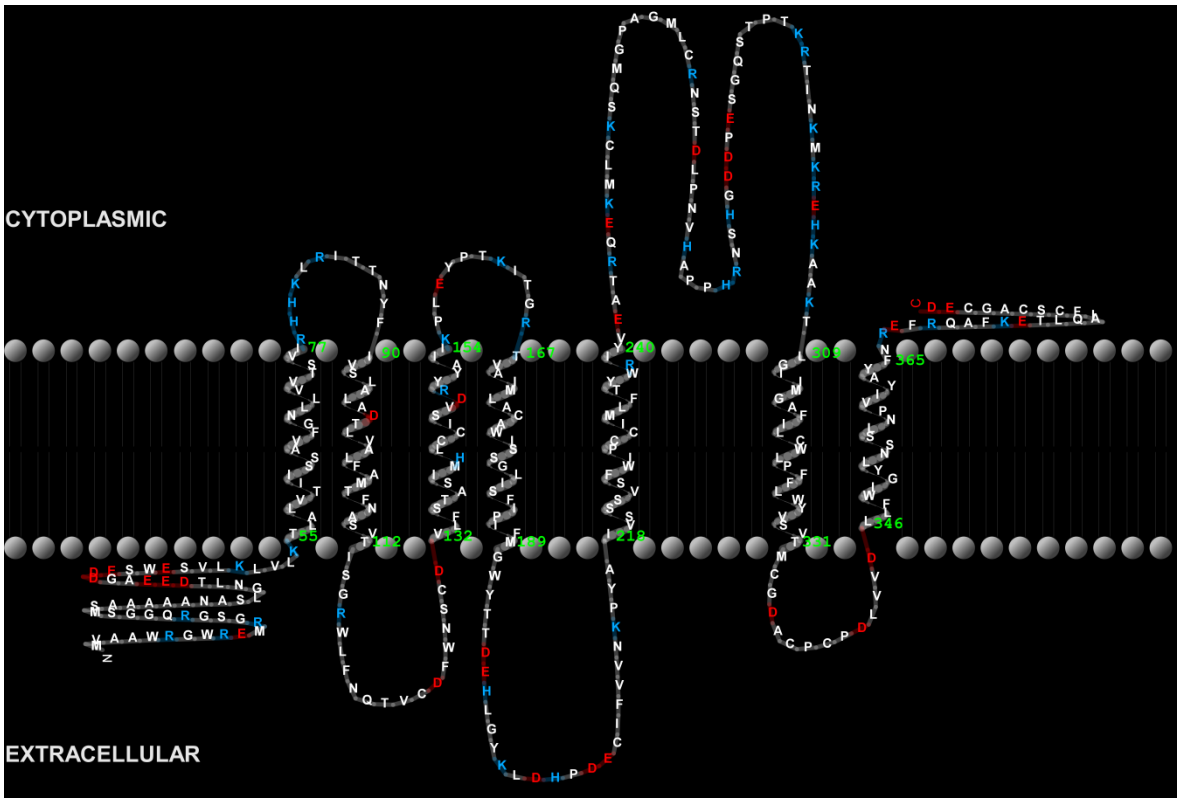


Figure 6.2: Predicted 2-D structure of the *βAOR* protein created using TMRPres2D (Spyropoulos *et al*., 2004).

It was found that A263C and C264A caused the same amino acid substitution, located directly on the first Intracellular loop (IL1) within the cytoplasmic domain, indicated by the red arrow (figure 6.3).

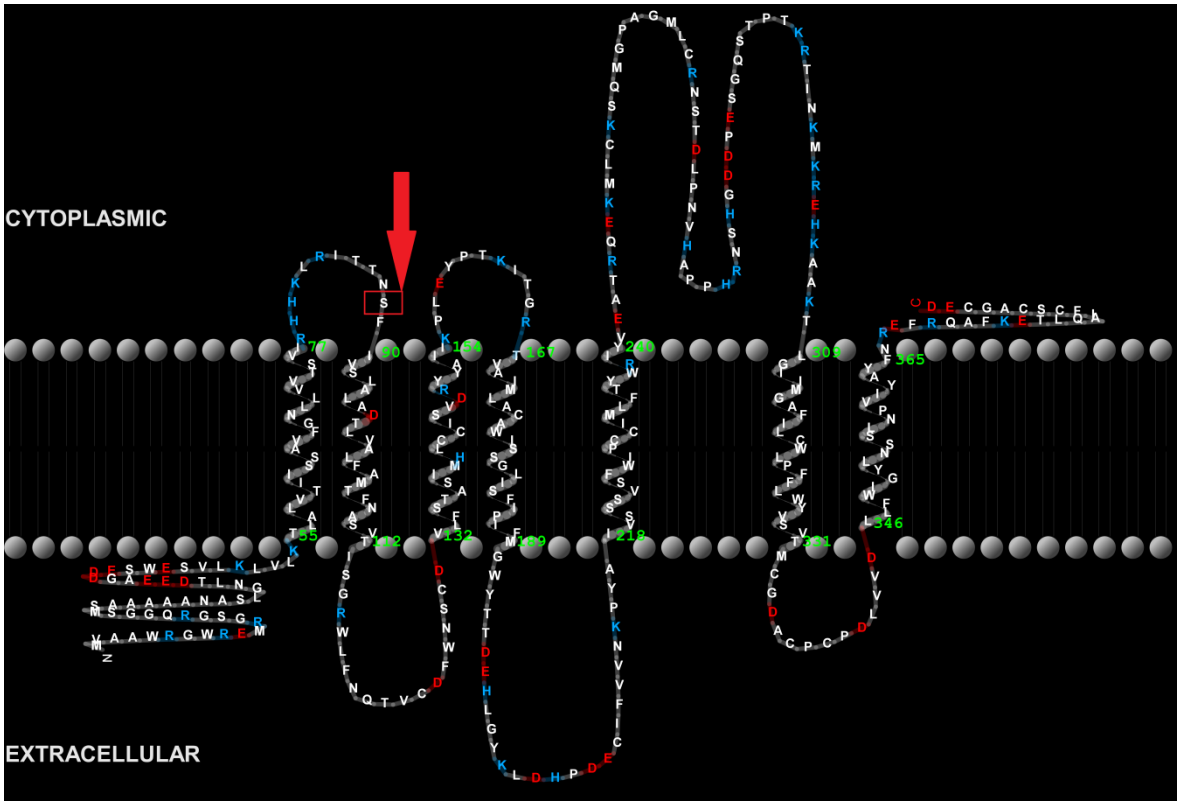


Figure 6.3: Predicted 2-D structure of the *βAOR* protein showing the consequence for A263C and C264A, highlighted in red box, indicated by the red arrow created using TMRPres2D (Spyropoulos *et al*., 2004).

T265A was found to cause an amino acid substitution directly next to A263C and C264A, it too was located directly on IL1, indicated by the red arrow (figure 6.4).

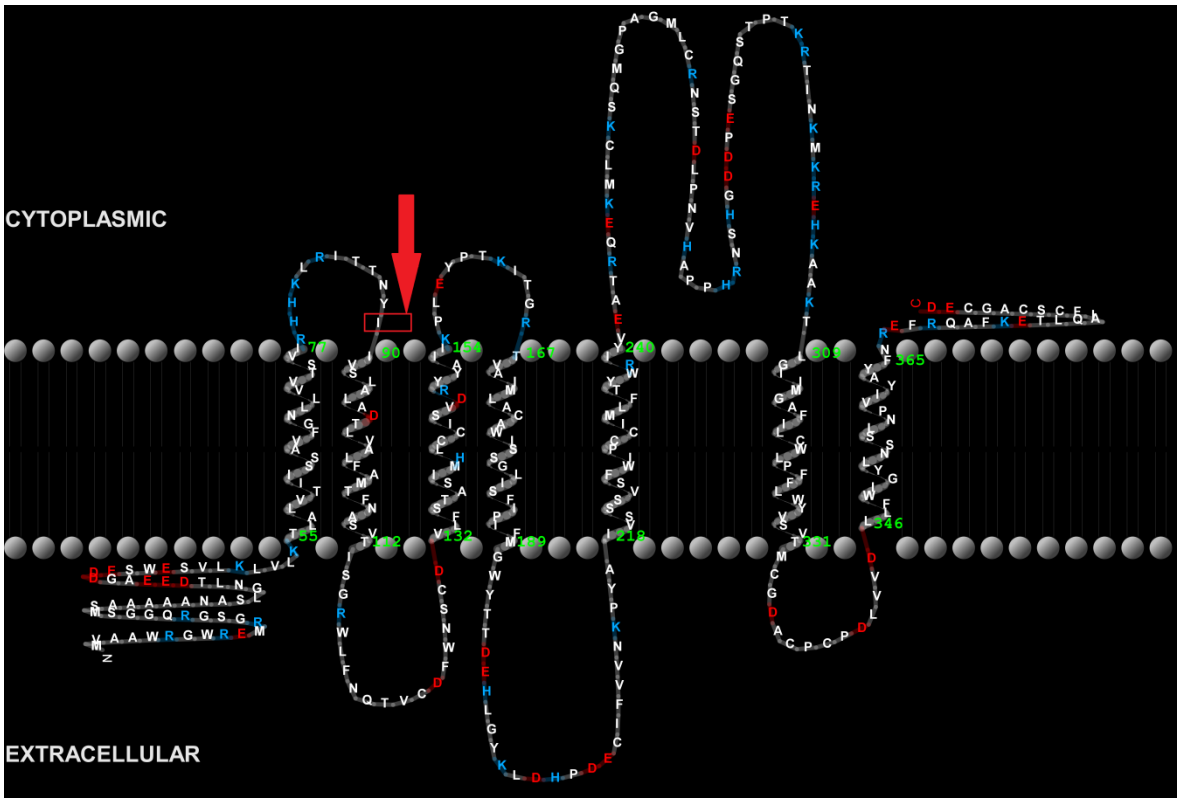


Figure 6.4: Predicted 2-D structure of the *βAOR* protein showing the consequence for T265A, highlighted in red box, indicated by the red arrow created using TMRPres2D (Spyropoulos *et al*., 2004).

Any shape change in the protein structure was not observed by either substitution in the 2-D images created (figure 6.3 and 6.4). This does not discredit the potential for shape change caused by the SNPs as what is presented here is a prediction in one plain, shape change may be occurring in a different field of view.

A chi-square test was performed to determine the frequency of mutations in the susceptible and resistant populations, excluding populations of unknown acaricide resistance status (table 6.4). Results showed that populations that are resistant are highly significantly likely to have one of the SNPs.

Table 6.4: Chi-square test results indicating the frequency of mutations in the susceptible and resistant populations.

Pearson Chi-Square	DF	P-value
22.426	1	0.000
Likelihood ratio Ch-Square	**DF**	**P-value**
25.227	1	0.000

Discussion

Eight putative SNPs were found, three of which had been previously described (Corley *et al*., 2013; Koh Tan *et al*., 2016) but there was no grouping of polymorphisms according to either geographic or proposed taxonomic origin. A225G was previously described by Koh Tan *et al*., 2016 in other species of *Rhipicephalus* (*R. appendiculatus*, *R. evertsi* and *R. sanguineus*) and had not been previously described in *R.(B) decoloratus*. Two other SNPs; A181T and T185C had been previously described by Corley *et al*., 2013 in Australian populations of *R.(B) microplus,* one of which was known to be resistant to amitraz (A181T) and one of which came from a region in which resistance to amitraz is common (T185C). In this study A181T and T185C were reported in two *R.(B) decoloratus* individuals as well as ticks from the South American isolates Jaguar, Juarez and Sao Gabriel, all of which are known to be resistant populations.

The other five SNPs found had not been previously described. Two of the SNPs were synonymous and thus had no translational effect on the protein structure of *βAOR*. The remaining three SNPs were found to have consequence on the amino acid structure. A263C and C264A both changed tyrosine to serine and T265A changed phenylalanine to isoleucine. When the 2-D structure of the *βAOR* protein was predicted for these SNPs it was found that they all caused a direct change to IL1 within the cytoplasmic domain of *βAOR*. Previous predictions of the 2-D structure of A225G found the location to be within the extracellular domain (Koh-Tan *et al*., 2016). The 2-D structure of the *βAOR* protein indicating the position of the 36 bp insertion, found it introduced 12 amino acids into the first transmembrane, thus extending the extracellular domain (Koh-Tan *et al*., 2016). If these changes are considered with regard to the function of GPCR, the extension of the extracellular domain was hypothesized to result in an obstruction of binding sites to formamidines and thus potentially confer resistance to them (Koh-Tan *et*

al., 2016). It has been reported that small changes in primary structure can have a large impact on the function of GPCRs, even when the change in structure occurs in areas that are not directly involved in the G-protein interaction (Broeck, 2001). Based on this it is not unreasonable to hypothesize that change in the primary structure observed in this study may change the shape of IL1 substantially enough to affect the function of this receptor. Whether this translates into Amitraz resistance directly, is unlikely, however it is potentially a step in a complicated process to developing Amitraz resistance in these ticks. Populations that are known to be resistant to Amitraz are significantly more likely to have one of these SNPs regardless of geographical location, which further supports the role of these SNPs in developing Amitraz resistance. This gene could be undergoing epigenetic selection pressures, however the question would be is it heritable? Based on the evidence gathered here, the suggestion is yes, as the development of acaricide resistance is occurring in a population.

The finding of A181T and T185C was novel, and its presence in a number of different populations is consistent with positive selection. To detect whether this is the case a number of different statistical tests could be used. Three main approaches could be used, either using divergence data, a combination of divergence and polymorphism data or by using polymorphism data (Zeng *et al*., 2006). Using the data genetrated in this study, a haploid test or a site by site frequency spectrum test could be used. A hapoloid test could be used to verify the neutral theory of molecular evolution, which predicts that genomic regions evolving at high rates will also have high levels of polymorphism within a species (Hudson *et al*., 1987) and thus tests wether or not the mutations are effected by positive selection. The problem with this approach is that all mutations are assumed to be selectively neutral, which in the case of *βAOR* is not a suitable assumption to make as it is unlikely to be undergoing genetic drift. A site by site frequency spectrum test looks at the distribution of the proportion of regions where the mutation is at frequency ‘x’ (Zeng *et al*., 2006). This approach assumes that there is no selection, accounts for the possibility for different sites of mutation and can be split into three main variant classes; low, intermediate and high into order to give bigger picture on the arrangement of polymorphisms (Zeng *et al*., 2006). For this test to be conducted, more samples would be needed than were generated in this study.

Conclusion

Overall it is clear that *βAOR* does not group based on geographic location or putative species. The shared SNPs between the Australian population and South American isolates support the evidence of the role of *βAOR* in Amitraz resistance, especially with the same SNP (A181T and T185C) arising differently at different sites within South America. The three novel SNPs that were reported also indicates a more direct role of *βAOR* in resistance as in previous studies the mutations reported were located in the extracellular domain. With amino acid substitutions occurring on the G-protein binding sites, it is possible to hypothesize that these mutations may cause conformational changes to the intracellular loop and so affect the way it interacts with the G-proteins.

CHAPTER 7

General discussion and conclusion

Summary introduction

The recently flagged changes in the taxonomic status of *R.(B) microplus* have assumed considerable practical importance. It has become common for scientists to use distinct species status of *R.(B) microplus* and *R.(B) australis* as an explanation for differences in the results among studies conducted in different regions on a tick that was previously considered to be a single species (Baron *et al*., 2015). Some have also begun to query the validity of reports that do not specify whether the subject is *R.(B) microplus* or *R.(B) australis* (e.g. Kaewmongkol *et al*. 2015). The belief that being classified as a distinct species should have any impact on the mechanisms of drug resistance or efficacy of a vaccine seems unreasonable, unless the taxa were genetically much more distant, than one would expect given their obvious phenotypic similarities. A generic expectation that the subject of a research report should be unequivocally defined as *R.(B) microplus* or *R.(B) australis* also presents a challenge to researchers if there is no effective, non-molecular means of definitive differentiation.

This project set out to apply genetic and morphological studies of tick populations from around the world to provide a phylogenetic analysis of *R.(B) microplus* and its nearest relations. We look to propose a credible evolutionary framework and determine the extent to which genetic divergence within the former species, among geographically distinct populations, is likely to lead to functional consequences for vaccine and acaricide development.

Two main approaches to the quantification and analysis of the variation within *Rhipicephalus (Boophilus)* clade were taken. The morphology study consisted of three main analyses; scoring based on published criteria, morphometrics and geometric morphometrics. The molecular study consisted of investigating the amount of variation for three genes of interest, the mitochondrial gene; *COX1* and two nuclear functional genes; *Bm86* the only gene currently used as a target for vaccines and *βAOR*, a gene with relevance to acaricide resistance.

General Discussion

Using morphological and phylogenetic analysis to resolve *Rhipicephalus (Boophilus) microplus* based on geographical location

Differentiation between the adult members of the Boophilid clade, based on a morphological scoring system was set up as four two-way analyses; *R.(B) australis* versus *R.(B) microplus*, *R.(B) microplus* versus *R.(B) annulatus*, *R.(B) microplus* versus *R.(B) decoloratus* and *R.(B) annulatus* versus *R.(B) decoloratus*. The morphological criteria assigned to the *R.(B) australis* versus *R.(B) microplus* analysis was based upon the published re-description outlined by Estrada-Peña *et al*. (2012). All subsequent analyses were based on morphological criteria, as described in Walker *et al*., 2003. Each morphological feature assessed was assigned a score of either 0 or 1, representing whether the feature was absent or present respectively. This preliminary study was designed to set the framework for the subsequent analyses in chapters 3-6, and enabled initial verification of specimen identification, which was important for the specimens that were collected from the field.

Results indicated that male and female *R.(B) annulatus* and *R.(B) decoloratus* are easily distinguished from one another, as are males and females of *R.(B) microplus* and *R.(B) decoloratus*. *R.(B) microplus* and *R.(B) annulatus* were also consistently identified for females correctly, however in the case of three *R.(B) microplus* males, the caudal appendage was absent despite having a long spur on coxa I, resulting in a score indicating a mixture of features. These findings were contradictory to previous reports that stated that due to the similarities in morphology observed between the Boophilid species and variability observed within species, differentiation was cumbersome and unreliable (Uilenburg, 1962, Lempereur *et al*., 2010).

R.(B) australis and *R.(B) microplus* were not clearly differentiated for either males or females. Among females, the Australian population was assigned almost evenly to *R.(B) australis* and *R.(B) microplus*, with 8 individuals showing a mixture of features. However, only Mozo was assigned solely to *R.(B) microplus*, the rest of the regions had ticks with mixed features and 6 ticks in South Africa, as well as 4 in Juarez were scored as *R.(B) australis*. Most male ticks, however, were assigned as either *R.(B) australis* or with a mixture of both species features, with a

surprisingly small number scoring as *R.(B) microplus*, for all geographical locations.

Barker and Walker (2014) stated that they could not unambiguously differentiate between *R.(B) microplus, R.(B) australis* and *R.(B) annulatus* using morphology independent of geography. However, the currently proposed distribution of *R.(B) australis* indicates that it is not solely an Australian tick. Populations of *R.(B) microplus* from Cambodia, Philippines, Indonesia, New Caledonia, Borneo, New Guinea, Tahiti and parts of Southeast Asia are all now proposed to be reinstated to *R.(B) australis* (Ali *et al*., 2016). This means that potentially in regions such as Thailand, *R.(B) australis* may occur alongside populations of *R.(B) microplus*. With populations of both species living within the same region it is possible that hybridization may be the reason for the mixture of features observed. However, this does not seem to be a reasonable explanation for the mixture of morphological features observed in some of the South American populations, especially considering that they are not from the field. The finding that proposed *R.(B) australis* specific features were found in South American populations, undermines the proposition that *R.(B) australis* is a morphologically distinct species from *R.(B) microplus* (Ali *et al*., 2016, Estrada-Peña *et al*., 2012).

The morphometric study was designed to expand the level of detail with regard to differentiation of the Boophilid ticks, by quantifying the phenotypic variation previously observed between *R.(B) microplus, R.(B) australis, R.(B) annulatus* and *R.(B) decoloratus*. It was also designed to test the criteria outlined by Estrada-Peña *et al*. (2012) on multiple populations of *R.(B) microplus*. Additional features were explored as potential points of differentiation between the populations of *R.(B) microplus* and *R.(B) australis*. The Fisher Pairwise comparison results for larvae showed Australian ticks trended towards a smaller body length, idiosoma length and scutum width, confirming the reported smaller body length and narrower scutum width proposed in the re-description of *R.(B) australis* (Estrada-Peña *et al*., 2012). For the rest of the morphological features, there was no obvious trend between the different populations/species and the PCA didn't yield any conclusive evidence that *R.(B) australis* is a separate species from *R.(B) microplus*, in fact there was no obvious grouping of species at all, even for *R.(B) decoloratus* and *R.(B) annulatus*.

The Fisher Pairwise comparison and PCA for males showed that Australian ticks were significantly different. No patterns were seen in the differentiation based on the other species. Based on the Fisher Pairwise comparison *R.(B) decoloratus* females tended to be smaller for most of the morphological features tested and *R.(B) annulatus* tended to be larger, with no differences observed between *R.(B) microplus* populations and *R.(B) australis.* However, this was not supported by the PCA, as grouping of Australian ticks was observed. The ambiguity and indeed variability observed in the morphological features tested here substantiate the claims that differentiation between Boophilid species is inconsistent (Barker and Walker *et al.*, 2003, Uilenburg, 1962, Lempereur *et al*., 2010).

The third part of this study intended to take a novel approach towards understanding tick morphology. Geometric morphometrics is the quantitative representation of shape using geometric coordinates instead of measurements. It aims to give the shape of the feature independent of size and so is useful for eliminating physiological changes. This could potentially be useful as it eliminates the bias of size, which can occur as a result of feeding. Previous studies that have used this technique and have found it to be a powerful approach to observing differences in morphology between species (Pretorius and Clarke, 2000, Pretorius and Clarke, 2001, Clarke and Pretorius, 2005).

Despite having a difference in scutum length, the overall scutal shape for larvae did not differ significantly based on species or population. Based on the Mahalanobis distance among groups however, Uruguay (Mozo) and Mexico (Yucatan) had the most divergent scutum shape (2.13, p=<0.001), whereas South African and Australian ticks had the most similarly shaped scutum (0.516, p=<0.001). Grouping of males for species and location was highly variable across the features tested. Overall *R.(B) decoloratus* had the most differently shaped morphology, particularly for adanal shield. Some grouping was also seen for *R.(B) annulatus*, mainly for conscutum shape, which can be explained by their lack of caudal appendage, although the lack of coxal spurs also resulted in a difference for coxa I shape. Grouping of *R.(B) decoloratus* and *R.(B) annulatus* was also observed in shape of the basis capituli and coxa I shape, respectively for females, but grouping based on location was not clear. Porose shape was found to differ in shape across the species/locations, in particular *R.(B) decoloratus* and *R.(B)*

annulatus grouped away from *R.(B) microplus* and *R.(B) australis*. These findings indicate that the geometric morphometric analysis did not clearly and consistently enable the differentiation of any of the sample populations of ticks in this study. Each feature differed among samples in different sets of pairwise relationships. It's success in previous studies (Pretorius and Clarke, 2000, Pretorius and Clarke, 2001, Clarke and Pretorius, 2005) indicated its potential as an approach, however based on the findings here, the lack of consistent morphological difference previously observed for the Boophilid ticks is once again confirmed (Barker and Walker *et al.*, 2003, Uilenburg, 1962, Lempereur *et al*., 2010). With this three-way approach to understanding the morphological differences of the Boophilid ticks covered in this work we can draw the following conclusions. *R.(B) australis* and *R.(B) microplus* larvae and males are distinguishable from one another with regard to size, and the differences observed here are supportive of the previous findings of Estrada-Peña *et al*. (2012). The ventral spur on palp article i on males, which was reported by Estrada-Peña *et al*. (2012) is present, but inconsistently, and is not limited to the Australian population. Females are indistinguishable from one another based on setae patterns, linear measurements and shape. This inconsistency in the resolution of the taxonomic status of *R.(B) microplus* confirms the views of Barker and Walker (2014), however we found no evidence that the geographical location impacts upon the ability to differentiate between *R.(B) microplus* and *R.(B) australis*.

Within the last 25 years the genus *Rhipicephalus* has undergone a degree of re-organization. The common evolutionary ancestor for this genus was thought to be unique to the *Rhipicephalus* genus, however with the development of molecular techniques it has been found that some Rhipicephaline species are more related to Boophilid species (Murrell and Barker, 2003b). The strongest support for the paraphyly of *Rhipicephalus* and *Boophilus* has come from combining 12S rDNA and *COX1* (Murrell and Barker, 2003b, Murrell *et al*., 2000) and as a result *Boophilus* is now considered to be a sub-genus of *Rhipicephalus*. With regard to *R.(B) microplus,* Burger *et al* (2014) presented results that indicated that *COX1* could resolve complex regional groupings, indicating that *R.(B) microplus* was composed of two clades and a separate grouping of *R.(B) australis* was also reported.

In this study, we built upon results of previous work by Burger *et al.* (2014) by using a larger number of ticks from more geographical locations, most of which have not been previously included in a taxonomic study of this nature. In doing so we aimed to confirm that *COX1* can be used to resolve complex relationships within the *R.(B) microplus* clade and to determine whether specimens clustered according to region. Maximum likelihood trees were constructed with a bootstrap analysis and a relaxed clock Bayesian analysis was undertaken to estimate topology and divergence timings. The maximum likelihood trees indicated that *R.(B) microplus* is a clade composed of five groups including *R.(B) australis* and *R.(B) annulatus*. The first group has a world-wide distribution, including *R.(B) microplus* from South America, North America, South Africa, Thailand and published sequences from Cambodia, Malaysia and one from China. *R.(B) australis* makes up the second group. The third group consists of published Indian and Malaysian samples. The fourth group is composed exclusively of Chinese samples. *R.(B) annulatus* was found to share a common ancestor with the Chinese group. These groupings support the observations made previously (Burger *et al.,* 2014), where it was proposed that *R.(B) microplus* is a complex made up of *R.(B) australis, R.(B) annulatus* and two *R.(B) microplus* clades; A: Brazil, China and Cambodia; B: China. The later findings of Low *et al*. (2015) proposed an additional clade, named clade C, made up of Malaysian and Indian specimens are also supported. With the addition of the sequences generated in this study, it is possible to expand on this proposed complex, increasing the size of *R.(B) microplus* clade A, with the addition of multiple locations.

R.(B) decoloratus appears to share a common ancestor with *R.(B) microplus* and *R.(B) annulatus*, diverging approximately 29 Mya. However, it appears to be monophyletic with *R. bursa, R. evertsi* and *R. pravus*. This supports the findings from previous studies that proposed the synonymizing of *Boophilus* with *Rhipicephalus* (Klompen *et al*., 1997, Murrell and Barker, 2003b, Murrell *et al*., 2001a).

The relationship that these groups have with one another within the *R.(B) microplus* clade needs to be examined in finer detail. A possible suggestion is that the *R.(B) microplus* species complex is made up of 5 sub-species. The sub-species should be less divergent than species (Won and Hey, 2005). Based on

the dates reported in this study, the divergence of the groups discussed, including *R.(B) australis* and *R.(B) annulatus*, happened too long ago. Another interpretation of subspecies is a group that can successfully interbreed with another group, producing fertile offspring, but do not in practice due to geographical isolation (Mayr and Ashlock, 1991). If this definition is accepted, then further work in the form of a mating study, where several crosses spanning the group would be needed. Csordas *et al*. (2016) report on one of the few studies that made insights into variability of *R.(B) microplus* within Brazil. Broadly speaking, the Brazilian *R.(B) microplus* have been reported to fall within clade A, however the molecular characterization, using *COX1*, of *R.(B) microplus* for five different regions within Brazil concluded that different regions have populations with distinct haplotypes (Csordas *et al*., 2016).

Variation in genomic nucleotide sequences of the *Bm86* and *βAOR* genes in Boophilid ticks from multiple geographical locations

The concealed antigen, *Bm86*, is the basis of the only commercially available vaccine against ticks. Since the introduction of commercial vaccines based on the antigen, variability in the efficacy against populations of *R.(B) microplus* in different regions have been reported (García-García *et al*., 1999, Willadsen *et al*., 1995, García-García *et al*., 2000). A proposed divergence in the amino acid sequence between recombinant *Bm86* vaccine and native *Bm86* expressed in ticks from different geographical locations has been suggested as one possible cause for efficacy variation (Freeman *et al*., 2010). It has also been proposed that there is an inverse correlation between efficacy of the vaccine and variation in the *Bm86* sequence (García-García *et al*., 1999). At present, there is no clarity on the link between sequence variation and function for *Bm86*. Only transcript sequences and expressed sequence-tag (EST) are available in the public domain, as yet there is nothing available on the *Bm86* gene genomic sequence. The genomic DNA sequence in a limited region of the gene was isolated for examination to determine whether there was variation in the gene that clustered according to the proposed new classification. The amplification of the *Bm86* gene was attempted from genomic DNA extracted from three Boophilid tick species; *R.(B) microplus* (multiple locations), *R.(B) annulatus* (USA) and *R.(B) decoloratus* (South Africa).

Our attempts to sequence *Bm86* from gDNA extracted from ethanol preserved ticks collected for this study were not successful. It was hypothesized that this was a result of inadequate DNA quality. However, analysis conducted on published cDNA sequences revealed a large number of polymorphisms. This suggests that without knowing the exon/intron boundaries, the primer set which was designed may not have worked.

Bm86 published sequences did not demonstrate grouping of *R.(B) microplus* according to geographical location, except for *R.(B) microplus* from Thailand. There was also a large amount of sequence variation between the Thai samples, as reported previously (Kaewmongkol *et al.,* 2015). *R.(B) decoloratus* and *R. appendiculatus* demonstrated distinct groups from *R.(B) microplus* and *R.(B) annulatus*. *R.(B) annulatus* also formed a distinct group with 87% Bootstrap support, but was also found to group closely with the Thai samples. Again, this finding was previously reported (Kaewmongkol *et al.,* 2015). The primers were also used on six *R.(B) microplus* tick cell lines from the Tick Cell Biobank at The Pirbright Institute and were found to contain further nucleotide variation for cell lines BME/CTVM23 and BME/CTVM30. This could have been caused by the fact that the cell lines are composed of multiple individuals, and as previous studies have indicated (Kaewmongkol *et al*., 2015), there can be variation in the *Bm86* sequence from ticks of the same species from the same geographical location. Additional sequence variants were observed when cell line sequences were aligned with published sequences. BME/CTVM2 was the only cell line that appeared to align in partial segments. With a lack of information regarding the size and arrangement of the *Bm86* gene and the number and length of introns and exons, an unreliable primer set can result. It is also potentially a transcript variant that hasn't previously been isolated; however, this is just a speculation without further annotation available on the gene.

The amount of variation observed in the rest of the *R.(B) microplus* specimens indicates that the gene is highly polymorphic, and the Australian isolate is divergent from the rest of the samples (>50% Bootstrap support). Previous work demonstrating relationships between species and region grossly show consensus with the trees created in this study (Nijhof *et al*., 2010). These studies indicated that *Bm86* grouped mainly based on species (*R.(B) microplus, R.(B) annulatus,*

R.(B) decoloratus and *R. appendiculatus*) but regional variation was not obvious (Nijhof *et al.,* 2010). Some regional variation was observed with Mexican and Brazilian samples grouping away from Mozambique and Australian samples with 96% Bootstrap support. This was again similar to what was found in this study, however we included many more sequences, expanding upon the amount of variation observed in the *Bm86* gene. The Indian Chennai isolate was previously found to have 97% identity with the reference Australian strain (Anbarasi *et al*., 2014), however when aligned with all the sequences, it was found to only be partial and was omitted. Polymorphisms have also been documented in South American strain of *R.(B) microplus* (Sossai *et al*., 2005). In this study, it was found that Alegre and Betim strains were the most divergent from both the *Bm86* reference and *Bm95*. Alegre and Betim strains diverged with only 52% Bootstrap support but with such a low level of support were considered unresolved. It is clear there is still a large amount of information on the *Bm86* gene still lacking in annotation.

Previous studies in insects have demonstrated that octopamine receptors are the target for formamidine-based acaricides, such as amitraz (Baxter and Barker, 1999; Hollingworth and Lund, 1982). In insects, octopamine receptors can be classified into three main types; tyraminergic, alpha-adrenergic-like and beta-adrenergic-like (Evans and Maqueira, 2005). Corley *et al*. (2012) identified 8 distinct GPCR from *R.(B) microplus* using a targeted, degenerate primer PCR approach and presented a phylogenetic analysis of the family of genes. They argued that the product of *βAOR* was the most likely target of amitraz. Subsequent work (Corley *et al*., 2013) showed that the entire coding sequence lay within a single exon of 1644 bp, and identified two SNPs, both non-synonymous, within the gene. One of these SNPs (A181T) was tightly associated with resistance to amitraz in an extended field trial. The second SNP (T185C) was from a population of unknown resistance status. Further diversity in this gene was demonstrated by Koh-Tan *et al*. (2016), using *in vitro* cell cultures of ticks. In that study, a 36 bp insertion was identified at position 190 in a cell culture from a resistant isolate of ticks from Latin America. The existence of a new, related gene was also inferred from consistent multiple primer binding sites and ambiguous sequence information.

The overall aim of this study was to quantify regional and taxonomic variation in the *βAOR* gene among *R.(B) microplus* ticks from multiple geographical locations (table 6.1). We also included the North American *R.(B) annulatus* and South African *R.(B) decoloratus* samples by way of comparison.

Eight putative SNPs were found, three of which had been previously described (Corley *et al*., 2013; Koh Tan *et al*., 2016) but there was no grouping of polymorphisms according to either geographic, or proposed taxonomic origin. A225G was previously described by Koh Tan *et al*., 2016 in other species of *Rhipicephalus* (*R. appendiculatus*, *R. evertsi* and *R. sanguineus*) and had not been previously described in *R.(B) decoloratus*. Two other SNPs; A181T and T185C had been previously described by Corley *et al*., 2013 in Australian populations of *R.(B) microplus,* one of which was known to be resistant to amitraz (A181T) and one of which came from a region in which resistance to amitraz is common (T185C). We reported A181T and T185C in two *R.(B) decoloratus* individuals as well as ticks from the South American isolates Jaguar, Juarez and Sao Gabriel, all of which are known to be resistant populations.

The other five SNPs found had not been previously described. Two of the SNPs were synonymous and thus had no translational effect on the protein structure of *βAOR*. The remaining three SNPs; A263C, C264A and T265A were found to have consequence on the amino acid structure. A263C and C264A both changed tyrosine to serine and 265 changed phenylalanine to isoleucine. When the 2-D structure of the *βAOR* protein was predicted for SNPs it was found that they all caused a direct change intracellular loop (IL) 1 within cytoplasmic domain of *βAOR*. Previous predictions of the 2-D structure of A225G found the location to be within the extracellular domain (Koh-Tan *et al*., 2016). The 2-D structure of the *βAOR* protein indicating the position of the 36 bp insertion, found it introduced 12 amino acids into the first transmembrane domain, thus extending the N-terminal extracellular domain (Koh-Tan *et al*., 2016). If these changes are considered with regard to the function of a GPCR, the extension of the extracellular domain was hypothesized to result in an obstruction of binding sites to formamidines and thus potentially confer resistance to them (Koh-Tan *et al*., 2016). Previous studies have shown that small changes in primary structure can have a large impact on the function of GPCRs, even when the change in structure occurs in areas that are

not directly involved in the G-protein interaction (Broeck, 2001). Based on this it is not unreasonable to hypothesize that change in the primary structure observed in this study might change the shape of IL1 substantially enough to affect the function of this receptor. Populations that are known to be resistant to amitraz are significantly more likely to have one of these SNPs regardless of geographical location, which further supports the role of thesis SNPs in developing amitraz resistance.

Final Conclusions

In this work, we found that ticks from USA, Mexico, Brazil, Uruguay, South Africa and Thailand belonged to clade A of the *R.(B) microplus* species complex. Although the presence of clade A in North and South America is not surprising, we expanded the detail of its reported range in the Americas and further extended it to Thailand. The presence of *R.(B) microplus* clade A in Thailand raises potential questions about how these clades could be overlapping geographically to the extent that they appear to. The presence of *R.(B) australis* is reported to be in Australia, New Caledonia, Sumatra and Java (Indonesia), Cambodia, Philippines, Tahiti, Papua New Guinea and elsewhere in Asia (Barker and Walker, 2014). However, based upon the findings from *COX1*, specimens sequenced from Cambodia grouped with *R.(B) microplus* clade A. This along with the Thai specimens, suggests that in some countries in Asia, *R.(B) microplus* and *R.(B) australis* are both present. Based on morphology, it is not possible to differentiate between the *R.(B) microplus* clades and *R.(B) australis* consistently, however the mixture of morphological features observed in Thailand, which was not consistent in males and females, may elude to cross-breeding of the two groups in areas where both are present. However, the presence of the spur on the ventral surface of palp article i, a feature reportedly unique to *R.(B) australis* males, was also observed in lab colony male specimens from parts of South America. The presence of this spur in these specimens would not occur if it was indeed a *R.(B) australis* specific feature. Additional overlapping of geography can also be observed for the other clades of *R.(B) microplus*. Proposed clade A and B from Burger and Barker (2014) demonstrates distinct overlapping in China and potentially in other parts of Asia. Members of clade B was also suggested to be present in Northern India (Barker and Walker, 2014), which would overlap in India with the presence of clade C, proposed by Low *et al*. (2015).

Burger and Barker (2014) first suggested that *R.(B) annulatus* was a clade of the *R.(B) microplus* species complex. The evidence seen from phylogenies inferred from *COX1*, combined with the reported ambiguity in the morphology, resulting in inconsistent differentiation between *R.(B) annulatus* and *R.(B) microplus* (Uilenburg, 1962, Lempereur *et al*., 2010) supports the assigning of *R.(B) annulatus* to the species complex. In contrast, the first part of the morphological study conducted in this work, found that *R.(B) annulatus* and *R.(B) microplus* could be differentiated unambiguously for both males and females, based on the morphological criteria outline by Walker *et al* (2003). However, a more thorough examination of the morphology in the subsequent techniques confirmed previous reports on the inconsistent and highly variable morphology, although some differentiation was still possible. When all three approaches towards understanding, morphology is combined, it is evident that *R.(B) annulatus* is closely related to *R.(B) microplus. Rhipicephalus (Boophilus) decoloratus* on the other hand is morphologically and molecularly distinct from both *R.(B) annulatus* and *R.(B) microplus*.

The support for a *R.(B) microplus* species complex has now been established in three separate studies. Based upon *COX1, R.(B) microplus* comprises Clade A (Burger and Barker, 2014): China (Yunnan Province), Brazil, Cambodia, Africa, with the addition of: Mexico, USA (Texas), Uruguay, Thailand, Panama and Malaysia; Clade B (Burger and Barker, 2014): China (Henan, Hubei and Guizhou Provinces), Sri Lanka, northern India and elsewhere in Asia; Clade C (Low *et al*., 2015): Malaysia and India; *R.(B) australis* and *R.(B) annulatus*. Separation of clades A-C is not supported by morphology (i.e. they are morphologically indistinct from one another), however it does support the inclusion of *R.(B) australis* and *R.(B) annulatus* within the species complex.

The groupings of *R.(B) microplus* discussed above were found to have no relationship with the variation observed in both the *Bm86* and *βAOR* sequences. Variations observed in the *Bm86* gene, as expected, were independent of the proposed species and regional classification. The work done in this study shows that the *Bm86* gene is highly polymorphic, is probably expressed as different isoforms, and previous studies on vaccine efficacy show results that are unlikely to be completely explained by simple polymorphisms in the cDNA sequences that

have been published so far. The apparent high degree of intronic variation, observed in the cell lines, might provide an alternative explanation in terms of gene expression for variation in vaccine efficacy.

The shared SNPs between the Australian population and South American samples support the likely role of *βAOR* in amitraz resistance, especially with the same SNP arising differently at different sites within South America. The possibility that amitraz-resistant ticks designated as *R.(B) microplus* and *R.(B) australis* might share the same mutation that has previously been associated with amitraz resistance (and other, similar mutations) reinforces the fact that the evolutionary forces of selection, gene flow and genetic drift are likely more important from a functional perspective than their arbitrary species or subspecies distinction.

APPENDIX

Tick morphology

The body of an unfed tick is flattened dorsoventrally and divided into two sections. The anterior gnathosoma features the mouthparts including; the hypostome, palps and basis capituli (figure A1) (Walker *et al*., 2003). The palps of the gnathosoma are composed of 4 segments called palp articles, numbered i-iv (see glossary of terms) (Walker *et al*., 2003). The palps function as sensory organs that assist in finding a host. The hypostome is located between the palps and is the feeding apparatus of the tick (Walker *et al*., 2003). At the proximal tip of the hypostome are two highly sclerotized segmented appendages called the chelicerae. On the chelicerae are a number of tooth-like structures arranged on a triangular-shaped plate which act as the cutting tool, creating the opening in the host's skin allowing the hypostome to be inserted. The basis capituli of the gnathosoma is a large fused coxa that allows movement of the mouthparts independent from the body (Walker *et al*., 2003).

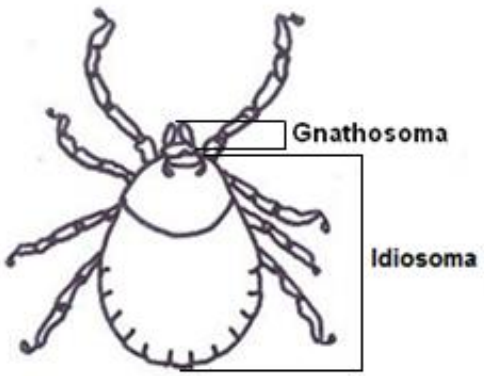


Figure A1: Dorsal surface of an Ixodid tick, showing the major divisions of the body (Source: Authors own drawing).

The posterior idiosoma includes the main bulk of the body, to which the legs are connected. On the dorsal surface, a sclerotized plate can be observed. In adult tick this plate can be used to distinguish between the sexes; on males, the plate dominates the dorsal surface and is called the conscutum, in females the plate is called the scutum and is relatively small, taking up approximate a third of the dorsal surface and thus allowing for full engorgement. If eyes are present they are located on the lateral border of the scutum/conscutum, normally at the level between the second and third legs. As well as the scutum/conscutum the dorsal surface may also have a number of grooves, either located anteriorly (cervical grooves) or posteriorly (posteromedial grooves). The posterior wall of the idiosoma may also have a number of uniform grooves giving it a 'pie-crust' effect, which are called festoons. Along with the festoons, other protrusions may be seen, in the case of certain members of the *Rhipicephalus* genus, caudal appendages may be observed, as well as the spurs from the ventral plates (figure A2) (Walker *et al*., 2003).

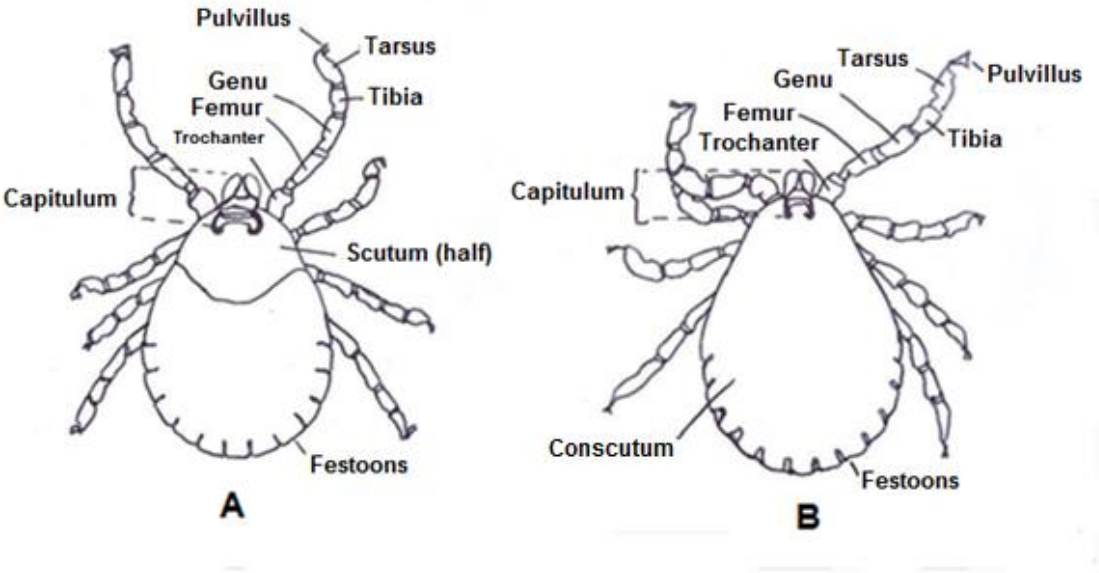


Figure A2: Dorsal view of a female (A) and male (B) Ixodid tick, showing the visible features. (Source: Authors own drawing).

On the ventral surface of the idiosoma, the legs are attached via coxa. In females the genital opening is located centrally between coxa III or coxa IV. In both sexes the anus is located posteriorly to coxa IV (figure A3). As with the dorsal surface, there are a number of grooves on the ventral surface; the genital groove is located in the anterior portion of the surface, in females this groove goes around the genital groove anteriorly (figure A3 drawing A). The anal groove is associated with the anus and in certain genera this will circle the anus posteriorly and in others it will circle anteriorly. Large openings surrounded by a tear-shaped sclerotized plate, called spiracles are located posterior-lateral to coxa IV. These structures are the site of respiration. Additional sclerotized plates may be seen on certain males; these plates are called adanal shields and can vary in number and may have a number of posterior projecting spurs of varying length on them (figure A3 drawing B). Festoons and caudal appendages are also visible from the ventral surface (figure A3) (Walker *et al*., 2003).

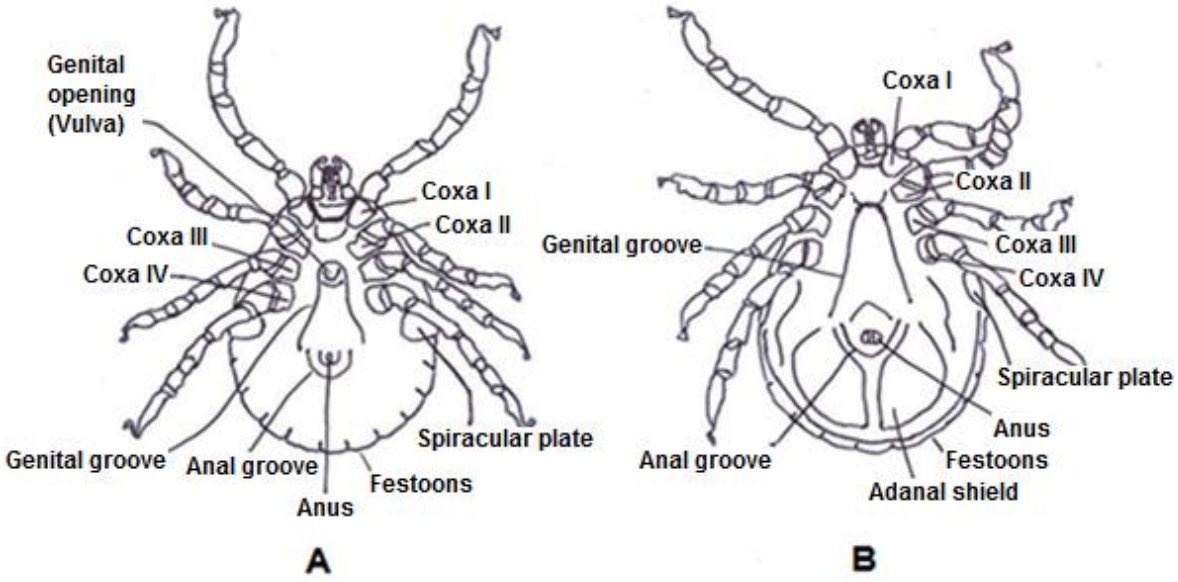


Figure A3: Ventral view of a female (A) and male (B) Ixodid tick, showing the visible features. (Source: Authors own drawing).

Key morphological features

Adanal shields (Ventral plates)

The adanal shields are a group of plates that can be absent, indistinct or distinct on the ventral surface of the idiosoma in males. They are paired plates located laterally to the anus and are often accompanied by a secondary pair of smaller plates called accessory shields which are located laterally to the adanal shields (figure A4). There are a number of different variations that can be observed in these plates. Firstly the posterior margin of the main adanal plates can be rounded or squared, as seen in figure A4 image A; the overall shape may be narrow trapezoid or broad and curved, as seen in figure A4 images B and C. The accessory adanal shields may be absent, small or large (accessory adanal shields are present in figure A4 images A-C) (Walker *et al*., 2003). An additional pair of ventral plates may be observed in some males of the *Hyalomma* genus, these plates are located posteriorly to the adanal shields (Not picture in figure A4) (Walker *et al*., 2003).

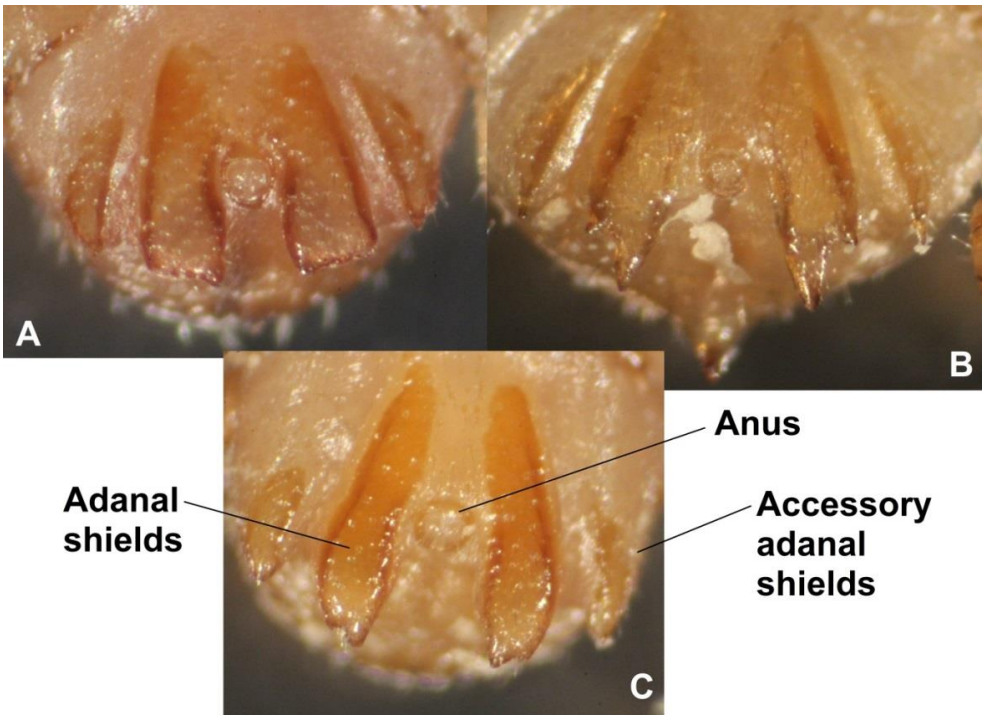


Figure A4: Examples of the different types of ventral plates; A: adanal and accessory plate present, posterior border of adanal plates are squared with spurs are indistinct (*Rhipicephalus (Boophilus) annulatus)*. B: adanal and accessory plate present, posterior border of adanal plates are squared with distinct spurs that are visible from the dorsal surface (*Rhipicephalus (Boophilus) decoloratus)*. C: adanal and accessory plate present, posterior border of adanal plates are rounded with spurs indistinct (*Rhipicephalus (Boophilus) microplus)*. (Source: Authors own photograph).

As well as the size and shape of the plates themselves, spurs may be present on the posterior border of the ventral plates. These spurs may be distinct (figure A4, image B and C) or indistinct (figure A4, image A) and depending on their length may be visible from the dorsal surface (Walker *et al*., 2003). The function of these plates and the spurs that can be associated with them is currently not known. Their position and the presence of spurs could be hypothesized to have a role in the mating, however due to the positioning of the male tick during mating; this surface does not seem to come into contact with the ventral surface of the female.

Anal groove

The anal groove is a small depression in the integument that is either absent or indistinct and is located on the ventral surface. It forms a loop around the anus either posteriorly (figure A5, image B and C) to the anus or in the case of *Ixodes*, anterior to the anus (figure A5, image A) (Walker *et al*., 2003). This feature can be found in both male and female ticks. As well as its position, its alignment can also be used in identification; possible shapes include long and parallel/diverging (figure A5, image B) or short and converging (figure A5, image C) (Walker *et al*., 2003). No functional significance is known.

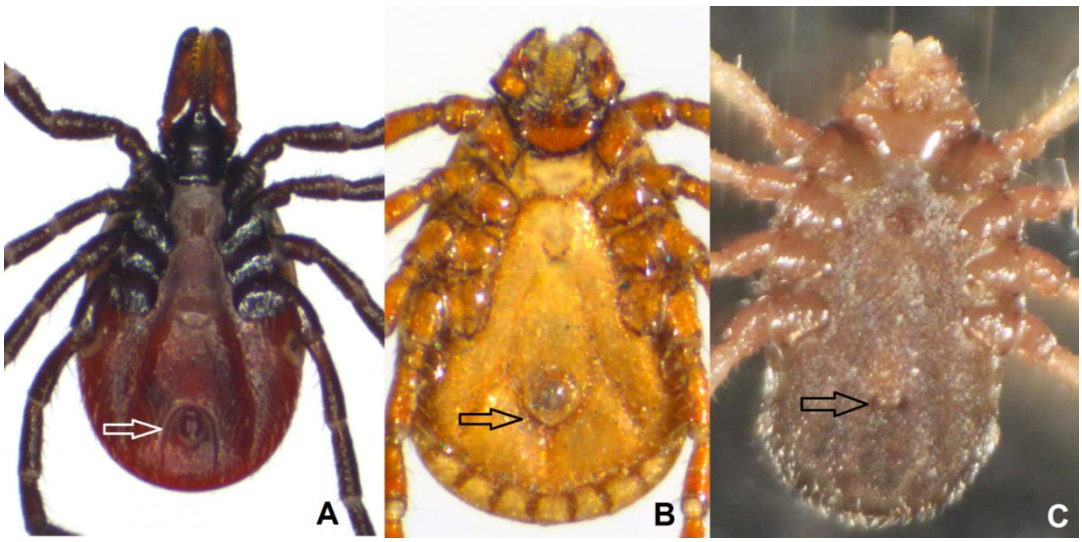


Figure A5: Examples of the different types of anal grooves; A: posterior groove (*Ixodes ricinus)*. B: anterior groove (*Haemaphysalis intermedia*). C: anal groove absent (*Rhipicephalus (Boophilus) microplus)*. (Source: Authors own photograph).

Auriculae

Auriculae are paired lateral protrusions located on the lateral margins of the ventral basis capituli. They can either be distinct (figure A6, picture B) or indistinct (figure A6, picture A) but can also vary greatly in shape. This feature can be useful, when it is present, in distinguishing between closely related species (e.g. *Ixodes*) (figure A6) (Walker *et al*., 2003). No functional significance is known.

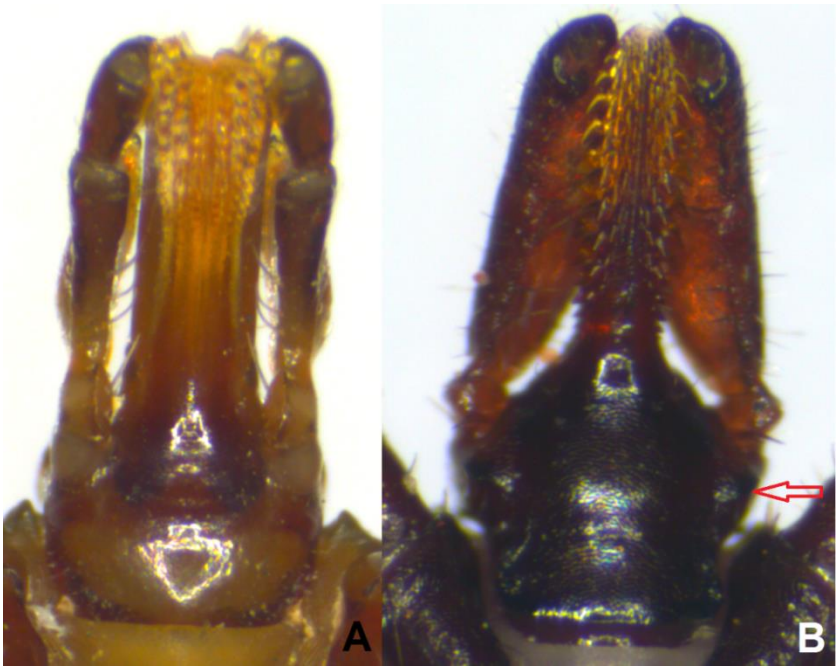


Figure A6: Position of the auriculae on the ventral surface of the basis capituli; A: auriculae is absent (*Amblyomma variegatum*). B: auriculae are present and indicated by the red arrow (*Ixodes ricinus*). (Source: Authors own photograph).

Basis capituli

The basis capituli is located posterior to the palps/hypostome and anterior to the idiosoma. The lateral margin of the basis capituli can be either straight (as in *Dermacentor,* 'Der', figure A7), medium angular (as seen in *Amblyomma*, 'Am', figure A7) or distinctly angular (as seen in *Rhipicephalus,* 'Rhi', figure A7), which can also vary between the sexes. The basis capituli also has other features used in identification including porose areas and cornua on the dorsal surface and the auriculae on the ventral surface (Walker *et al*., 2003). The variability in the form of the basis capituli may be linked to the size and arrangement of the plaps and thus to the preferred host and the type of hide that the mouthparts have to pierce. Based upon this, it is possible to hypothesise that those tick species feeding onhosts with a thick hide may benefit from having a wider, sturdier basis capituli. However the functional significance of the variation seen in figure A7 has not been determined.

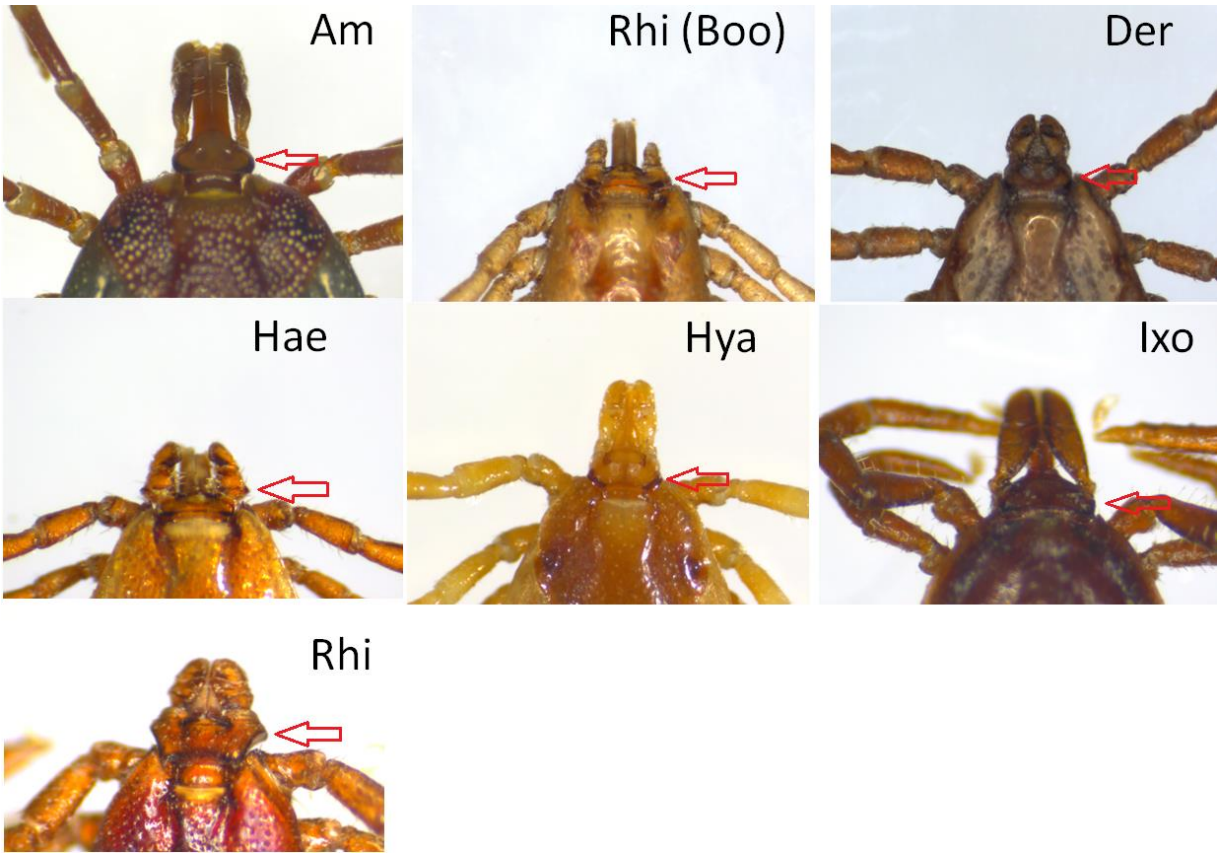


Figure A7: Examples of the different shapes of basis capituli between seven genera; Am: *Amblyomma*. Rhi (Boo): *Rhipicephalus (Boophilus)*. Der: *Dermacentor*. Hae: *Haemaphysalis*. Hya: *Hyalomma*. Ixo: *Ixodes*. Rhi: *Rhipicephalus*. (Source: Authors own photograph).

Caudal appendage

The caudal appendage is a protrusion located on the posterior border of the idiosoma. It can be variable in size and is visible from the dorsal and ventral sides. This feature is either absent (figure A8, image C); narrow (figure A8, image A) or broad (figure A8, image B) (Walker *et al*., 2003). The functioinal significane of the caudal appendage is not known.

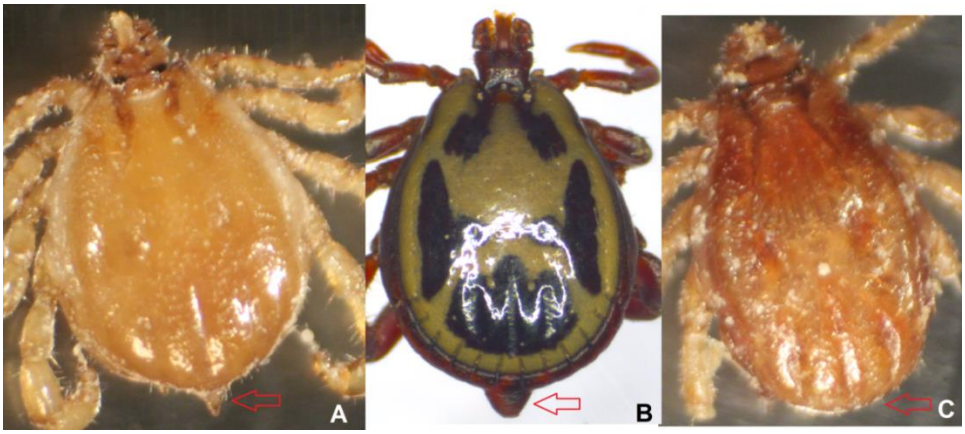


Figure A8: Examples of caudal appendage; A: narrow (*Rhipicephalus (Boophilus) microplus*). B: Broad (*Rhipicephalus pulchellus*). C: absent (*Rhipicephalus (Boophilus) microplus*). (Source: Authors own photograph).

Cornua

Cornua are a pair of projections located on the posterior border of the basis capituli. When they are present they are either distinct (figure A9, image B) or indistinct (Walker *et al*., 2003). Figure A9, image A shows the absence of the cornua. There appear to be no functional relevance to the presence of the cornua.

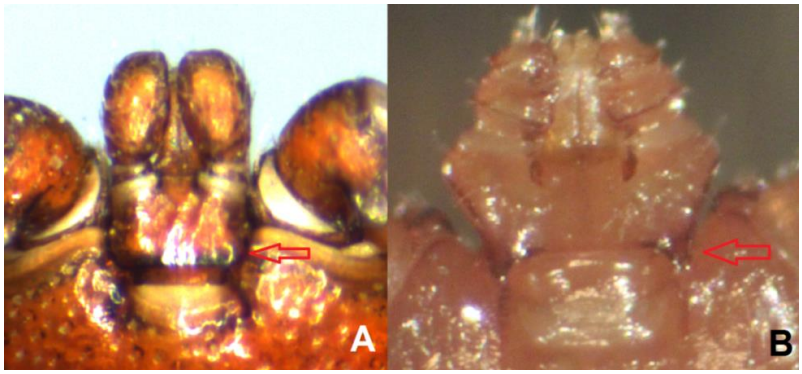


Figure A9: Position of cornua on the dorsal basis capituli; A: Indistinct (*Haemaphysalis inermis*). B: distinct (*Rhipicephalus (Boophilus) microplus*). (Source: Authors own photograph).

Coxae

In adults there are four coxae (I-IV) located on the ventral surface of the idiosoma. Spurs may be present on all or some of the coxae and there are either internal, external or both types of spur present (figure A10) (Walker *et al*., 2003).

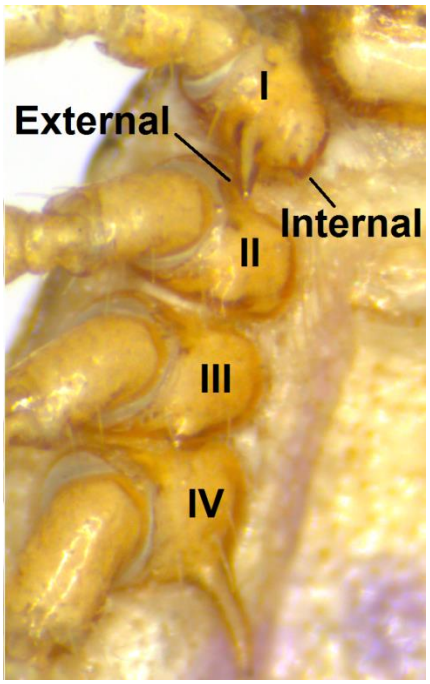


Figure A10: Arrangement of the four coxae and the position of the internal and external spurs (*Amblyomma americanum*). (Source: Authors own photograph).

Coxal spurs can be either long (figure A11, image B) or short (figure A11, image A) and either indistinct (figure A11, image A) or distinct (figure A11, image B) (Walker *et al*., 2003).

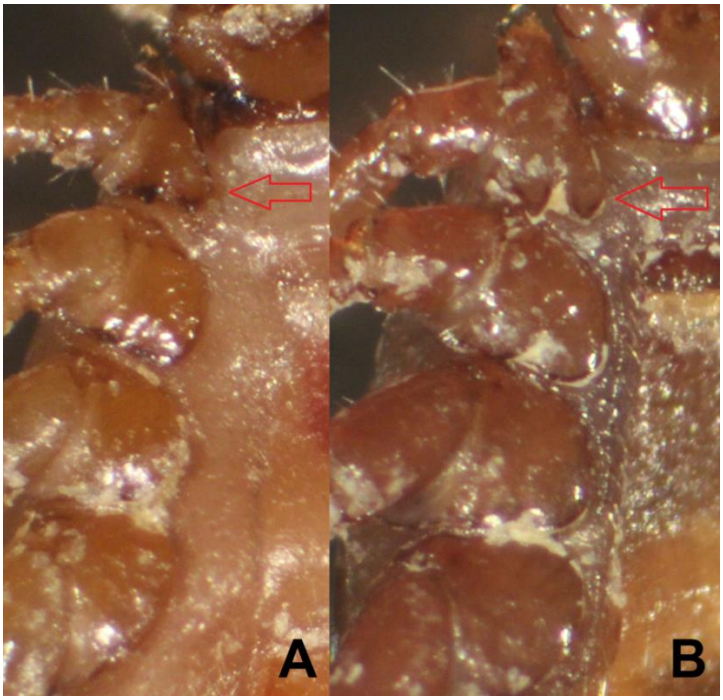


Figure A11: Types of spurs that can be observed indicated by the red arrow; A: short and indistinct (*Rhipicephalus (Boophilus) microplus*). B: Long and distinct (*Rhipicephalus (Boophilus) decoloratus*). (Source: Authors own photograph).

Coxa I can have the most variation with regard to the spurs. A spur on the anterior surface of coxal I (figure A12, image A) may be visible from the dorsal aspect (figure A12, image B) (Walker *et al*., 2003).

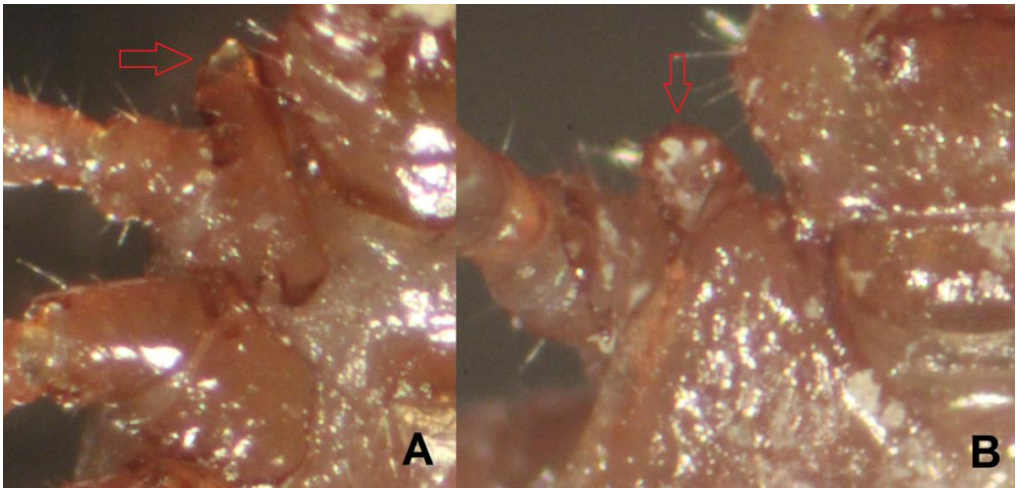


Figure A12: Anterior spur on coxa I, indicated by the red arrow, from the ventral aspect (A) and dorsal aspect (B) (*Rhipicephalus (Boophilus) decoloratus*). (Source: Authors own photograph).

The pairing of the internal and external spurs on coxa I can also be indicative in identification. Spurs can either be single, varying in length (figure A13, image A),

paired and equal (figure A13, image B) or paired and unequal (figure A13, images D and E), or absent altogether (figure A13, image C) (Walker *et al*., 2003).

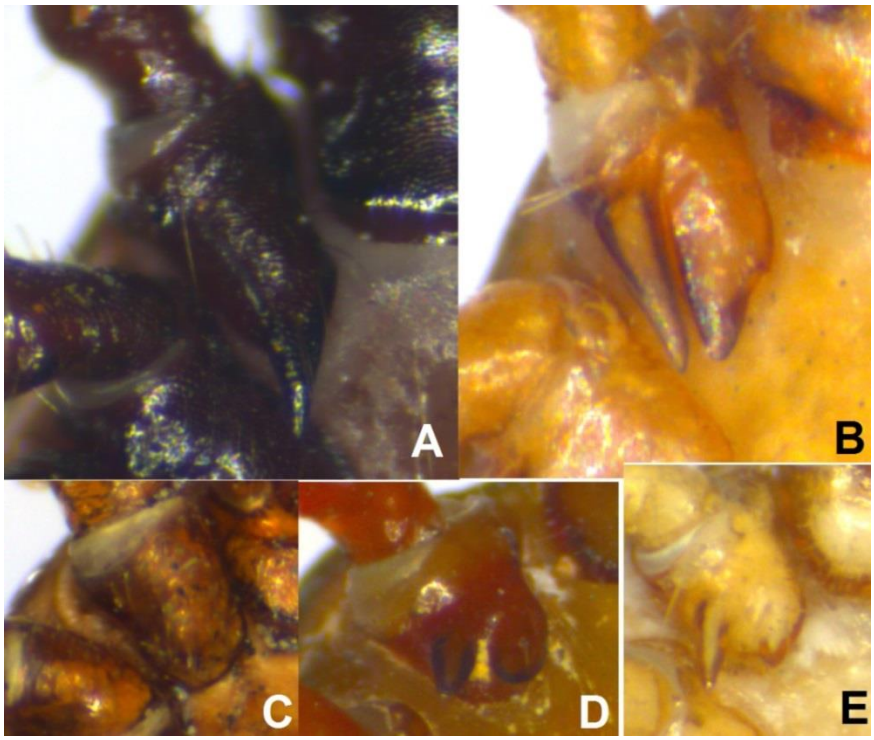


Figure A13: Variations observed in the pairing of spurs on coxa I; A: single spur (*Ixodes ricinus*). B: equal and paired (*Hyalomma marginatum*). C: absent (*Haemaphysalis inermis*). D: small and unequal (*Amblyomma variegatum*). E: unequal (*Amblyomma americanum*). (Source: Authors own photograph).

The size of coxa IV can also vary from species to species. In some males this coxa can be very large compared to the rest of the coxae (figure A14, image A). Additionally the internal spur on this coxa, when present, can range in length from short to long (figure A14, image D and E) (Walker *et al*., 2003). The coxae are articulation points for the legs, the functional relevance of the spurs however, is not known.

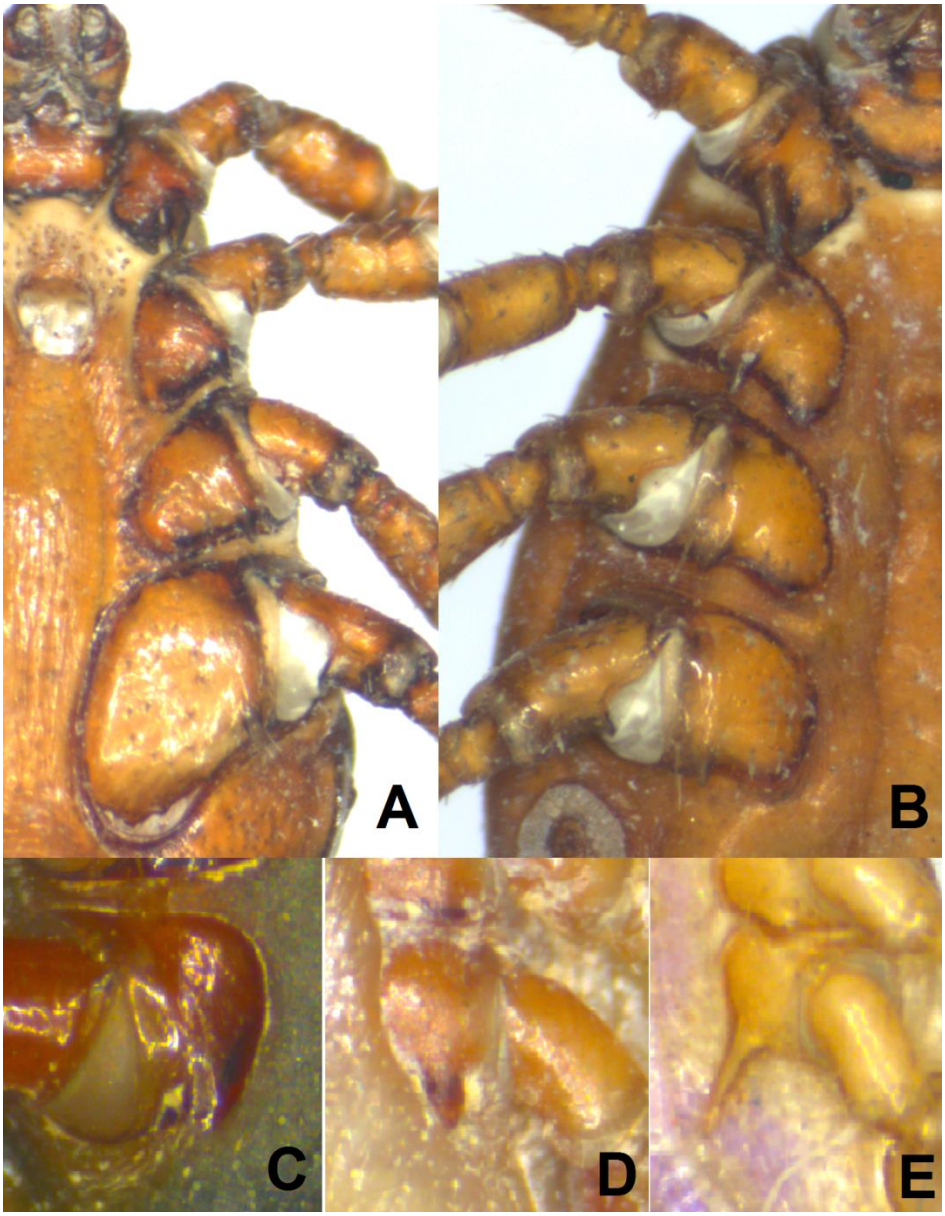


Figure A14: Variation observed on coxa IV; A: enlarged coxa IV (*Dermacentor andersoni* male). B: Normal sized coxa IV (*Dermacentor andersoni* female). C: spur absent from coxa IV (*Amblyomma variegatum*). D: short spur on coxa IV (*Amblyomma testundinarium*). E: Long spur on coxa IV (*Amblyomma americanum*). (Source: Authors own photograph).

Dorsal idiosoma grooves

A number of grooves are present on the dorsal surface of the idiosoma. Cervical and scapular grooves are located on the anterior portion of the idiosoma. Cervical grooves are located medially, with the scapular grooves positioned in a more lateral position. These two sets of grooves form the borders of two depressions called the cervical field (figure A15). The cervical field depression can vary in the depth of the depression, the shape and the texture. With regard to the texture, a number of pits called punctations varying in size and distribution may be present

(Walker *et al*., 2003). In the image shown in figure A15, the scapular groove shown is distinctly visible, and a large number of punctations varying in size can also be observed.

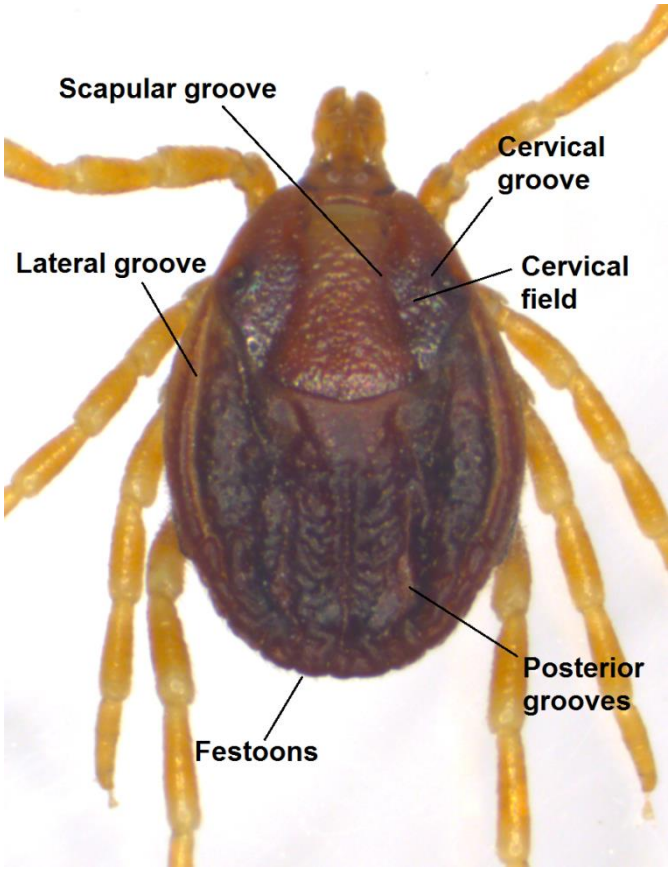


Figure A15: Position of the grooves located on the dorsal idiosoma (*Hyalomma marginatum*). (Source: Authors own photograph).

Additional grooves may also be present along the lateral borders of the idiosoma, running posteriorly. These lateral grooves can vary in length (long and short), depth (indistinct and distinct) and texture (smooth with no punctations, wrinkled or distinctly punctate). In the case of the image shown in figure A15, these lateral grooves are long, distinct, and wrinkled. A number of posterior grooves may also be present, if present these can be indistinct or distinct and in some *Hyalomma* males may cause ridges in the conscutum surface. Additional grooves located on the posterior margin called festoons may be present. The festoons can vary in number and size, in some species the central festoon appearing larger than the rest (Walker *et al*., 2003). The functional significance of these grooves are not known, it could however be surmised that they play some sort of role in preventing the ticks body from drying out, acting to trap moisture to the integument.

Hypostome

The hypostome is a pair of blades used for feeding on the host. It is located within the palps and contains a number of hypostomal teeth on the ventral surface, the number and arrangement of which can be used in some identifications. A 3+3 (figure A16, image B) or 4+4 (figure A16, image A) arrangement of the teeth is normally observed (Walker *et al*., 2003).

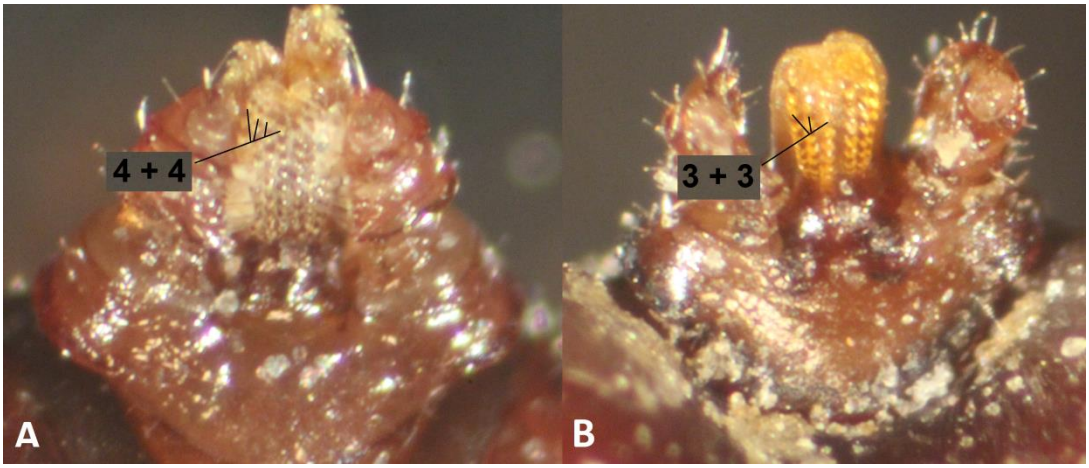


Figure A16: Position and arrangement of the hypostomal teeth, *Rhipicephalus (Boophilus) microplus* image A and *Rhipicephalus (Boophilus) decoloratus*, image B. (Source: Authors own photograph).

Legs

There are four pairs of legs attached to the ventral surface of the idiosoma by the coxae. The legs are composed of a number of articulating limbs starting at the body with the coxae and then the trochanter, femur, genu, tibia, tarsus and pulvillus (figure A17). The legs themselves can vary in colour and size but key features can be observed on the trochanter and the tarsus. On trochanter I there may be a large triangular-shaped spur pointing posteriorly which can be either short or long when present. Tarsus I may vary in shape around the position of the Haller's organ, a feature often used in distinguishing between females of *Ixodes* (Walker *et al*., 2003). The front pair of legs are important for host seeking. Ticks will climb up vegetation and quest for host, waving their front two legs in the air. Ticks have a small pore located on the tarsus of the first pair of legs called the Haller's organ (figure A17). This sensory pit is composed of chemoreceptors that allow the questing tick to detect slight changes in chemical composition of the air

around them, such as would be caused by the presence of a host and exhaled host carbon dioxide (Needman and Teel, 1991).

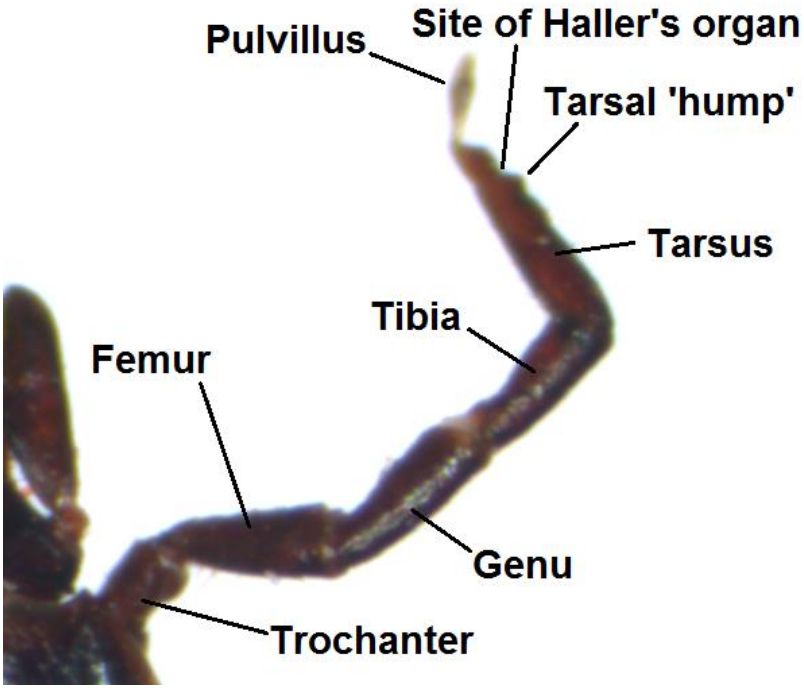


Figure A17: Structures of the leg (Leg I shown). Spur on trochanter I is absent, in this species of *Ixodes* a 'hump' is present near the Haller's organ, in other specimens this feature may be tapered or stepped. (Source: Authors own photograph).

Palps

Palps are made up of four segments called articles which can vary in shape and size. Palp article i is located at the base of the palp and is the articulation point of the palp with the basis capituli. In some species a protuberance may be observed on the internal margin of this palp. Additionally the internal margin may be long and slightly concave or short and distinctly concave. Article ii can vary greatly in size in relation to the size of the other articles from all small, article ii broad or article ii long. It may also have a dorsal spur, ventral spur or a lateral extension. All these features may be present or absent. Article iii can have a ventral spur that projects posteriorly towards articles ii and i and finally article iv is located on the ventral aspect of article iii. Article iv does not have any features useful for identification and is used as a sensory organ (figure A18) (Walker *et al*., 2003). Although not involved in the uptake of blood during feeding, the palps contain sensory organs thought to be involved in taste and smell (Waladde and Rice, 1982).

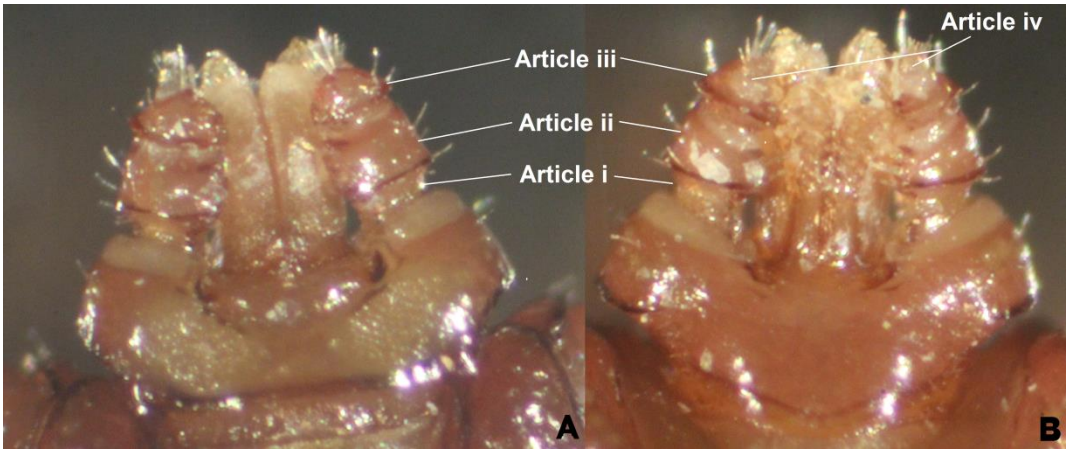


Figure A18: Position of the palp articles; A: dorsally. B: ventrally (*Rhipicephalus (Boophilus) microplus*). Variation in the palp articles between genera can be seen in figure A7. (Source: Authors own photograph).

Porose areas

The porose areas are pitted areas located on the dorsal aspect of the basis capituli of female ticks. Their shape and areas of separation can be used in distinguishing among females of closely related species. The shape of the porose area can vary from narrow oval (figure A19, image B) to broad oval (figure A19, image A) and the area separating them can be narrow (figure A19, image B) or broad (figure A19, image A) (Walker *et al*., 2003). The porose areas are a feature specific to females, theiy function as openings of the accessory glands, which secrete a substance that is thought to have lubricant (Feldman-Muhsam and Havivi 1963) and potentially anti-autoxidant properties (Atkinson and Binninton 1973).

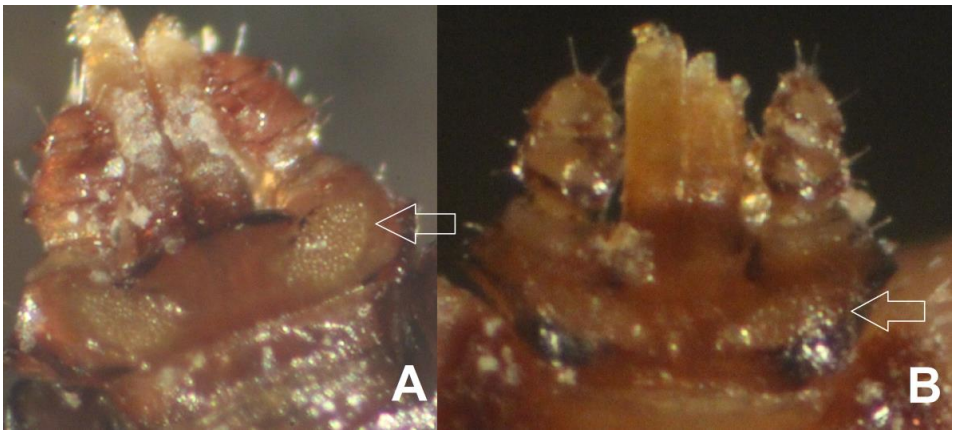


Figure A19: Position of the porose area indicated by white arrow and variation in shape; A: broad oval (*Rhipicephalus (Boophilus) microplus*). B: narrow oval (*Rhipicephalus (Boophilus) decoloratus*). (Source: Authors own photograph).

Setae

A number of hair-like structures called setae can be found all over the body of an Ixodid tick, the thickness, length and number of which can be used in identification. However this feature is easily damaged during specimen collection. It can also become brittle and easily removed when specimens are stored in ethanol. Setae can be found in a number of locations, firstly, a group of setae may be located on a protuberance on the internal margin of palp article i. This group of setae is seen in *R.(B) decoloratus* and can be used to distinguish it from *R.(B) annulatus* and *R.(B) microplus* when visible. Postpalpal setae are located on the ventral basis capituli, caudal to the palps. Medial alloscutal setae (figure A20) are located on the dorsal idiosoma, posterior to the scutum and may be thin and colourless (figure A20, image B) or thick and white (figure A20, image A) and arranged in dense rows of 4-6 (figure A20, image A) or thinner rows of 2-3 (figure A20, image B). Scutal setae may also be present (not pictured) on the bulk of the scutum, behind the eyes or in males on the lateral margins of the capitulum. These setae can appear long and pale or short and slender (Walker *et al*., 2003). The function of the setae is not fully understood, however it can be surmised that they may play a role in preventing the tick from losing too much water by acting to trap moitures to the integument.

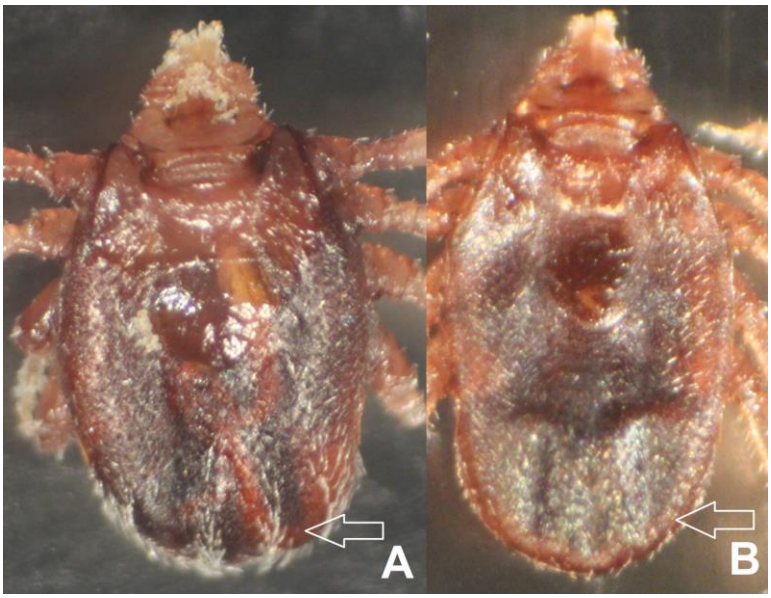


Figure A20: Example of variation of the medial alloscutal setae indicated by the white arrows; A: long, pale and abundant (*Rhipicephalus (Boophilus) australis*). B: short, slender and sparse (*Rhipicephalus (Boophilus) microplus*). (Source: Authors own photograph).

Scutum/ Conscutum

The sclerotized plate located on the dorsal aspect of the idiosoma may dominate the entire surface in males (conscutum) (figure A21, images B and D) or take up approximately a third of the dorsal aspect in females (scutum) (figure A21, images A and C). The scutum can vary in shape amongst the females of different genera; being narrow (figure A21, image C) or broad (figure A21, image A) at the base, having either smooth (figure A21, image C) or wavy (figure A21, image A) lateral edges and either straight (figure A21, image C) or convex (figure A21, image A) overall appearance. For both males and females ornamentation (figure A21, images A and B) may be observed with the potential addition of enameling (figure A21, images C and D) (Walker *et al*., 2003). The function of the scutum/conscutum is not fully understood, due to its thickness and toughness it is possible to suggest that its role may be to protect the tick, particularly if the tick gets groomed off by the host.

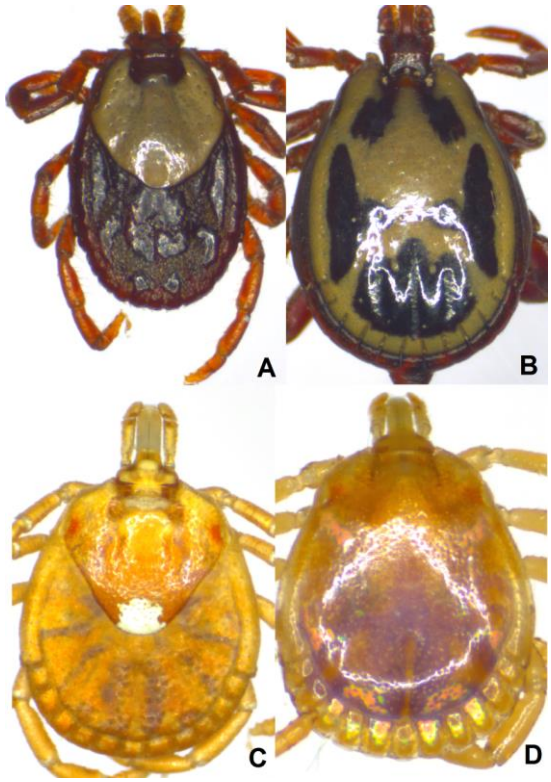


Figure A21: Examples of scutum/conscutum variation; A+B: female and male, ornamentation with enamel absent (*Rhipicephalus pulchellus*). C+D: female and male, minor ornamentation with enameling present (*Amblyomma americanum*). Scutum shape: A: broad posterior tip with convex and wavy lateral borders. C: narrow posterior tip with straight and smooth lateral borders. (Source: Authors own photograph).

Table A1: Published primer sequences available for *COX 1*.

Gene/Locus	Species	GenBank accession number	Forward primer name	Forward primer	Reverse primer name	Reverse primer	Reference
COX 1	*R.(B) annulatus*	AF132825	TW-J-1302	GTTAACAAACTAGTAGCCTTCAAAG	C1-N-2009	GATCAAACAAATAAGGGTA	(Murrell *et al*., 2000)
COX 1	*R.(B) decoloratus*	AF132826	TY-J-1449	AATTTACAGTTTATCGCCT	C1-N-2312	CATACAATAAAGCCTAATA	(Murrell *et al*., 2000)
COX 1	*R.(B) microplus*	AF132827	C1-J-2180	AACATTTATTTTGATTTTT	C1-N-2191	(Simon *et al*., 1994)	(Murrell *et al*., 2000)
COX 1			TY-J-1460	(Simon *et al*., 1994)			(Murrell *et al*., 2000)
COX 1			C1-J-1718	(Simon *et al*., 1994)			(Murrell *et al*., 2000)
COX 1			TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC	C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC	(Simon *et al*., 1994)
COX 1			C1-J-1718	GGAGGATTTGGAAATTGATTAGTTCC			(Simon *et al*., 1994)
COX 1			C1-J1632	TGATCAAATTTATAAT		(Simon *et al*., 1994)	(Kambhampati and Smith, 1995)
COX 1					C1-N-2191	GGTAAAATTAAAATATAAACTTC	(Simon *et al*., 1994)

Table A2: Primer design, adapted from (Green *et al*., 2012).

Property	Optimal design
Base composition	G + C content should be between 40% and 60%, with an even distribution of all four bases along the length of the primer (e.g. no polypurine or polypyrimidine tracts and no dinucleotide repeats). If possible, avoid GC-rich stretches, which are prone to forming secondary structures.
Length	The region of the primer complementary to the template should be 18-30 nucleotides in length. Members of a primer pair should not differ in length by >3 bases. Primers shorter in length than 18 nucleotides will tend to bind non-specifically to complex template DNAs (e.g. genomic DNAs). Primers >30 nucleotides in length have an increased probability of forming secondary structures such as hairpin loops.
Internally repeated and self-complementary structures	Ensure that the primers contain no inverted repeat sequences of self-complementary sequences >3 bp in length. Sequences of this type tend to form hairpin structures that can suppress binding of the primer to its target sequence.
Complementarity between members of a primer pair	The 3'-terminal sequences of one primer should not be able to bind to any site on the other primer. Because primers are present in high concentrations in PCR, even weak complementarity between them can cause hybrid formation and the consequent amplification of primer dimers. These molecules can be real nuisances because they can compete for DNA polymerase and dNTPs and can suppress amplification of the true target DNA. Formation of primer dimers can be reduced by careful primer design and by using hot start or touchdown PCR and/or by the use of specially formulated DNA polymerases (e.g. AmpliTaq Gold; Applied Biosystems). If all else fails try adding formamide or dimethyl sulfoxide (DMSO) to the PCR mix and re-optimize the concentration of Mg^{2+} in the PCR by setting up a series of test PCRs containing different amounts of the divalent cation.
Melting temperature (T_m)	The optimum T_m of the duplex formed between a primer and its target is between 55ºC and 60ºC. The T_ms of the primers in a PCR should not differ by more than 2-3 centigrade degrees. Most software for primer design uses equation-based nearest-neighbour thermodynamic theory. A first-order approximation of the melting temperature of oligonucleotides with >25 bases can be calculated from the Wallace rule (Wallace *et al*., 1979): $T_m = 2ºC(A+T) + 4ºC(G+C)$ Where A, G, C and T are the number of occurrences of each nucleotide.
GC clamp	The presence of G or C bases within the last 5 bases from the 3' end of primers helps promote tight binding of the 3' end of the target sequence because of the stronger hydrogen-bonding of G and C bases. Priming efficiently and specificity are increased if the 3'-terminal residue is G. However, greater than three Gs or Cs should be avoided in the last five bases at the 3' end of the primer.
Adding restriction sites and other useful sequences to the 5' termini of primers	Useful sequences not complementary to the target DNA can be added to the 5' termini of oligonucleotide primers. However, terminal and subterminal restriction sites are cleaved poorly by restriction enzymes; thus, the length of the primer should be extended by at least 3 nucleotides beyond the restriction site. The NEB catalog contains information on the efficiency with which different restriction enzymes cleave sites near the termini of DNA molecules.
False priming	Target sequences should be searched (http://www.ncbi.nlm.nih.gov/BLAST/) for cross-homology with the oligonucleotide primers. False priming at cross-homologous sites increases the level of nonspecific amplification.

Table A3: Published sequences, and where available primer sequences for Bm86.

Reference	Gene/Locus	Species	Strain	GenBank acension number	Forward primer name	Forward primer	Reverse primer name	Reverse primer
(Kaewmongkol *et al*., 2015)	Bm86	*R. australis/ R.microplus*	Yeerongpilly, Australia	M29321	For	ATGCGTGGCA TCGCTTTATT	Rev	GTTTAGCCCA ACTATCTTTAT TTGACATC
	Bm86	*R. microplus*	S1	KJ995882				
	Bm86	*R. microplus*	C1 (M1)	KJ995883				
	Bm86	*R. microplus*	C2 (M2)	KJ995884				
	Bm86	*R. microplus*	C3 (M3)	KJ995885				
	Bm86	*R. microplus*	N1	KJ995886				
	Bm86	*R. microplus*	N2	KJ995887				
	Bm86	*R. microplus*	N3	KJ995888				
	Bm86	*R. microplus*	N4	KJ995889				
	Bm86	*R. microplus*	N5	KJ995890				
	Bm86	*R. microplus*	N6	KJ995891				
	Bm86	*R. microplus*	N7	KJ995892				
	Bm86	*R. microplus*	N8	KJ995893				
	Bm86	*R. microplus*	N9	KJ995894				
	Bm86	*R. microplus*	N10	KJ995895				
	Bm86	*R. microplus*	N11	KJ995896				
	Bm86	*R. microplus*	NE1	KJ995897				
	Bm86	*R. microplus*	NE2	KJ995898				
	Bm86	*R. microplus*	NE3	KJ995899				
	Bm86	*R. microplus*	NE4	KJ995900				
	Bm86	*R. microplus*	NE5	KJ995901				
	Bm86	*R. microplus*	NE6	KJ995902				
	Bm86	*R. microplus*	NE7	KJ995903				

	Bm86	*R. microplus*	NE8	KJ995904				
	Bm86	*R. microplus*	NE9	KJ995905				
	Bm86	*R. microplus*	NE10	KJ995906				
	Bm86	*R. microplus*	NE11	KJ995907				
	Bm86	*R. microplus*	NE12	KJ995908				
	Bm86	*R. microplus*	NE13	KJ995909				
	Bm86	*R. microplus*	NE14	KJ995910				
(Cunha *et al*., 2011)	Bm86	*R. microplus*	Campo Grande	EU352677.1	BmCG-EcoRI-F1	CGGAATTCTCATCCATTTGCTC	BmCG-NotI-R1	GCGGCCGCAGCACTTGACTT
(Freeman *et al*., 2010)	Bm86	*R. microplus*	Yeerongpilly, Australia	M29321.1	For	ATGCGTGGCATCGCTTTGTT	Rev	GGTGTTCGATGTAAGCGTGATG
	Bm86	*R. microplus*	Maverick1, South Texas (USDA-APHIS-VS)	HQ014400	Bm86for685	GACGAAAGAAGCTGGGTT	Bm86rev605	CCAGGAGAGCAATAGGAGTC
	Bm86	*R. annulatus*	Kinney1, South Texas (USDA-APHIS-VS)	HQ014401	Bm86for1650	GTACCACATGCAACCCTAAA		
	Bm86	*R. annulatus*	Starr3, South Texas (USDA-APHIS-VS)	HQ014385	Bm86 internal forward	ATCGACAAAGCTGCTATTGTCC	Bm86 internal reverse	TTTCTCTGCTATGAGTCTTGCC
	Bm86	*R. microplus*	Zapata2, South Texas (USDA-APHIS-VS)	HQ014386				
	Bm86	*R. microplus*	Webb1, South Texas (USDA-APHIS-VS)	HQ014387				
	Bm86	*R. microplus*	Starr5, South Texas (USDA-APHIS-VS)	HQ014388				
	Bm86	*R. microplus*	Zapata3, South Texas (USDA-	HQ014389				

			APHIS-VS)					
	Bm86	*R. microplus*	Webb2, South Texas (USDA-APHIS-VS)	HQ014390				
	Bm86	*R. microplus*	Zapata5, South Texas (USDA-APHIS-VS)	HQ014391				
	Bm86	*R. microplus*	Zapata10, South Texas (USDA-APHIS-VS)	HQ014392				
	Bm86	*R. microplus*	Zapata1, South Texas (USDA-APHIS-VS)	HQ014393				
	Bm86	*R. microplus*	Zapata12, South Texas (USDA-APHIS-VS)	HQ014394				
	Bm86	*R. microplus*	Hidalgo1, South Texas (USDA-APHIS-VS)	HQ014395				
	Bm86	*R. microplus*	Starr1, South Texas (USDA-APHIS-VS)	HQ014396				
	Bm86	*R. microplus*	Starr2, South Texas (USDA-APHIS-VS)	HQ014397				
	Bm86	*R. microplus*	Zapata11, South Texas (USDA-APHIS-VS)	HQ014398				
	Bm86	*R. microplus*	Dimmit1, South Texas (USDA-APHIS-VS)	HQ014399				
(Nijhof *et al*., 2009)	Bm86	*R. microplus*	Australia	M29321 (AAA30098)	(AMN-For)	CGTCCCGACT TGACCTGC	(AMN-Rev)	AGGAGCGGCT GAACAGTTTG
	Bm86	*R. microplus*	Mozambique	FJ809946				

	Bm86	*R. annulatus*	Mozambique	EU191621 (ABY58969)				
	Bm86	*R. annulatus*	Mozambique	EU191620 (ABY58968)				
	Bd86-2	*R. decoloratus*		DQ630524 (ABG21131)				
	Ra86-1	*R. appendiculatus*		FJ809944				
	Ra86-2	*R. appendiculatus*		FJ809945				
(Canales *et al*., 2008)	Bm86	*R. microplus*		EU191620	CZABM5	*ACTCGAGAAA AGAGAGTCAT CCATTTGCTCT GACTTCGG*	CZABM3	GGCAGGTCTG TTTTTGCTCA
	Ba86	*R. annulatus*		EU191621				
	Bd86	*R. decoloratus*		EU191622				
(Sossai *et al*., 2005)	Bm86	*R. microplus*		M29321	S	GGCAGGTCTG TTTTTGCTCA	AS	TGAAAGTAAC CCGAGCAAGG
	Bm86	*R. microplus*	Venda Nova do Imigrante (VNI)	AY766041				
	Bm86	*R. microplus*	Alegre2	AY766042				
	Bm86	*R. microplus*	Alegre1	AY766043				
	Bm86	*R. microplus*	Caraibas	AY766045				
	Bm86	*R. microplus*	La Paz	AY766046				
	Bm86	*R. microplus*	Las Cejas	AY766047				
	Bm86	*R. microplus*	SJose´1	AY785783				
	Bm86	*R. microplus*	SJose´2	AY785784				
	Bm86	*R. microplus*	Mozo	AY785785				
	Bm86	*R. microplus*	Paraiba do Sul (PSul)	AY785786				
	Bm86	*R. microplus*	SLuiz	AY785787				
	Bm86	*R. microplus*	SSAlto	AY785788				

	Bm86	*R. microplus*	MClaros1	AY785789				
	Bm86	*R. microplus*	Coxim	AY785790				
	Bm86	*R. microplus*	Itaqui	AY785791				
	Bm86	*R. microplus*	SLivra	AY785792				
	Bm86	*R. microplus*	Butia	AY785793				
	Bm86	*R. microplus*	SGabriel	AY785794				
	Bm86	*R. microplus*	PAlegre	AY785795				
	Bm86	*R. microplus*	Betim1	AY785796				
	Bm86	*R. microplus*	Betim2	AY785797				
	Bm86	*R. microplus*	Bugre1	AY785798				
	Bm86	*R. microplus*	Bugre2	AY785799				
	Bm86	*R. microplus*	Vicosa (UFV)	AY785800				
	Bm86	*R. microplus*	Sao Miguel do Anta (SMA)	AY785801				
	Bm86	*R. microplus*	Jaboti	AY785802				
	Bm86	*R. microplus*	SCarlos	AY785803				
	Bm86	*R. microplus*	Guara´	AY785804				
	Bm86	*R. microplus*	Teresina	AY785805				
	Bm86	*R. microplus*	Tucuruı´	AY785806				
	Bm86	*R. microplus*	COeste	AY785807				
	Bm86	*R. microplus*	MClaros2	AY787167				
	Bm86	*R. microplus*	Vene A	AY848824				
	Bm86	*R. microplus*	Vene B	AY848825				
(de la Fuente *et al*., 2000a)	Bm86	*R. microplus*	Yeerongpilly, Australia		oligo 2888	ATTGAGCGTACCACATGCAACCCTAA	oligo 629/bmt-2	CCAGATCTTTAAGCACTTGACTTTCCAGGATC
	Bm86	*R. microplus*	Camcord		oligo 2888	ATTGAGCGTACCACATGCAACCCTAA	oligo 629/bmt-2	CCAGATCTTTAAGCACTTGACTTTCCAGGATC

	Bm86	*R. microplus*	Tuxpan		oligo 2888	ATTGAGCGTACCACATGCAACCCTAA	oligo 629/bmt-2	CCAGATCTTTAAGCACTTGACTTTCCAGGATC
	Bm86	*R. microplus*	Aldama		oligo 2888	ATTGAGCGTACCACATGCAACCCTAA	oligo 629/bmt-2	CCAGATCTTTAAGCACTTGACTTTCCAGGATC
	Bm86	*R. microplus*	Mora		oligo 2888	ATTGAGCGTACCACATGCAACCCTAA	oligo 629/bmt-2	CCAGATCTTTAAGCACTTGACTTTCCAGGATC
	Bm86	*B. annulatus*			oligo 2888	ATTGAGCGTACCACATGCAACCCTAA	oligo 629/bmt-2	CCAGATCTTTAAGCACTTGACTTTCCAGGATC
(Rodríguez *et al*., 1994)	Bm86	*B. microplus*			bmt-1	GTCTAGAGGAATCATTTGCTCTGACTTC	bmt-3	CCTTGATTTCCATGGACAATAGCAGC
	Bm86	*B. microplus*			bmt-4	GCTGCTATTGTCCATGGAAATCAAGG	bmt-2	CCAGATCTTTAAGCACTTGACTTTCCAGGATC
(García-García *et al*., 1999)					1572-1598	ATTGAGCGTACCACATGCAACCCTAA		

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